METHANOGENIC AND NON-METHANOGENIC ACTIVITIES AND ARCHAEAL COMPOSITION OF A FULL-SCALE ANAEROBIC EGSB REACTOR TREATING A BREWERY WASTEWATER

by

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To my family…

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ABSTRACT

Anaerobic treatment has widely been used especially for treatment of medium and high strength industrial wastewaters such as brewery wastewater which are highly polluted and considered to be medium-high strength wastewaters. Water and wastewater management constitutes a practical problem for the food and beverage industry including the brewing industry. For this reason, different reactor configurations have been developed.

Expanded Granular Sludge Bed (EGSB) reactor is considered a desirable treatment option for the brewery wastewaters and other high strength organic wastewaters produced by industries. The reactor performance is usually evaluated in terms of process efficiency and stability through estimation of organic matter removal, VFA levels, quantity and composition of biogas produced, etc. However, the changes in the activity of methanogenic species could have not been determined by the conventional parameters which can only provide information about the current conditions inside the reactors. These parameters do not indicate the most suitable organic loading rates should be applied to anaerobic reactors. The specific methanogenic activity (SMA) test, therefore, has been used to determine the maximum methanogenic activity. In addition, non-methanogenic activity tests can explain each biodegradation phase of anaerobic treatment processes.

Research into engineered-design system using rRNA-based molecular techniques has provided detailed descriptions of the complex bacterial and archaeal populations present, obviating the need for anaerobic culture techniques. For accurately describing microbial populations, rRNA-based approaches utilizing the techniques of fluorescent in situ hybridization (FISH) with nucleic acid probes, together with other genetic analyses, have dramatically increased our knowledge of many ecosystems and have yielded a clearer overall picture of microbial diversity.

In this study, methanogenic and non-methanogenic activities and archaeal population dynamics in a full-scale EGSB reactor treating a brewery wastewater were determined for a three months period (April, May and June 2007), using SMA Test and FISH, respectively.

In this study, the SMA tests were carried out in the samples taken only from the bottom of the EGSB reactor. The tests results showed that the maximum acetoclastic methanogenic activity was found to be 457 mL CH_4/g VSS.d in April sample. In addition, the maximum specific methanogenic activity with butyrate and propionate were 460 mL CH_4/g VSS.d and 250 mL CH_4/g VSS.d in June and April samples, respectively. Finally, maximum overall methanogenic activity using a VFA mixture (2000 mg/L acetate, 500 mg/L butyrate and 500 mg/L propionate) was found to be 400 mL CH_4/g VSS.d in April sample. Regarding to non-methanogenic activity measurements, a method based on substrate utilization rate was adopted. June sample had the maximum acidogenic activity of 2.84 mg COD/mg VSS.d. The hydrolytic step was dominant in 3000 and 4000 mg/L sucrose concentrations in the three samples.

Methane archaeal composition of the biological sludge samples taken from three different heights along the anaerobic EGSB reactor was determined by FISH .FISH results supported activity test results particularly acetoclastic methanogenic activity tests results. *Methanosaeta spp.* were found to be the predominant methanogens in the EGSB reactor at all heights. However, predominance of *Methanosaeta spp.* tended to change to hydrogenotrophic methanogens along the anaerobic reactor during the three months sampling. In addition, the numbers of the methanogenic population decreased apparently from bottom to top of the anaerobic EGSB reactor.

ÖZET

Anaerobik arıtım, özellikle orta kuvvette endüstriyel atık suların arıtımında yaygın bir şekilde kullanılır. Örneğin çok kirli ve orta kuvvetli sayılan bira endüstrisi atık suyu için anaerobik arıtım tercih edilen bir sistemdir. Su ve atıksu yönetimi gıda ve bira endüstrisini de içeren maya endüstrisi için bir problemdir. Bu sebeple, çeşitli reaktör konfigürasyonları geliştirilmiştir.

Genişlemiş granüler çamur yataklı reaktör, bira endüstrisi ve diğer kuvvetli atıksuya sahip endüstriler için arzu edilen arıtımı gerçekleştirecek bir seçenek olarak kabul edilir. Reaktör performansı, genellikle organik madde giderimi, uçucu yağ asitleri seviyesi, miktar ve üretilen gazın kompozisyonu açısından değerlendirilir. Bunun yanında, reaktör içinde bulunan metan arkeyal türlerin aktiviteleri ve zamana bağlı değişimleri, sadece reaktörün o anki durumu hakkında bilgi verebilen geleneksel parametreler ile belirlenemez. Bu parametreler anaerobik reaktöre uygulanması gereken optimum organik yükleme oranını göstermez. Spesifik metanojenik aktivite testi (SMA) maksimum metanojenik aktiviteyi belirlemek üzere kullanılan bir testtir. Ayrıca, non-metanojenik aktivite testleri de anaerobik sistemlerde gerçekleşen biyodegredasyon adımları hakkında bilgi verebilir.

Anaerobik arıtma sistemlerinin mühendislik araştırmalarında, rRNA bazlı moleküler tekniklerin kullanımı sistemde var olan kompleks bakteriyel ve arkeyal popülasyonun detaylı bir tanımını sağlamakta ve anaerobik kültür tekniklerinde ihtiyaç duyulan eksiklikleri gidermektedir. Mikrobiyal popülasyonun doğru tanımlanması için nükleik asit probları kullanılan rRNA bazlı florasanlı yerinde hibritleşme metodunun diğer genetik analizlerle beraber birçok ekosistem ve mikrobiyal çeşitlilik hakkında bilgimizi fazlasıyla artırmış ve net bir bakış açısı sağlamıştır.

Bu çalışmada, bira endüstrisi atıksuyunu arıtan anaerobik genişlemiş granüler çamur yataklı reaktörden (EGSB) sırasıyla; Nisan, Mayıs, Haziran 2007 aylarında metanojenik, non-

metanojenik aktiviteleri ve arkeyal popülasyon dinamikleri, spesifik metanojenik aktivite testi ve florasanlı yerinde hibritleşme metodu kullanılarak belirlenmiştir.

SMA testleri EGSB reaktörün sadece dip seviyesinden alınan numuneler kullanılarak yapılmıştır. Test sonuçları maksimum metanojenik aktivitenin 457 mL CH_4/g UAKM.gün olduğunu göstermiştir. Buna ek olarak, substurat olarak bütirat ve propiyonat kullanılması ile spesifik metanojenik aktivite 460 mL CH_4/g UAKM.gün (4000 mg/L bütirat için) ve 250 mL CH_4/g UAKM.gün (3000 mg/L propiyonat için) olarak Nisan ve Haziran ayları için sırası ile tespit edilmiştir. Son olarak, uçucu yağ asitleri karışımı (2000 mg/L asetat, 500 mg/L bütirat and 500 mg/L propiyonat) kullanılarak toplam aktiviteye bakılmış ve 400 mL CH_4/g UAKM.gün aktivite değeri en yüksek Nisan örneğinde ölçülmüştür. Ayrıca, alınan biyolojik çamur numunelerinde substurat tüketim hız değerleri kullanılarak non-metanojenik aktivite ölçülmüştür. Haziran örneği 2.84 mg KOİ/mg UAKM.gün ile en yüksek asitojenik aktiviteyi göstermiştir. Hidrolitik fazın, 3000 ve 4000 mg/L sükroz için üç numune periyodunda da baskın olduğu tespit edilmiştir.

Anaerobik EGSB reaktörün üç farklı seviyesinden alınan biyolojik çamur numunelerinin metan arkeyal kompozisyonu Florasanlı Yerinde Hibritleşme Testi kullanılarak tesbit edilmiştir. FISH sonuçları özellikle asetoklastik metanojenik aktivite sonuçları olmak üzere aktivite sonuçlarını desteklemiştir. *Methanosaeta spp.* EGSB reaktörde en baskın tür olarak belirlenmiştir. Fakat, reaktör boyunca baskın olan *Methanosaeta spp.* türünün örnekleme periyotlarında hidrojenotrofik metanojenlere doğru değişme eğiliminde oldukları tesbit edilmiştir. Ayrıca, metanojenik mikroorganizma popülasyonunun sayısı reaktörün alt kısmından üst kısmına doğru azalma göstermiştir.

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AMP Actual Methane Production

BOD Biological Oxygen Demand

COD Chemical Oxygen Demand

CWO Catalytic Wet Oxidation

DGGE Denaturating Gradient Gel Electrophoresis

EGSB Expanded Granular Sludge Bed

FISH Fluorescent in situ Hybridization

HRT Hydraulic Retention Time

OLR Organic Loading Rate

PCR Polymerase Chain Reaction

PFA Paraformaldehyde

PMP Potential Methane Production

rRNA Ribosomal RNA

SMA Specific Methanogenic Activity

SRB Sulphate Reducing Bacteria

SS Suspended Solids

TS Total Solids

TVS Total Volatile Solids

UASB Upflow Anaerobic Sludge Blanket

VFA Volatile Fatty Acids

VSS Volatile Suspended Solids

1. INTRODUCTION

Anaerobic treatment has widely been used especially for treatment of high strength industrial wastewaters. It is an energy-efficient method. Up to now, the complex microbial ecology of the biological sludge, symbiotic relationships and the effect of microbial diversity on performance of anaerobic digestion systems were poorly understood. Over the last two decades, modern full-scale anaerobic treatment plants have been designed, monitored and controlled with a high degree of confidence in their performance characteristics.

Water and wastewater management constitutes a practical problem for beverage industry including brewing industry. Brewery wastewaters are highly polluted and considered to be medium-high strength wastewaters. In spite of significant improvement over the last 20 years, water consumption and disposal remain critical from an environmental and economic standpoint. There are several possible strategies to treat such wastewaters including concentration/incineration (Randall and Knopp, 1980), anaerobic treatment (Driessen, 1994; Austermann-Haun Seyfield, 1994; Ince et al, 1995) non-catalytic wet oxidation with steam generation followed by aerobic polishing or catalytic wet oxidation (CWO) using either homogeneous catalysts such as dissolved transition metal cations (Belkacemi et al., 2000). Anaerobic treatment is mostly preferred for the treatment of such types of wastewater. Anaerobic EGSB and UASB reactors are considered the desirable treatment options for brewery industry wastewaters and other high strength industrial wastewaters. (Connaughton et al., 2006; Zoutberg et al., 1996, Driessen, 1994).

Performances of anaerobic biological reactor systems are usually evaluated in terms of process efficiency and stability through estimation of organic matter removal; mostly parameters such as chemical organic demand (COD), VFA levels, quantity and composition of biogas produced, etc., generally are used. However, the changes in activity of methanogenic species could have not been determined by the conventional parameters which can only provide information about the current conditions inside the reactors. The specific

methanogenic activity (SMA) test, however; was developed Monteggia, (1991) and modified and used to determine the maximum acetoclastic methanogenic activity (Ince et al., 1995). The SMA test has been reported to be a control parameter and a means of determining the optimum operating conditions of anaerobic treatment systems (Monteggia, 1991; Ince et al., 1994, 2001). In addition, overall methanogenic, acidogenic and hydrolytic activity tests were carried out to understand system performance of each biodegradation step by several researchers (Henze and Herremoes, 1983, Hutnan et al., 1999, and Soto et al., 1993).

For accurately describing viable microbial populations, rRNA-based approaches utilizing the techniques of fluorescent in situ hybridization (FISH) with nucleic acid probes, together with other genetic analyses, have dramatically increased our knowledge of many ecosystems and have yielded a clearer overall picture of microbial diversity (Amann et al., 1990; Head et al., 1998; Hugenholtz et al., 1998). rRNA-based molecular techniques have provided detailed descriptions of the complex bacterial and archaeal populations. An obvious advantage of using FISH with rRNA-targeted nucleic acid probes is that metabolically active cells are detected, so descriptions of the physiologically important population members can be identified (Poulsen et al., 1993). It also allows categorizing and quantifying methanogens at different levels of phylogenetic depth and localizing individual community members in their natural spatial positions and providing a basis to estimate the *in situ* growth rates of methanogens in natural populations.

The aim of this study was determination of methanogenic and non-methanogenic activities and archaeal population dynamics in a full-scale anaerobic EGSB reactor treating a brewery wastewater. Methanogenic and non-methanogenic activities were determined by SMA test unit and archaeal methanogenic population were identified by FISH during a three months operating period, May, June and July 2007.

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 methane (CH_4), carbondioxide (CO_2) and trace amounts of hydrogen in the absence of oxygen. In the first two phases, organic pollutants are hydrolyzed and/or fermented into intermediate short-chain fatty acids (e.g., lactate, butyrate and propionate), which are further degraded to acetate and H_2/CO_2. In the last phase, acetate and H_2/CO_2 are converted into methane (Liua et al., 2001).

Anaerobic process is typically utilized as a stabilization technique to reduce the volume of waste activated sludge and produce bio-gas (Chu et al., 2005).

Anaerobic processes are also methanogenic, i.e. most of the carbon atoms originating in the waste material are reduced to methane (CH_4), the ultimate product of biological metabolism in anaerobic environments (Huges, 1979).

Methane production is a common phenomenon in several diverse natural environments ranging from glacier ice, sediments, marshes, rumen, and oil fields. The process is a combination of two phases namely acidogenesis and methanogenesis. Each of these steps is carried out by two groups of microorganisms which are acidogenes and methanogens respectively. In acidogenesis phase, soluble organic compounds are fermentized into volatile organic acids, CO_2 and H_2. Methanogenesis phase occurs in two ways. First way is decarboxylation of acetate and the other way is reduction of the CO_2.

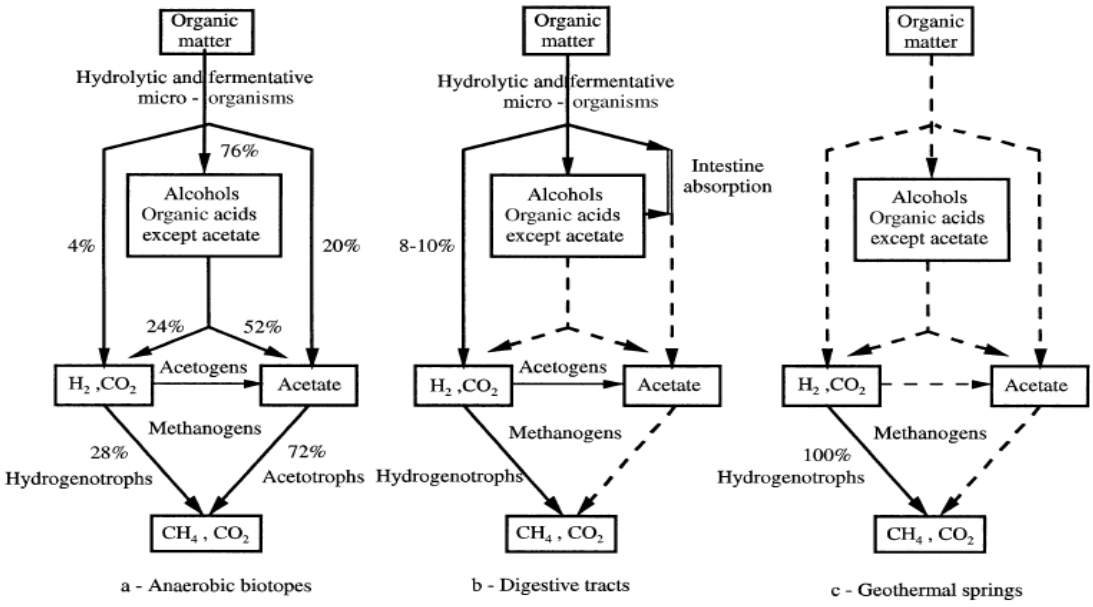


Figure 2.1. Schematic Diagram Showing the Three Different Methanogenic Ecosystems Operating in Nature (Garcia et al., 2000)

During methanogenesis, amount of energy is released relatively low compared to other terminal electron accepting processes. As a result of this, the amount of biomass produced is much less than that of other processes. Therefore, methanogenesis has been used as the treatment process for wastewater, sewage and other complex wastes because sludge yields are low and most of the energy in the original substrates is retained in the energy reach fuel, methane. Anaerobic treatment by methanogenesis is often a net energy producer, resulting in significantly lower operating costs compared to aerobic treatment (Lettinga, 1995). Although low cell yields associated with anaerobic treatment make it optional for wastewater treatment, it is also one of its main disadvantages because large reactor volumes and long retention times are needed to achieve the required treatment efficiency (McCarty, 1971). However, with recent developments in the field of environmental sciences on anaerobic treatment, the quality of the equipments used in the system, much cost-effective reactor configurations and operations are being achieved. This has allowed the description of the most sensitive steps in the process and the development of strategies to enhance operational stability of anaerobic treatment systems (Lettinga, 1995).

The biochemical steps of the anaerobic digestion have been modeled by various scientific researches such as Nine-stage Model (Harper and Pohland, 1986), Six-stage Model (Hutnen et al., 1999) and Three-stage Model (Gerardi, 2003). According to Gerardi (2003), anaerobic digestion could be considered as a three-stage process:

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

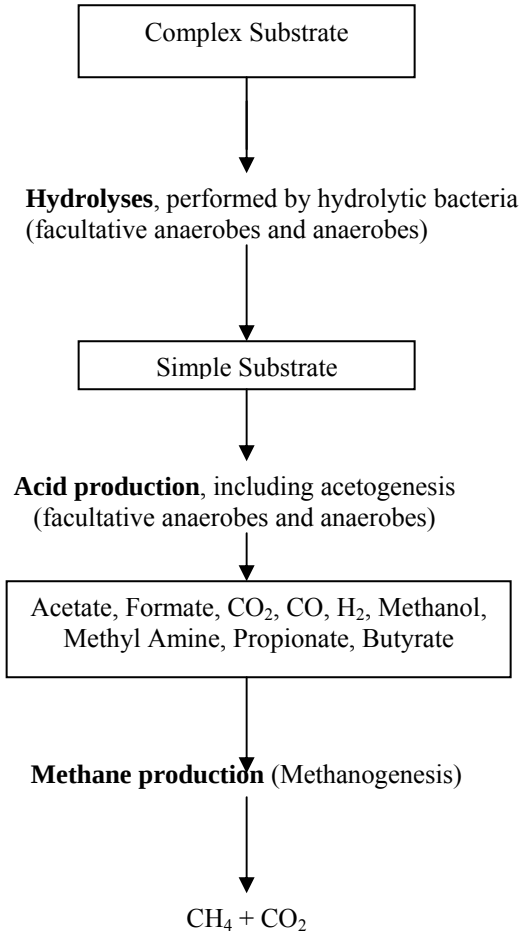


Figure 2.2. Three Stage Process Anaerobic Digestion (Gerardi, 2003)

In the six-stage model given by Stronach et al. (1986), biochemical reactions are classified into 6 parts which are given below.

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.

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 2.3.

1. Hydrolysis of organic polymers to intermediate organic monomers,
2. Fermentation of organic monomers,
3. Oxidation of propionic and butyric acids and alcohols by obligate H2 producing acetogens,
4. Acetogenic respiration of bicarbonate by homoacetogens,
5. Oxidation of propionic and butyric acids and alcohols by sulphate reducing bacteria (SRB) and nitrate reducing bacteria (NRB),
6. Oxidation of acetic acid by SRB and NRB,
7. Oxidation of hydrogen by SRB and NRB,
8. Acetoclastic methane formation,
9. Methanogenic respiration of bicarbonate.

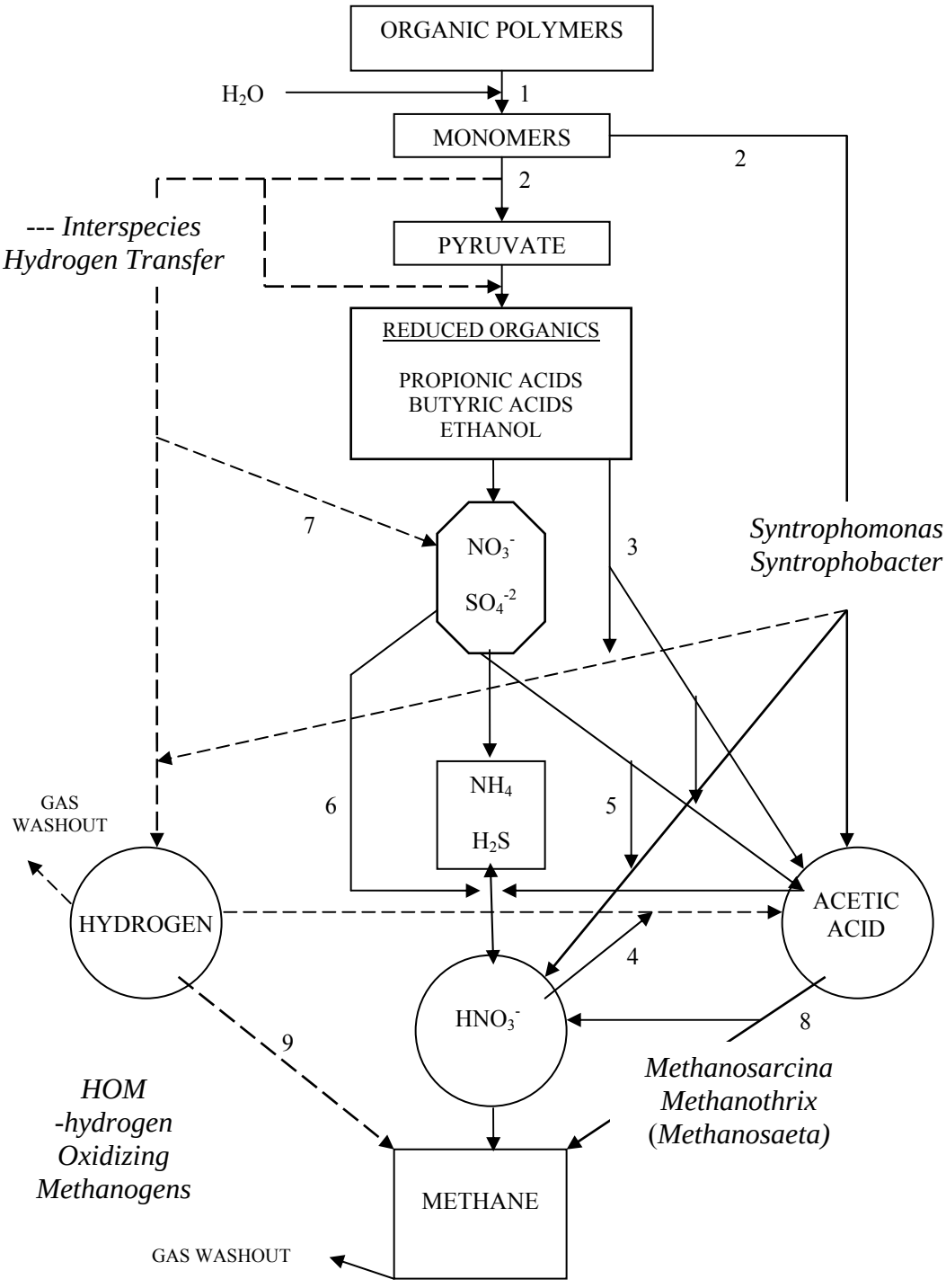


Figure 2.3. Substrate Conversion Patterns Associated with the Anaerobic Digestion (Harper and Pohland, 1986).

In the anaerobic digestion complex insoluble compounds such as particulate and colloidal wastes undergo hydrolysis. Particulate and colloidal wastes consist of carbohydrates, fats, and proteins. These wastes are polymeric substances; that is, large insoluble molecules consisting of many small molecules joined together by unique chemical bonds. The small molecules are soluble and quick go into solution once the chemical bonds are broken. 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 contrast to glucose and starch, the main problems encountered with the biological conversion of lignocellulose arise from its inaccessible structure. The sugar availability of polymers is low and, generally, hydrolysis of the cellulose and hemicellulose is the rate- limiting step. In addition to this structure-related property, the lignocelluloses must be free from contamination with heavy metals, pathogens, parasite eggs, xenobiotics, etc., to allow proper and safe bioprocessing (Claassen et al., 1999).

In the acid forming stage, soluble compounds produced through hydrolysis are degraded by a large diversity of facultative anaerobes and anaerobes through many fermentative processes. The degradation of these compounds results in the production of CO_2, H_2, alcohols, and volatile fatty acids which are acetic, butyric and propionic acids. This step is accomplished by acidogens. Second group of the acid forming step is acetogens. All volatile fatty acids are oxidized to acetic acid. This process is called ß oxidation.

In the last step, previous products are converted into methane and CO_2 by the *Archaea* called methanogens. Methanogenesis is the slowest and sensitive process and the specific environmental conditions are required for the growth of methanogens such as optimum pH and temperature.

2.2. Biochemistry and Microbiology of Anaerobic Digestion

During anaerobic degradation of organic matter in environments in which the availability of inorganic electron acceptors is limiting, organic material serves as both electron donor and electron acceptor, resulting in the production of CO_2 and methane. Anaerobic digestion process was first discovered in lake bottoms and swamps (Boone, 1991). From the end of the last century onwards, anaerobic digestion has been also applied in man-made environments for both energy production and as a cost-effective method for waste stabilization (Lettinga 1996; Lettinga 1999; Van Lier et al., 1998). The latter refers to the dual energy benefit of anaerobic digestion: no energy is required for stabilizing wastes. Quite the contrary, an energy-rich end-product is produced. The positive energy balance on one hand and the increasing energy demand on the other have generated a growing interest in anaerobic digestion. During anaerobic degradation, the chemical energy present in organic compounds is largely conserved as methane. If glucose is fermented in methanogenic fermentation the Gibbs free energy change under standard conditions is (ΔG_0) 404 kJ/mol. However, oxidation of the 3 mol methane formed per mol glucose will yield a (ΔG_0) of - 760.2 kJ/mol (Thauer et al., 1977). So in total:

$$\text{Glucose} \rightarrow 3HCO^- + 3CH_4 + 3H^+ \qquad -404 \text{ kJ} \qquad (2.1)$$

$$3CH_4 + 6O_2 \rightarrow 3CO_2 + 6H_2O \qquad -2280.5 \text{ kJ} \qquad (2.2)$$

From a technological point of view it is important to note that a complete methanogenic conversion occurs by mixed microbial communities yielding methane as the sole reduced organic product.

The anaerobic digestion of organic compounds to methane and carbon dioxide is a multistage process involving different physiological groups of microorganisms (Pretorius, 1994). However, according to Pfeffer (1979) the process can be considered as a three stage process in its simplest form.

Anaerobic digestion involves numerous interactions between four major metabolic groups that are generally accepted as present in anaerobic digesters; hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotophic methanogens, and acetoclastic methanogens (Chynowth and Pullammanappallil 1996; Zinder et al., 1984). These microorganisms possess a unique biochemistry which enables them to derive metabolic energy from the methanogenic pathway (Whitman et al., 1992; Thauer, 1998). Most of the described species of methanogens are rather specialized. *Methanobrevibacter spp.* is only able to use H_2 + CO_2 for growth, whereas *Methanosaeta spp*. only uses acetate as their energy substrate. *Methanosarcina sp*p. are more versatile; they can use H_2+CO_2, acetate, methanol, methylated amines and pyruvate for growth and methane production (Whitman et al., 1992; Jetten et al., 1992).As a consequence of the limited range of substrates utilised by methanogens, the anaerobic breakdown of organic matter is carried out by communities of different physiological types of anaerobic bacteria (Stams, 1994; Schink, 1997; Stams and Oude Elferink, 1997).

Figure 2.4 illustrates the different phases of the anaerobic digestion process. Biopolymers like polysaccharides, proteins, nucleic acids and fats are first hydrolysed by extracellular enzymes. The monomers and oligomers which are formed, such as sugars, amino acids, purines, pyrimidines and glycerol are fermented by a wide variety of different types of bacteria. The products that are formed include on the one hand hydrogen, formate and acetate, which in their turn can be converted by methanogens, and on the other hand propionate, butyrate and higher fatty acids. These higher fatty acids have to be anaerobically oxidized to methanogenic substrates prior to further conversion to methane and CO_2, but the (ΔG_0) of this conversion is highly positive.

In the first stage, a group of anaerobic microorganisms, primarily cellulolytic bacteria convert organic polymers to the individual monomers by hydrolysis.

These monomers are fermented to various intermediates, primarily acetate, propionate and butyrate by acidogenes. Additional acetate is produced by acetogenic bacteria. In the

acetogenesis stage which is probably the most important, acetic acid becomes the substrate for a group of strictly anaerobic methanogenic microorganisms. These microorganisms ferment the acetic acid to methane and carbon dioxide. This methane, along with the methane formed by microorganisms that reduce carbon dioxide by using hydrogen gas or formate produced by other species, accounts for the methane produced in this process .

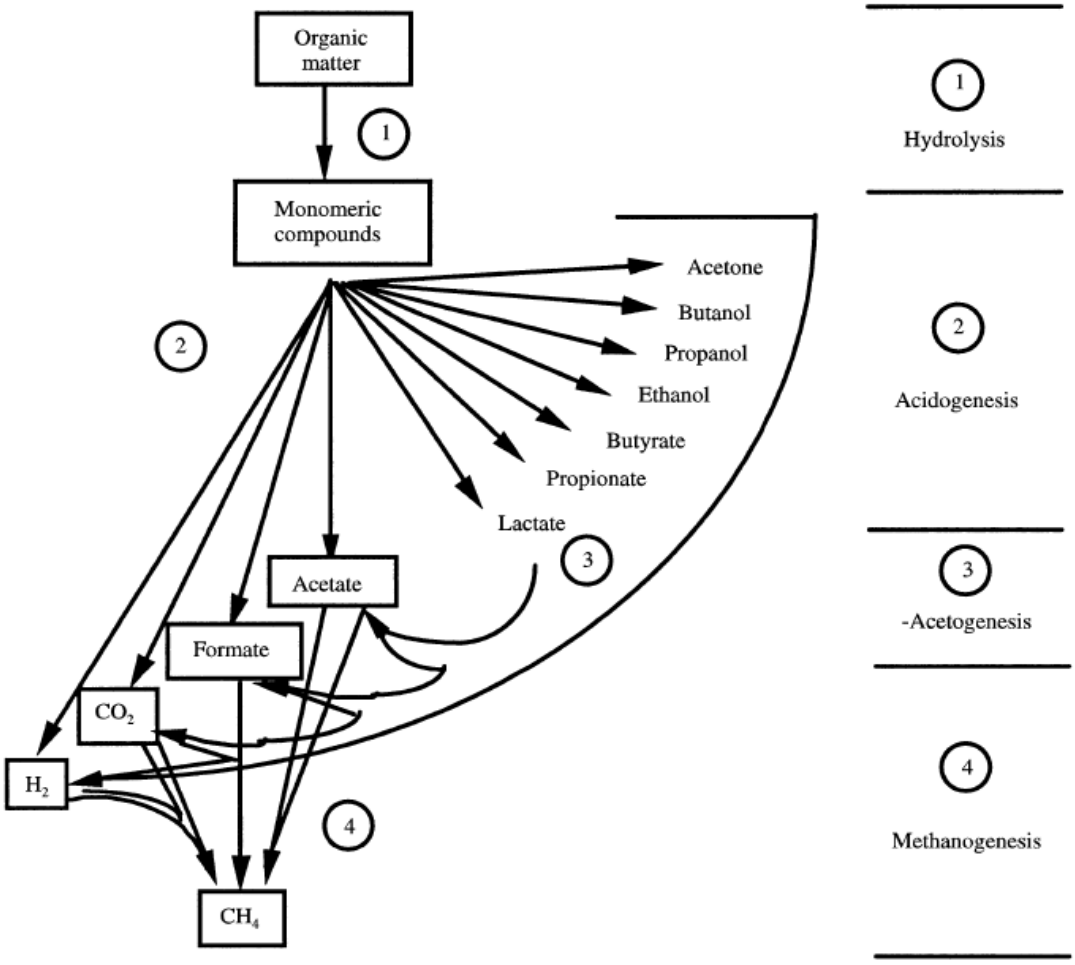


Figure 2.4. Schematic diagram showing anaerobic degradation of organic matter

(Garcia et al., 2000)

2.2.1. Hydrolysis

In methane fermentation of waste waters containing high concentrations of organic polymers, the hydrolytic activity relevant to each polymer is of paramount significance, in that polymer hydrolysis may become rate-limiting step for production of simpler bacterial substrates to be used in subsequent degradation steps. (Stronach et al., 1986). It was stated that Clostridium is responsible for degradation of compounds containing cellulose and starch while Bacillus play role in the degradation of proteins and fats (Lema et al., 1991; Noike et al., 1985).

Polymeric materials such as lipids, proteins, and carbohydrates are primarily hydrolyzed by extracellular, hydrolases, excreted by hydrolytic microorganisms. These 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). Hydrolytic enzymes, (lipases, proteases, cellulases, amylases, etc.) hydrolyze their respective polymers into smaller molecules, primarily monomeric units, which are then consumed by microbes.

Lipases convert lipids to long-chain fatty acids. A population density of 10^4 - 10^5 lipolytic bacteria per mL of digester fluid has been reported. Clostridia and the micrococci appear to be responsible for most of the extracellular lipase producers. The long-chain fatty acids produced are further degraded by p-oxidation to produce acetyl CoA.

Proteins are generally hydrolyzed to amino acids by proteases, secreted by *Bacteroides, Butyrivibrio, Clostridium, Fusobacterium, Selenomonas,* and *Streptococcus.* The amino acids produced are then degraded to fatty acids such as acetate, propionate, and butyrate, and to ammonia as found in *Clostridium, Peptococcus, Selenomonas, Campylobacter,* and *Bacteroides.*

Polysaccharides such as cellulose, starch, and pectin are hydrolyzed by cellulases, amylases, and pectinases. The majority of microbial cellulases are composed of three species: (a) endo-(3-l, 4-glucanases; (b) exo-p-l, 4-glucanases; (c) cellobiase or p-glucosidase. These three enzymes act synergistically on cellulose effectively hydrolyzing its crystal structure, to produce glucose. Microbial hydrolysis of raw starch to glucose requires amylolytic activity, which consist of 5 amylase species: (a) a-amylases (b) p-amylases (c) amyloglucosidases (d) debranching enzymes (e) maltase. Pectins are degraded by pectinases, including pectinesterases and depolymerases. Xylans are degraded with xylanase and xylosidase to produce xylose.

Hexoses and pentoses are generally converted to C_2 and C_3 intermediates and to reduce electron carriers (e.g., NADH) via common pathways. Most anaerobic bacteria undergo hexose metabolism via the Emden-Meyerhof-Parnas pathway (EMP) which produces pyruvate as an intermediate along with NADH. The pyruvate and NADH thus generated are transformed into fermentation endo-products such as lactate, propionate, acetate, and ethanol by other enzymatic activities which vary tremendously with microbial species.

2.2.2. Acidogenesis and Acetogenesis

Although some acetate (20%) and H_2 (4%) are directly produced by acidogenic fermentation of sugars, and amino acids, both products are primarily derived from the acetogenesis and dehydrogenation of higher volatile fatty acids.

Obligate H_2-producing acetogenic bacteria are capable of producing acetate and H_2 from higher fatty acids. Only *Syntrophobacter wolinii,* a propionate decomposer and *Sytrophomonos wolfei,* a butyrate decomposer have thus far been isolated due to technical difficulties involved in the isolation of pure strains, since H_2 produced, severely inhibits the growth of these strains. The use of co-culture techniques incorporating H_2 consumers such as methanogens and sulfate-reducing bacteria may therefore facilitate elucidation of the biochemical breakdown of fatty acids.

H_2 production by acetogens is generally energetically unfavourable due to high free energy requirements. However, with a combination of H_2-consuming bacteria co-culture systems provide favourable conditions for the decomposition of fatty acids to acetate and CH_4 or H_2S. In addition to the decomposition of long-chain fatty acids, ethanol and lactate are also converted to acetate and H_2 by an acetogen and *Clostridium formicoaceticum,* respectively.

The effect of the partial pressure of H_2 on the free energy associated with the conversion of ethanol, propionate, acetate, and H_2/CO_2 during methane fermentation. An extremely low partial pressure of H_2 (10^{-5} atm) appears to be a significant factor in propionate degradation to CH_4. Such a low partial pressure can be achieved in a co-culture with H_2-consuming bacteria as previously described.

Acetogenic microorganisms utilize mostly H_2 and CO_2 (Eq. 2.3), H_2O and carbon monoxide (CO) (Eq. 2.4), methanol and CO_2 (Eq. 2.5) and six-carbon sugars (Eq. 2.6) to produce acetate (Gerardi, 2003):

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad (2.3)$$

$$4CO + 2H_2O \rightarrow CH_3COOH + 2CO_2 \quad (2.4)$$

$$4CH_3OH + 2CO_2 \rightarrow 3CH_3COOH + 2H_2O \quad (2.5)$$

$$C_6H_{12}O_6 \rightarrow 3CH_3COOH \quad (2.6)$$

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. 2.6 or chemolithotrophically through the reduction of CO_2 to acetate with H_2 as electron donor given in Eq.2.7.

$$2H_2CO_3 + 4H_2 \rightarrow CH_3COOH + 4H_2O \quad (2.7)$$

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 2.8.

$$3CH_3CH_2OH + 2CO_2 \rightarrow 4CH_3COOH + 2H_2 \quad (2.8)$$

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. 2.9.

$$2C_3H_3O_3^- + 2H_2O \xrightarrow{\text{NADH} \Downarrow 4H^+} 2CH_3COO^- + 2CO_2 \quad (2.9)$$

Two molecules of CO_2 generated in Eq.2.9 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.

Thus, in hydrolysis and acidogenesis, sugars, amino acids, and fatty acids produced by microbial degradation of biopolymers are successively metabolised by fermentation endo-products such as lactate, propionate, acetate, and ethanol by other enzymatic activities which vary tremendously with microbial species.

2.2.3. Methanogenesis

Methanogenesis is a common and important process in many anaerobic environments, for example, in anaerobic digesters (Raskin et al., 1994), cattle rumen (Miller et al., 1986), rice fields (Joulian et al., 1998), oil wells (Ollivier et al., 1997), landfills (Fielding et al., 1988), and a range of extreme habitats (Garcia et al., 2000). They play important roles in anaerobic treatment of organic wastes, formation of biogas as an alternative source of energy (Cicerone, R. J., and R. S. Oremland (1988)), generation of CH_4 as a greenhouse gas (Hayhoe et al., 2002) polluted environments. In the absence of other electron acceptors such as oxygen, nitrate, and sulfate, methanogens are involved in the terminal anaerobic breakdown of organic matter (Garcia et al., 2000). Anaerobic microorganisms are not able to directly use the organic wastes as energy sources. They catabolically rely on a restricted number of simple compounds, e.g., on CO_2 as oxidant with H_2 as electron donor or on acetate, methanol or formate (Zinder, 1993). Hence, they depend on other organisms such as fermenting or sulfate-reducing bacteria for their substrates.

Methanogenic microorganisms all belong to the domain *Archaea* with different physiological types mostly belonging to different phylotypes (Zinder, 1993). For example, while most species of the *Methanobacteriaceae* and *Methanomicrobiaceae* prefer H_2 and CO_2 (or formate) as substrates for methanogenesis, *Methanosaeta*, a genus within the *Methanosarcinaceae*, is known to generate energy only from acetate fermentation. Most of the other *Methanosarcinaceae* can use methanol and related substrates for the generation of CH_4.

Table 2.1. Substrates Converted to Methane by Various Methanogenic *Archaea* (Madigan et al., 2002)

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

Methanogens are physiologically united as methane producers in anaerobic digestion. Although acetate and H_2/CO_2 are the main substrates available in the natural environment, formate, methanol, methylamines, and CO are also converted to CH_4.

$$CO_2+4H_2 \rightarrow CH_4+ 2H_2O \qquad \Delta G°= 131 \text{ kJ} \qquad (2.10)$$

Since methanogens, as obligate anaerobes, require a redox potential of less than -300 mV for growth, their isolation and cultivation was somewhat elusive due to technical difficulties encountered in handling them under completely O_2-free conditions. However, as a result of a greatly improved methanogen isolation techniques developed by Hungate more than 40 strains of pure methanogens have now been isolated. Methanogens can be divided into two groups: H_2/CO_2- and acetate-consumers. Although some of the H_2/CO_2-consumers are capable of

utilizing formate, acetate is consumed by a limited number of strains, such as *Methanosarcina spp*. and *Methanothrix spp*. (now, *Methanosaeta),* which are incapable of using formate. Since a large quantity of acetate is produced in the natural environment, *Methanosarcina* and *Methanosaeta* play an important role in completion of anaerobic digestion and in accumulating H_2, which inhibits acetogens and methanogens. H_2-consuming methanogens are also important in maintaining low levels of atmospheric H_2.

H_2/CO_2-consuming methanogens reduce CO_2 as an electron acceptor via the formyl, methenyl, and methyl levels through association with unusual coenzymes, to finally produce CH_4. The overall acetoclastic reaction can be expressed as:

$$CH_3COOH \rightarrow CH_4 + CO_2 \qquad (2.11)$$

Since a small part of the CO_2 is also formed from carbon derived from the methyl group, it is suspected that the reduced potential produced from the methyl group may reduce CO_2 to CH_4.

2.3. Characteristics of Methanogens

Methanogens are strict anaerobes which share a complex biochemistry for methane synthesis as part of their energy metabolism. The discovery of the unique biochemical and genetic properties of these organisms led to the concept of Archaebacteria at the end of the seventies and the proposal in 1990 for the domain *Archaea.*

Methanogens, the first *Archaea* was sequenced and studied in some methane as their final product (Woese and Wolfe, 1985). Methanogens are oxygen-sensitive, fastious anaerobes and free-living terrestrial and aquatic organisms. According to Gerardi (2003), the oxygen sensitivity of methanogens is not disadvantageous because they are found in contain high degradable organic environments. In these habitats, oxygen is rapidly removed by microbial activity.

Methanogens have unrigid cell wall and unique cell membrane lipid. They can degrade substrates such as organic wastes and produce methane by their specialized coenzymes. Coenzymes that are unique to methane forming microorganisms are coenzyme M and the nickel containing coenzymes. Coenzyme M is used to reduce CO_2 to methane. The nickel-containing coenzymes are important hydrogen carriers in methanogens.

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

Methanogenic *Archaea* has 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 2.2.

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

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

2.3.1. Taxonomy of methanogens

Woese et al. (1990) proposed a new classification for living organisms, dividing life on earth into three major domains: Bacteria, *Archaea* and Eukarya (Figure 2.5).

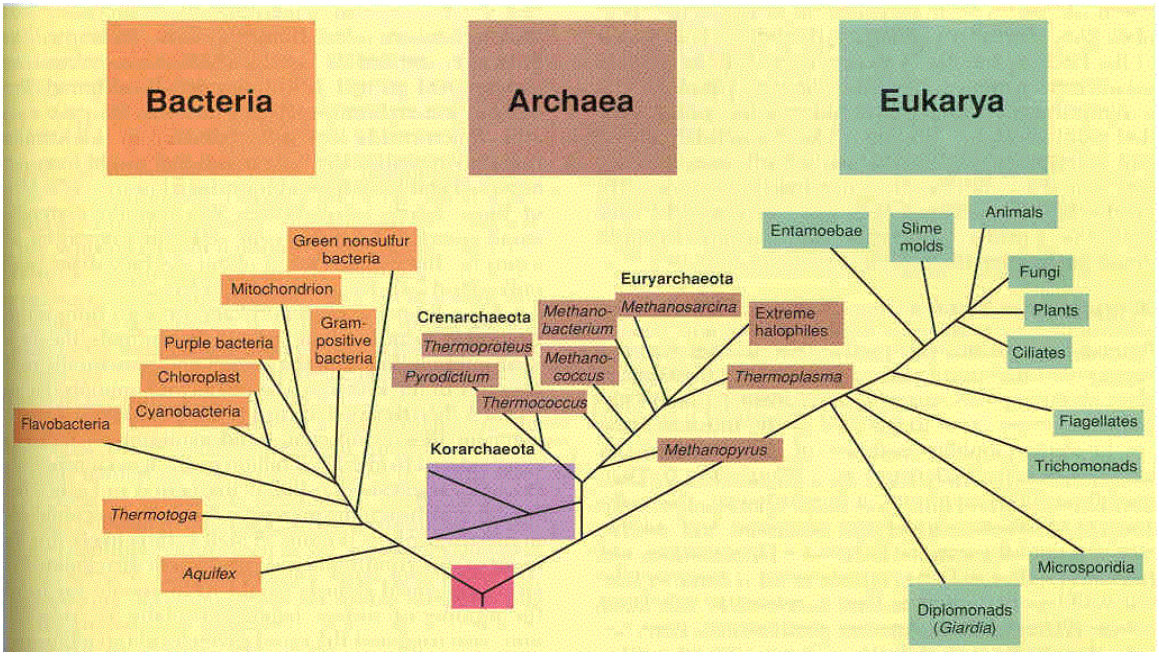


Figure 2.5. Universal phylogenetic trees (Madigan et al., 2002).

Methanogens were categorized together with nonmethanogens based on morphological properties. Recognition of methanogens physiological unity, their taxonomy was reorganized by Bryant (1974). *Archaea* was one of the major phylogenetic groups. Even though they had similar characteristics to the bacteria, not only their phenotypical characteristics but also their phylogenetic characteristics were different. They were 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).

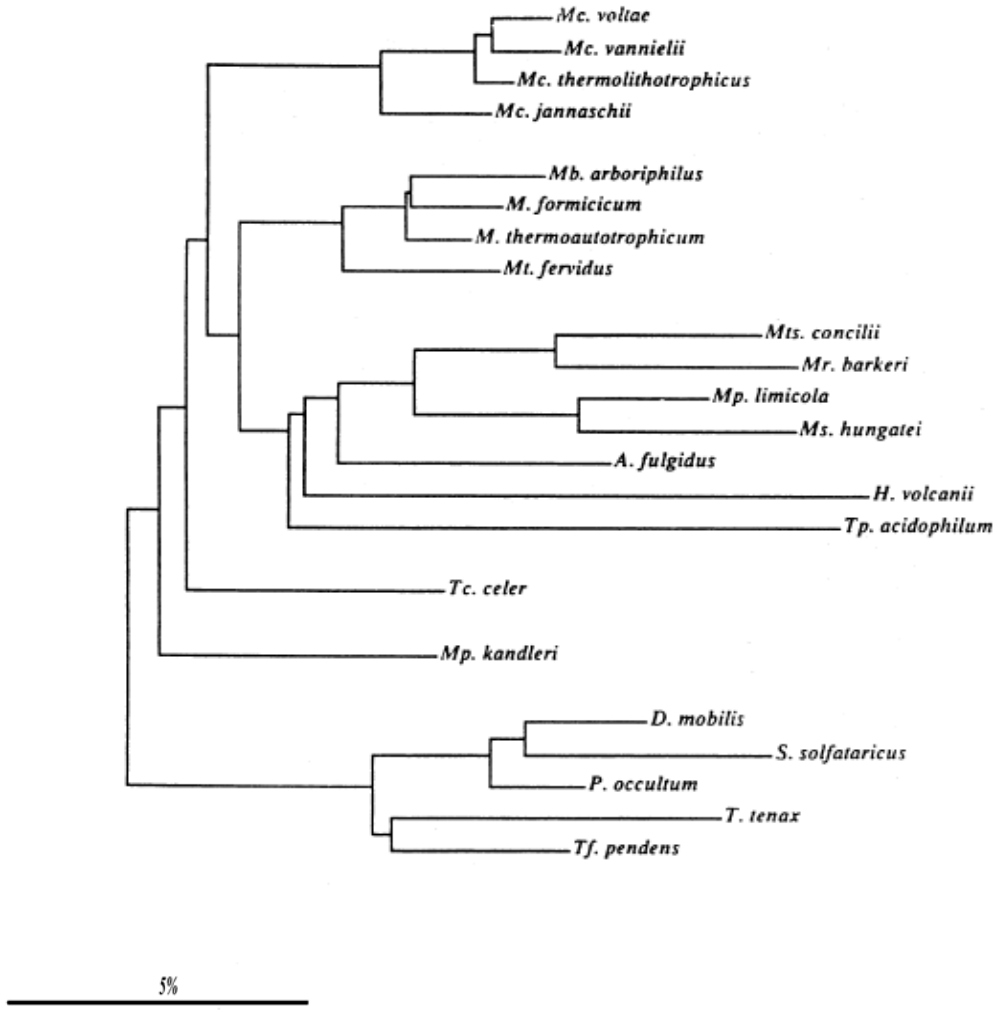


Figure 2.6. Representative phylogenetic tree for *Archaea* based upon 16S rRNA sequences. The scale bar measures five nucleotides changes per 100 residues.

The analysis of phylogenetic markers such as the 16S rRNA gene may efficiently complement traditional microbiological analysis by distinguishing different phylogenetic groups of prokaryotes (Rizzi et al., 2006). On the basis of phylogenetic information derived from comparative 16S rRNA analysis provides real information about phylogenetic relationships. All cells have rRNA which has specialized 16S region of each species or order. Not only 16S region but also 23S and 18S rRNA region provide enough genetic information to classify organisms.

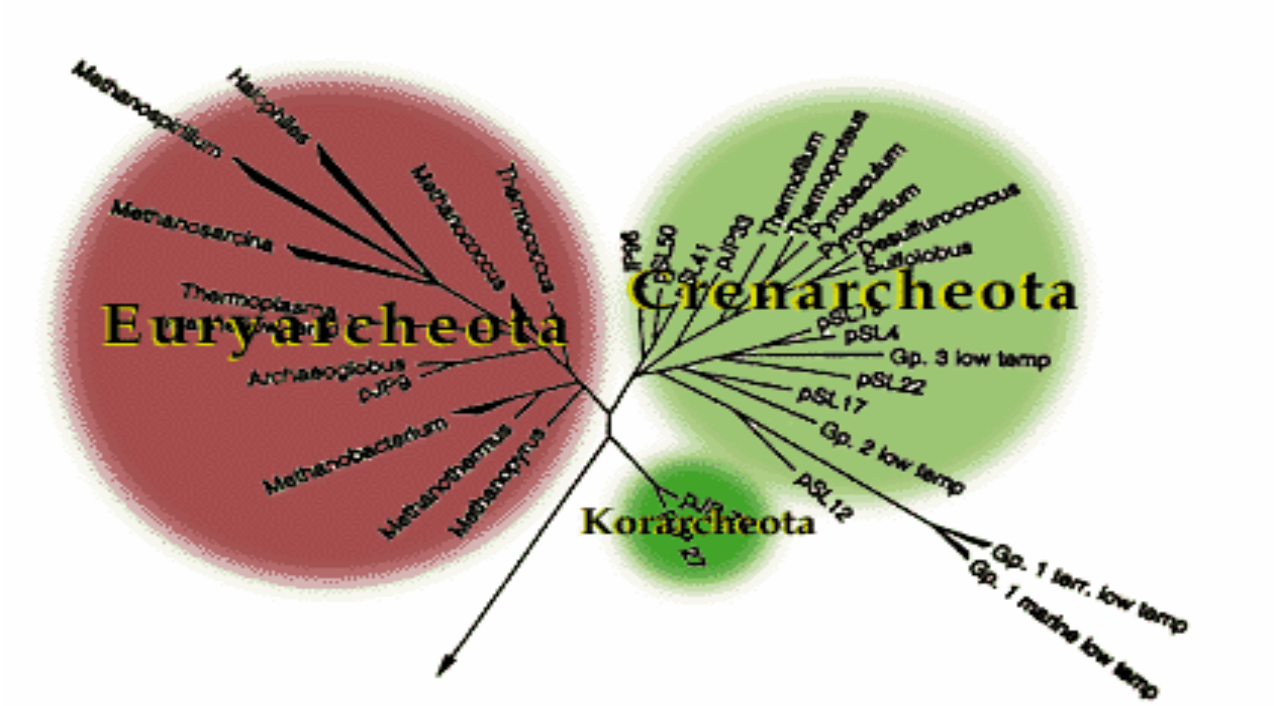


Figure 2.7. Major lineages of *Archaea*: Crenarchaeota, Euryarchaeota and Korarchaeota

The first kingdom, Crenarchaeota derived from being phylogenetically close to ancestor or source of *Archaea* (Woese et al., 1990).

The Euryarchaeota is a heterogenous group comprimising a broad spectrum of organisms with varied patterns of metabolism from different habitats. It includes extreme halophiles, methanogens, and some extreme thermophiles so far (Madigan et al., 2002).

Moreover, a third archaeal kingdom has recently been discovered which is reported isolation of several archaeal sequences evolutionary distant from all *Archaea* known to date by Barns et al. 1994 and then in 1996. The new group was placed on phylogenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota (Madigan et al., 2002).

Phylogenetically methanogens are divided into five orders (Lange and Ahring, 2001, Garcia et al., 2000).

1. The Methanobacteriales,
2. The Methanococcales,
3. The Methanosarcinales,
4. The Methanophyrales,
5. The Methanomicrobiales.

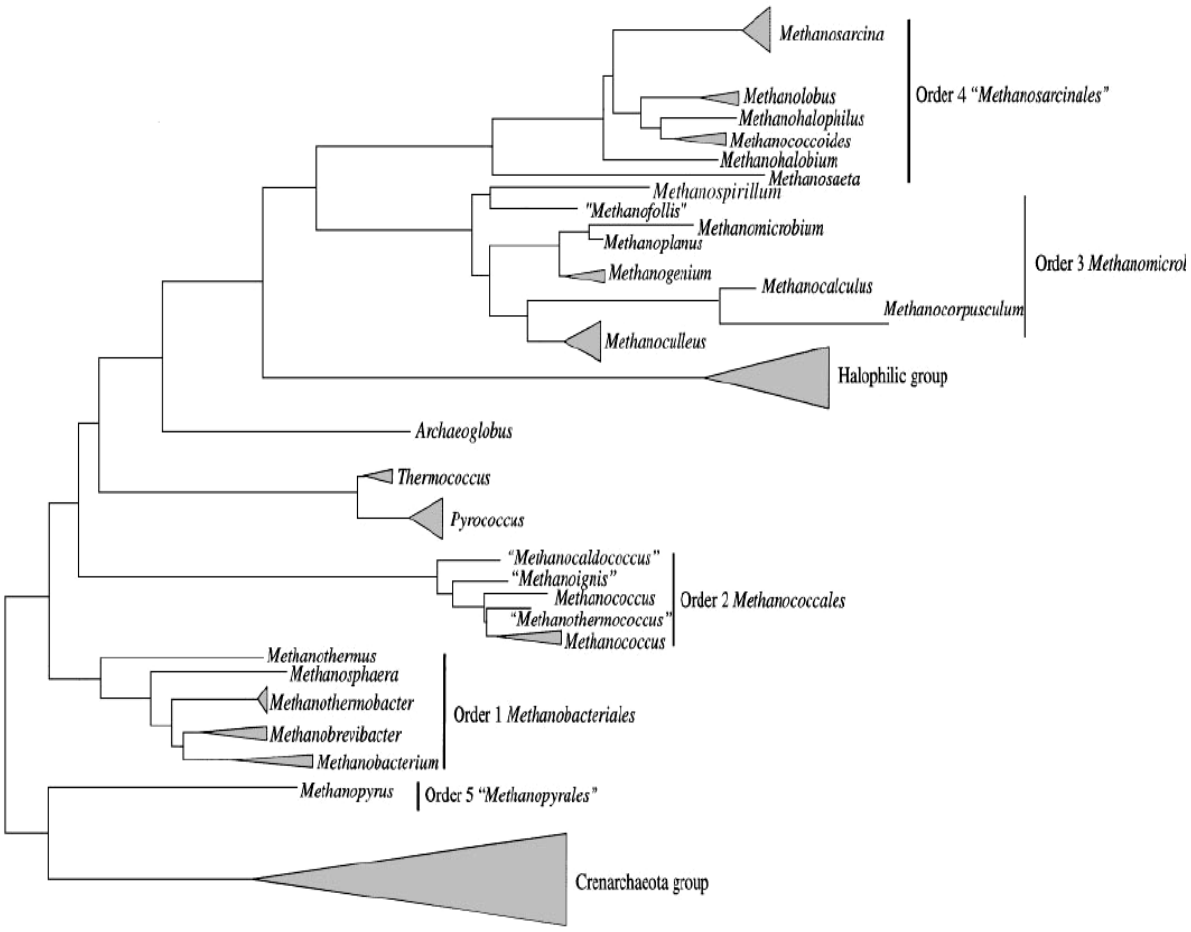


Figure 2.8. Updated phylogeny of methanogens, domain *Archaea.*

Figure 2.8 showed updated phylogeny of methanogens. Genus and family names shown in inverted commas identify changes proposed by Boone et al. Non-methanogens are indicated by their group names (large triangles) in the figure. *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).

2.3.2. Identification of Methanogens by Classical Methods

The ecology of microbial populations and communities in natural and engineered anaerobic systems remains largely unexplored. For example, complete identification and quantification of all contributing populations in complex anaerobic systems, which are needed to establish the link between microbial structure and function (i.e., metabolic activity), have not yet been achieved.

Conventional analysis of microbial communities depends on culture techniques. However, a limitation of the culture techniques is that it is possible that only a fraction of the bacteria present is detected because of cultivation bias. Furthermore, the presence in wastewater treatment systems of bacterial cells, which retain a certain detectable activity despite showing an extremely low level of culturability when conventional cultivation methods are used, was suggested.

The lack of studies rigorously connecting microbial structure and function is, at least in part, due to the limitations of traditional identification and enumeration techniques, such as selective enrichment, pure-culture isolation, most-probable-number estimates and determinative identification schemes. The major limitations of these traditional techniques are that only a relatively small fraction of the microorganisms making up a natural community can generally be cultured, and those that can be cultured are often difficult to identify. These problems are exacerbated in studies of fastidious anaerobes; because of their low growth rates

and obligate anaerobiosis, methanogens are among the microorganisms that are most difficult to study by culture-based techniques (Raskin et al., 1994).

Selective enrichment methods can also be used, however, before applying this method, physiological properties of the microorganisms 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.

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).

Morphology and ultrastructure have been used extensively in scanning or transmission electron microscopy studies to show the location of certain microorganisms' anaerobic granules by Macleod et al., (1990) and Morgan et al., (1991). Information gained from morphology-based studies is, however, ambiguous and limited since most microorganisms are small in size and simple in morphology and ultrastructure (Ahring, 2003).

2.3.3. Molecular Identification Methods

The drawbacks of the existing conventional methods, such as incomplete knowledge about their physiological (nutritional and physical–chemical) needs and the complex syntrophic and symbiotic relations, which are abundant in nature, make it impossible to obtain pure cultures of most microorganisms in natural environments. Moreover, most culture media tend to favor the growth of certain groups of microorganisms, whereas others that are

important in the original sample do not proliferate. It is therefore generally accepted nowadays that the number of known prokaryotic species (including the two domains: Bacteria and *Archaea*) is very small compared to the diversity of microorganisms and illustrates how difficult it is to get a full picture of the bacterial diversity of an ecosystem by relying only on conventional methodology (Sanz and Köchling, 2006).

The diversity of methanogenic *Archaea* in the environment may be monitored by using molecular methods such as fluorescence in situ hybridization (FISH) and Quantitative-Polymerase Chain Reaction (Q-PCR). For example, using FISH, Ficker et al., (1999) found that 17% of total microorganisms in a toluene-degrading enrichment culture hybridized with a *Methanosaeta*-specific hybridization probe and 2% hybridized with a *Methanospirillum*-specific probe.

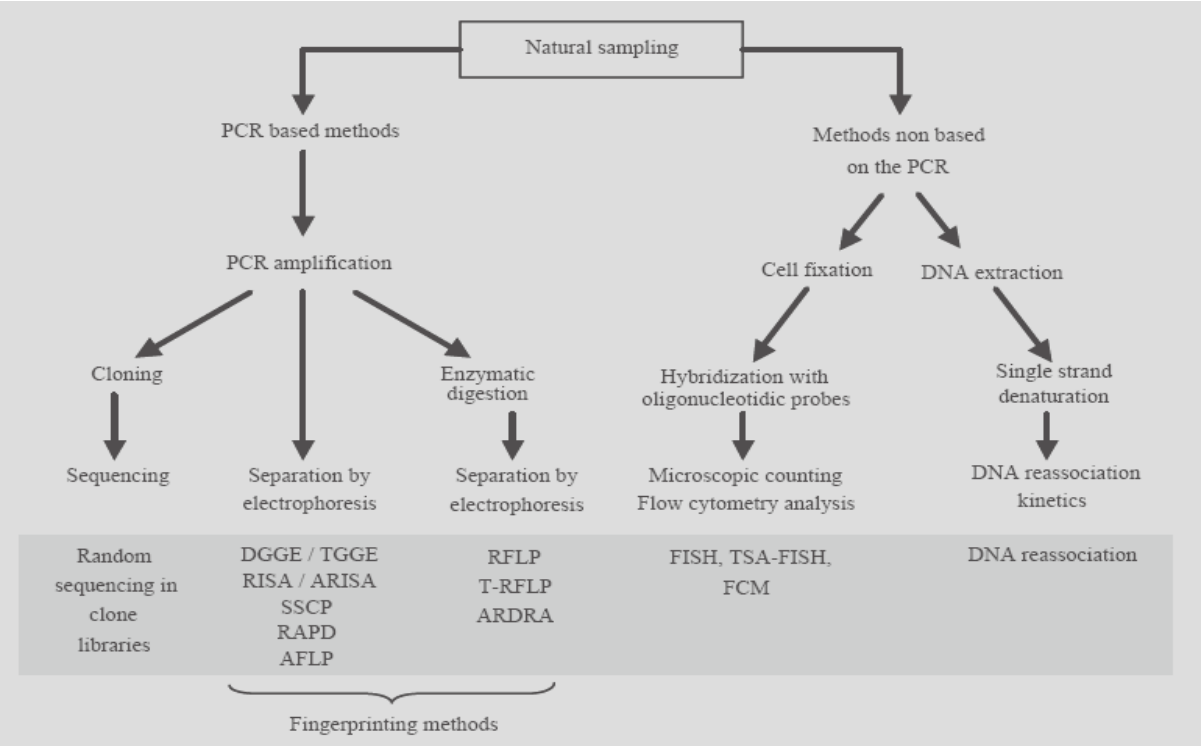


Figure 2.9. Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities. (Doriga et al., 2005).

Methods based on analyses of nucleic acids allow studying a wide range of microorganisms as they occur in nature without cultivation. The molecular-phylogenetic framework provided by comparative rRNA sequencing has given a basis for describing the community structure of natural microbial communities at the level of populations and even single cells without the limitations imposed by pure-culture isolation and biochemical identification. For example, oligonucleotide hybridization probes and PCR primers have been designed to identify individual species or members of phylogenetically coherent groups with either variable or conserved tracts of the rRNA sequence as hybridization targets.

Around 75% of the approximately 2000 anaerobic treatment systems presently in operation world wide correspond to Upflow Anaerobic Sludge Bed (UASB) reactors or to new configurations based on the same principle (EGSB, IC). In all of them, microorganisms form compact aggregates up to 2–4 mm in diameter, granular sludge, with high sedimentation velocity and high methanogenic activity (Diaz et al., 2003). Due to the huge structural complexity of granular sludge and the many trophic interactions among the microbial populations required to transform complex organic matter into biogas (CO_2 and CH_4), the microbial ecology (taxonomy, colonization, topological distribution of microbes, etc.) of this microecosystem is still not well understood.

Molecular ecology techniques, such as 16S rRNA gene cloning and sequencing (Amann et al., 1995), FISH (Amann, 1995; Amann et al., 1995), and Denaturing Gradient Gel Electrophoresis (DGGE) (Muyzer et al., 1993; Muyzer and Smalla, 1998), are nowadays the most powerful tools available to assess the diversity, abundance and distribution of microorganisms in natural and engineered ecosystems, superceding the restrictions and bias of conventional microbiology techniques (isolation, plate-counting, etc.) (Amann et al., 1995, Pace, 1997).

2.3.3.1. PCR-based molecular tools for the assessment of microbial diversity. Polymerase chain reaction is the first step for these tools. The amplification of the DNA is achieved by polymerase chain reaction (PCR). The choice of primers makes it possible to target the

sequence at different taxonomic levels (strain, species, genus, etc.). The final PCR products obtained contain a mixture of multiple copies of the same fragment amplified at the chosen taxonomic level (strain, species, genus, etc.).

Random sequencing in clone libraries: PCR products are cloned at the random sequencing in clone library then these clones are carried out random sequencing within the clone library. Identification of the dominant copies present in the initial PCR products is possible using sequence analysis. Comparing these sequences with the available in sequence databases (GenebankTM or EMBL) gives information about the identity or relatedness of the new sequences to known species.

Denaturing gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE): Denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE) have been used to assess the diversity of microbial communities, and to monitor their dynamics (Muyzer, 1999; Muyzer et al., 1996; Muyzer and Smalla, 1998). They typically involve amplifying the genes encoding the 16S rRNA and then, separating these fragments in a polyacrylamide gel. DGGE/TGGE exploits the fact that DNA molecules that have the same length, but differ at least by one nucleotide, can be separated by electrophoresis through a linear gradient of increasing chemical denaturants of urea and formamide (DGGE), or through a linear temperature gradient (TGGE). DGGE/TGGE approaches have been used in a huge number of studies of eubacterial, archaeal and eukaryotic communities in freshwater and coastal waters in relation to herbicide exposure and/or spatio-temporal variations (Øvreås et al., 1997; van Hannen et al., 1998; El Fantroussi et al., 1999; Casamayor et al., 2000, 2001, 2002; Lindström, 2000; Riemann and Middelboe, 2002; Zwart et al., 2002; Lyautey et al., 2003; Schauer et al., 2003).

Single-strand conformation polymorphism (SSCP): Sequence variations among DNA fragments, which are usually PCR-amplified, 16S rRNA gene sequences, are detected using single-strand conformation polymorphism analysis (SSCP). SSCP was originally described by Orita et al. (1989), and was first used to assess the diversity of natural microbial communities

by Lee et al. (1996). A three-dimensional conformation determined by the intramolecular interactions that influence their electrophoretic mobility in a non-denaturing polyacrylamide gel will be adapted using single-stranded DNA at low temperatures. Ross et al. (2001) and Wenderoth et al. (2003) monitored changes in groundwater microbacterial communities resulting from various strategies of bioremediation of polluted aquifers by this method.

Terminal-restriction fragment length polymorphism (T-RFLP): T-RFLP analysis is a community fingerprinting technique that is based on the restriction digest of double-stranded fluorescently end-labeled PCR fragments (Liu et al., 1997; Marsh, 1999). In addition, phylogenetic assignments can be inferred from the sizes of the terminal restriction fragment (TRF) using web-based resources that predict T-RF sizes for known bacteria (Kent et al., 2003). T-RFLP analysis has been used to compare the dynamics both between and within microbial populations in soils and activated sludge (Bruce, 1997; Liu et al., 1997; Marsh et al., 1998; Moeseneder et al., 1999; Osborn et al., 2000), but there are also some recent publications concerning aquatic ecosystems (Inagaki et al., 2002; Takai et al., 2002; Vetriani et al., 2003).

2.3.3.2. Non based-PCR molecular techniques for assessing microbial diversity. *Fluorescence in-situ hybridization* (FISH): Hybridization was widely used in environmental microbiology studies (De Long, 1992; Raskin et al., 1994; Wagner et al., 2003). The fluorescence in-situ hybridization (FISH) using rRNA-targeted fluorescent probes were commonly used in microbiology to investigate overall taxonomic bacterial compositions communities or assemblages. Probes could be designed to be complementary to species, group, or kingdom-specific target sites. Cells were fixed to make them permeable to the probe, which then hybridizes its specific target site.

In FISH, initially cells were fixed by using fixatives such as ethanol and PFA. After fixation, most microorganisms were permeable to short oligonucleotide probes (Amann et al., 1990). Fluorescently labeled probes penetrate to cell wall and hybridize with thousand copies of rRNA. Cells incorporated with sufficient dye were visualized by fluorescence microscopy.

Quantification of the cells was possible by this technique. D. Zheng and L. Raskin (2000) quantified *Methanosaeta* species by FISH in anaerobic bioreactors. At the same time, the microbial community dynamics could be analyzed by FISH (Fernandez et al., 1999). By chancing the environmental factors, dominant members of the community could be monitored via FISH. Harmsen et al. (1996) applied FISH to identify syntrophic propionate-oxidizing bacteria, and this study revealed the distribution of bacteria and methanogens in anaerobic granular sludge systems resolved the phylogenetic affiliation and localization of important microbial populations in a full-scale UASB reactor treating brewery wastewater.

One of the disadvantages of FISH was the specificity of probes. Currently, most of the group-specific probes were designed on the basis of sequences of cultured organisms. It was possible to overlook some groups such as recently uncovered wealth of archaeal diversity. Various papers reported use of this method to explore bacterial communities in activated sludge, marine and freshwater environments, and in both pristine and contaminated aquifers, the structure of the floc in anaerobic system (Batston, Keller, Blackall, 2004, Wagner et al., 1993; DeLong et al., 1992; Borneman et al., 1996).

DNA re-association analysis: This technique was used for whole DNA comparisons between two communities, or for studying the sequence variety of a single community. In both cases, the total DNA was extracted and purified. When comparing two communities, the DNA of one community may be radioactively labeled and used as a template. Crosshybridization between the two DNA samples was then carried out, and the degree of similarity was monitored. This technique has been used by Torsvik et al. (1990) and Øvreås et al. (1998) to evaluate biodiversity in aquatic communities.

2.4. Environmental Factors Affecting Anaerobic Treatment Processes

It is often assumed that the rate limiting steps of the anaerobic treatment process and methane yield are determined by the efficiencies of depolimerization (Chynowth and Pullammanappallil, 1996; Eastman and Ferguson, 1981). The low growth rate of

methanogenic bacteria can make the anaerobic system sensitive to environmental changes (Xing et al., 1997) and disturbances in populations from one trophic level may affect the entire community (Raskin et al., 1996). By removing the metabolic products of syntrophic acetogens, methanogens play a regulative role in maintaining the overall efficiency of the process.

2.4.1.Temperature

Methane production has been documented under a wide range of temperatures. In municipal wastewater treatment plants, anaerobic digestion is carried out in the mesophilic range at temperatures from 25°C [77°F] to up to 40°C [104°F] with the optimum at approximately 35°C [95°F]. Thermophilic digestion operates at temperature ranges of 50-65°C [122°F-149°F]. It allows higher loading rates and is also conductive to greater destruction of pathogens. One drawback is its higher sensitivity to toxicants. Because of their slower growth as compared with acidogenic bacteria, methanogenic bacteria are very sensitive to small changes in temperature, which leads to a decrease of the maximum specific growth rate while the half-saturation constant increases. Thus, a mesophilic digester must be designed to operate at temperatures between 30°C [86°F] and 35°C [95°F] for their optimal functioning.

2.4.2. Retention Time

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

2.4.3. pH

Most methanogenic bacteria function in a pH range between 6.7 and 7.4, but optimally at pH 7.0-7.2 and the process may fail if the pH is close to 6.0. Acidogenic bacteria produce organic acids, which tend to lower the pH of the bioreactor (Malina and Pohland, 1992). Under normal conditions, this pH reduction is buffered by the bicarbonate that is produced by methanogens. Under adverse environmental conditions, the buffering capacity of the system can be upset, eventually stopping the production of methane. Acidity is inhibitory to methanogens than of acidogenic bacteria. An increase in volatile acid levels thus serves as an early indicator of system upset. Monitoring the ratio of total volatile acids (as acetic acid) to total alkalinity (as calcium carbonate) has been suggested to ensure that it remains below 0.1.

Table 2.3. 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	7.0-7.5
Methanococcoides	7.0-7.5
Methanohalobium	6.5-7.5
Methanolobus	6.5-6.8
Methanothrix	7.1-7.2

2.4.4. Toxicants

A wide range of toxicants is responsible for the occasional failure of anaerobic digesters. Inhibition of methanogenesis is generally indicated by reduced methane production and increased concentration of volatile acids.

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

Increasing the VFA concentrations and carbon dioxide concentrations cause decreasing the pH, gas production and methane content.

2.4.4.1. Sulfide Inhibitions. Introduction of the waste streams and/or the biological production in the anaerobic digestion may cause the sulfides via reduction of sulfates or other sulphure-contaioning inorganic compounds. Anderson et al. (1986) found that sulphate in the influent of an anaerobic digester could inhibit methanogenesis due to both the competition for acetate and hydrogen by SRBs and the production of sulfide from sulphate reduction by SRBs. While soluble sulfide concentrations between 50 and100 mg/L can be tolerated in anaerobic treatment with slightly or no acclimation, higher than 200 mg/L soluble sulfides does not show a significant inhibitory effect after some acclimation. Stronache et al. (1986) stated that sulphate concentrations in excess of 200 mg/L had a direct toxic effect on anaerobic systems.

2.4.4.2. Ammonia-Nitrogen Inhibition. Although ammonia is an important buffer in an anaerobic treatment high concentrations of ammonia may cause failure in the system

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

2.4.4.3. Volatile Fatty Acids (VFA) Inhibition. Anaerobic reactor effluents contains, low concentrations of higher fatty acids however it contains higher concentrations of mainly acetic acid, propionic and butyric acids. Studies show that two important fermentation types occur

complementary to each other. These two types of fermentations are butyric and propionic acid. During butyric acid fermentation butyrate, acetate, hydrogen and CO_2 are produced, while propionic acid type fermentation produces propionate, acetate and some valerate, with no significant gas production (Dinopolou et al., 1988).

The most common inhibition that inhibits the system in the anaerobic reactor is the accumulation of VFA produced by acidogenic bacterial culture. Inhibition is identified by its high accumulation of VFA is the system which is an indicator of failure of methanogenic population. This failure might be caused by negative impact of bad environment conditions namely shock loading, nutrient depletion or infiltration of inhibitory substances. High concentrations of VFA (i.e.; butyric and propionic acid) in a system is making toxic impact on the microorganisms in the reactor. It is reported that (Ionnati and Fisher, 1983) inhibition of microbial growth was observed at 35 mg/L acetic acid and excess of 3000 gm/l propionic acid concentrations. The same researchers indicated that butyrate has a toxic effect at 1000 mg/L concentrations minimum. The inhibition of VFA at acidic medium can be attributed to the existence of unionized VFA in significant quantities in the system (Andrew, 1969).

When the pH value drops, the equilibrium go to the left causing the increasing of unionized VFAs. Krocker (1979) reported that reactor failure can be generally expected at the concentrations above 10 mg/L of unionized acids.

2.4.4.4. Heavy Metal Inhibition. Heavy metal may cause toxic effect on anaerobic processes which are influenced by the oxidation–reduction potential, pH and ionic strength and the resultant speciation of the metals or metal complexes. Low but soluble concentrations of copper, zinc and nickel salts are toxic.

Mosey and Hughes (1975) stated that heavy metal ions inhibit metabolisms and kill organisms by inactivating their certain enzymes. However, existence of the heavy metals in trace amounts is essential for the bacterial activity.

2.4.4.5. Anthropogenic and Recalcitrant Compounds Inhibition. Some industrial effluents such as dye manufacturing contain high levels of aromatic and other complex organic compounds including insecticide surfactants and polymers.

Some chlorinated aromatic compounds can be degraded by anaerobic microorganism. As a result methanol is produced and it was stated that 11 species of methanogens can grow on methanol (Madigan et al., 2000).

2.4.5. 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 bacteria (Stronach et al., 1986).

2.4.6. Mixing

Mixing ensures the absolute contact between the reactor contents and the biomass. It is also minimizes the inhibitory effects of local build-up of VFAs and other digestion products. Another advantage of mixing is that it avoids settling which could lead to reduction of substrate and microorganism contact. Mixing could be energy consuming process but it is applied most of treatment systems.

2.5. Anaerobic Reactor Types

Biological treatment process could be simply anaerobic or aerobic. Anaerobic treatment is an engineered design that is used to describe waste treatment processes. Anaerobic treatment systems are mostly preferred in terms of several advantageous such as high efficiency, lower excess sludge and biogas production. Reactor configurations and unites should be designed to achieve best treatment. Reactor types are very important for cost efficiency, start-up, maintenance, management, and effluent quality. Reactors types for an anaerobic system are listed below such as:

Expanded granular sludge bed
Upflow anaerobic sludge blanket
Anaerobic fluidized bed reactor
Hybrid reactor

2.5.1. Expanded Granular Sludge Bed

An EGSB reactor is a variant of the UASB concept (Kato et al., 1994). The distinguishing feature is that a faster rate of upward-flow velocity is designed for the wastewater passing through the sludge bed. The use of effluent recirculation in a UASB (or a high height/diameter ratio) results in the EGSB reactor (Seghezzo et al., 1998). The higher upflow liquid velocity keeps the granular sludge bed in an expanded condition (Zoutberg and Frankin, 1996). The increased flux permits partial expansion (fluidization) of the granular sludge bed, improving wastewater-sludge contact as well as enhancing segregation of small inactive suspended particle from the sludge bed. The increased flow velocity is either accomplished by utilizing tall reactors, or by incorporating an effluent recycle (or both). The EGSB design is appropriate for low strength soluble wastewaters (less than 1 to 2 g soluble COD/L) or for wastewaters that contain inert or poorly biodegradable suspended particles which should not be allowed to accumulate in the sludge bed.

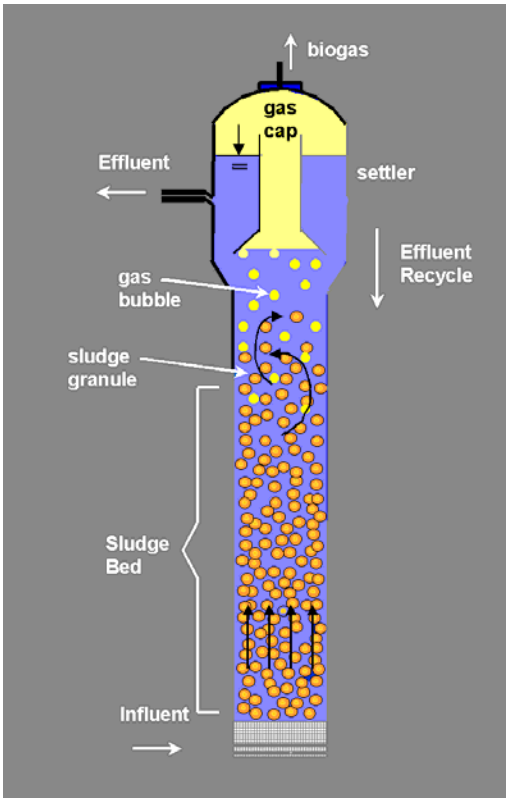


Figure 2.10. EGSB Reactor Configuration

In a recent survey (Frankin, 2001), 1215 full-scale high rate anaerobic reactors have been carefully documented, which have been built for the treatment of industrial effluents since the 1970's throughout the world. An overwhelming majority (72% of all plants) of the existing full-scale plants are based on the UASB or EGSB design concept developed by Lettinga in The Netherlands.

The biomass is present in a granular form. The granule size and inner structure seem to play a more relevant role in fully expanded EGSB reactors (Seghezzo, 1997). Accumulation of flocculent excess sludge between the sludge granules is also prevented (van der Last and Lettinga, 1992). Soluble pollutants are efficiently treated in EGSB reactors but suspended solids are not substantially removed from the wastewater stream due to the high upflow velocities applied. Recirculation of the effluent dilutes the influent concentration, but it was extensively proven that low strength wastewater can efficiently be treated in EGSB reactors (Kato et al., 1994; Kato, 1994).

2.5.2. Upflow anaerobic sludge blanket (UASB)

Successful construction of a UASB process capable of affording self-granulation (flocculation) of anaerobic microbes was first reported by Letting et al. Wastewater entering from the bottom of the reactor passes through a sludge bed and sludge blanket where organic materials are anaerobically decomposed. Gas produced is then separated by a gas-solid separator and the clarified liquid is discharged over a weir, while the granular sludge naturally settles to the bottom. Granules range in size from 0.5-2.5 mm, and in concentration from 50-100 kg VSS/m^3 at the bottom, to 5-40 kg VSS/m^3 in the upper part of the reactor. Bench- and pilot plant-scale experiments indicate that it is possible to operate this system at a COD loading of 40 kg/m^3.day at HRTs of 4-24 hours. COD concentration in the inlet waste water ranges from 2.000-20.000 mg/L at HRTs of 0.1-2 days (depending on the inlet COD concentration) in which the COD loads are 6-14 kg/m^3.day, resulting in a COD reduction of more than 80%. Studies on the application of this system to domestic sewage however indicate that the UASB reactor can operate at rather long HRT of significant parameters in the UASB operation are floe diameter, microbial density, and the structure of the gas-solid separator which effectively retains the microbial granules within the reactor. Granule formation in a UASB system is influenced by the growth of rod-type *Methanosaeta spp*. which produces spherical granules.

2.5.3. Anaerobic fluidized-bed reactor

In such systems, the medium to which the microbes adhere is fluidized within the reactor, resulting in conversion of organic materials to CH_4 and CO_2. Anaerobic microbes grow on the surface of the medium, expanding the apparent volume of the medium; hence this reactor is also designated an "expanded bed reactor". Use of artificial sewage in an AFBR, resulted in COD removal exceeding 80% at 20°C, and at a COD load of 2-4 kg/m^3.day this system was tolerant of shock loading for step changes of temperature from 13 to 35°C and from 35 to 13°C. In the case of COD shock loading from 1.3 to 24 kg/m^3.day, a steady state is

established after 6 days. The AFBR thus seems to be capable of performing at relatively low temperatures with both low and high COD waste waters, without significant shock loading effects.

2.5.4.Anaerobic Hybrid Systems

The hybrid systems 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. The hybrid systems combine a UASB and an anaerobic filter in the top part of the reactor and overcome the disadvantages of both of the configurations.

Although there will always be a net loss in energy in the whole system (the energy to grow the biomass is more than the output of the reactor), for the processing of waste organic material, anaerobic digestion is the preferable choice because it is environmentally friend. The biggest impacts on the environment include the energy and materials used to build the plant, transport costs and fuel use in transporting material to site and visual and audible impacts of the site operation. Odour can be a severe problem during emptying cycles. This is a particularly difficult issue for batch reactors.

3. MATERIALS AND METHODS

3.1. Characteristics of a Full Scale Expanded Granular Sludge Bed (EGSB) Reactor

A flow diagram of wastewater treatment plant of brewery wastewater, İzmir, is given in Figure 3.1. As seen, it is composed of two-stage anaerobic-aerobic biological treatment system. A full-scale anaerobic EGSB reactor was used for the first-stage of the biological treatment. The EGSB reactor is operated at a volumetric loading rate of 15 kg COD/ m^3 .d (data supplied by the industry). Diameter and total wet height of the anaerobic reactor are 14 m x 15.5 m with a total volume of 2280 m^3. The water level in the reactor is kept approximately at 14.6 m. The wastewaters are pumped through a screen having a pore size of 1 mm.

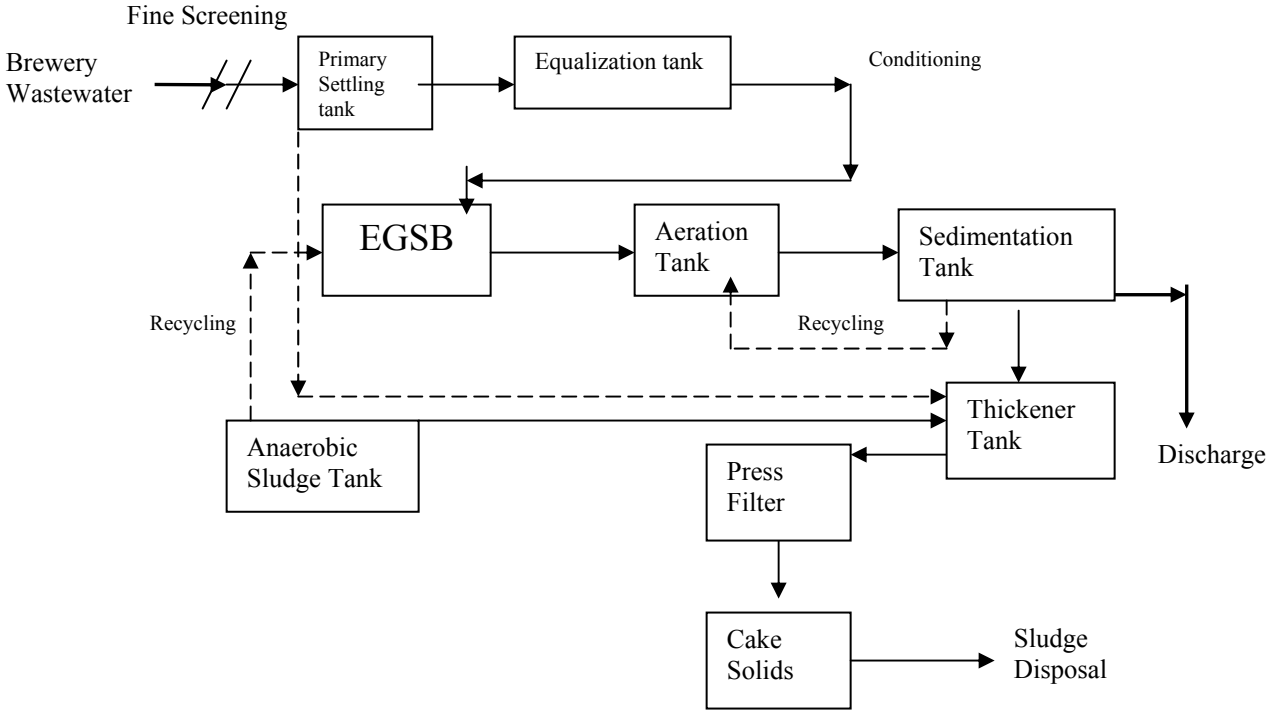


Figure 3.1. Flow Diagram of Two-stage Anaerobic-Aerobic Biological Treatment of Brewery Wastewater

3.2. Brewery Wastewater Characteristics

Brewery plants produce large quantities of wastewater containing high concentrations of degradable organic pollutants. Brewers are very concerned that the techniques they use are the best in terms of product quality and cost effectiveness. During production, beer alternately goes through three chemical and biochemical reactions (mashing, boiling, fermentation and maturation) and three solid-liquid separations (wort separation, wort clarification and rough beer clarification). The mashing process is one of the initial operations in brewery, rendering the malt and cereal grain content soluble in water. After extraction, the spent grains and wort (water with extracted matter) are called mash and need to be separated. 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. The amount of solid in the mash is typically 25-30%. General chemical characteristics of the wastewater are given Table 3.1

Table 3.1. General Chemical Characteristics of the Brewery Wastewater Used

Parameter	Brewery Wastewater
COD, m/L	5500-10500
BOD_5, mg/L	3000-4000
Total-N, mg/L	100-130
Total-P, mg/L	28-47
pH	5-7

3.3. Analytical Techniques

During this study pH, COD, BOD were monitored in representative intervals. Gas compositions for SMA tests were monitored via computerized unit. Gas compositions for SMA tests were determined using a Hewlet Packard 6850 gas chromatograph (GC) with a

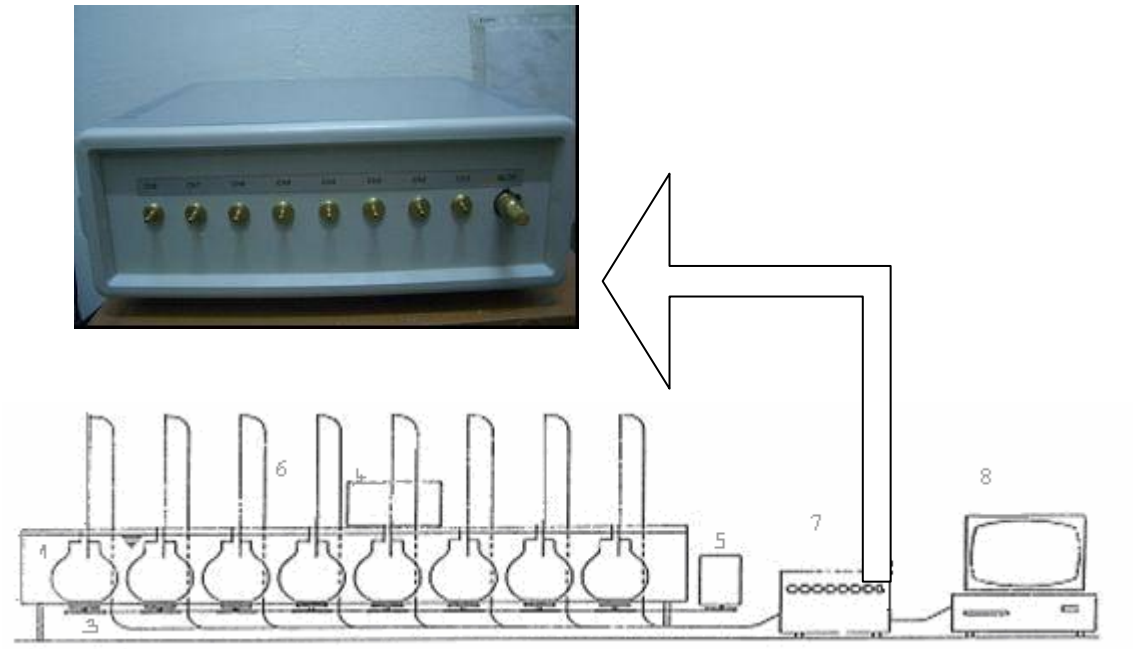
thermal conductivity detector (HP Plot Q column 30 m x 530 μm). Due to the granular characteristics of the reactor sludge, total solids and total volatile solids (TS/TVS) were measured. All analyses were carried out according to Standard Methods (APHA, 1997).

3.4. Methanogenic and Non-Methanogenic Activity Tests

3.4.1. Description of SMA Test Unit

In this study, a fully computerized specific methanogenic activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince (1995) was used to determine acetoclastic methanogenic activity. The SMA test unit consisted of eight 2 L digestion flasks which are placed into a water bath to control the temperature stability. Mixing is provided by magnetic stirrers, which run at a speed of 90 rpm. Gas measurement system contains pressure sensors, miniature valves and tubing for interconnection between the anaerobic reactor and the other units. This system has eight solenoid valves. The valve which has 3 ports is controlled with a pressure measurement device which is set to a pressure value of 100 kPa. As the pressure inside the system reached a set value, the control system sent an electrical signal to a control interface that activated the three-way solenoid valve, simultaneously closing the second port (to maintain the pressure inside the reactor) and opened the third port to the atmosphere. This made the connection of bulb to the atmosphere, releasing the excess gas accumulated during the build-up in pressure. The selenoid valve was set so that the two normally open ports (1 and 2) communicate with the pressure measurement device. When the third port was closed, the pressure in the system increased progressively. The valve was deactivated after an interval of time (3s for the complete release of the gases) and a new cycle was initiated. The test unit can 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 will be carried out by using a very sensitive Health Care Pump.



1. Digestion Flasks V= 2000 mL
2. Three-way Selenoid Valve
3. Magnetic Stirrer
4. Water Bath Heater
5. Stirrer Motor
6. Wiring
7. Selenoid Valve Controller Board
8. Computer with SMA Test Program

Figure 3.2. Experimental set-up for SMA test unit

3.4.2. Experimental Procedure for Specific Methanogenic Activity Test

The laboratory routine for SMA test is given as follows.

- The volatile suspended solid content (VSS) of the sludge sample to be analyzed must be determined before the test is started (preferably 12 hour in advance).

- The concentration of volatile suspended solid (VSS) in the reactors is brought about 2000 mg/L by diluting sludge sample with a mineral stock solution given in Table 3.2.

- The pH of the reactors should be adjusted to 7.

- Reactors should be flushed with helium gas about a period of 10 minutes to maintain anaerobic conditions in the reactor. The taps of the reactors must be closed immediately after flushing and all connections of the SMA test unit must be greased in order to prevent air leakage.

- Temperature of the reactor content should be maintained 35±0.5 °C by heating water bath.

- Acclimatize the test sample for 12-16 hours. Gas production during the time can be neglected.

- Acetate as substrate is introduced to the SMA reactor.

- Mixing system should be opened and data collection system should be reset.

- Biogas production is saved automatically for every hour.

- Methane concentration is determined at regular intervals by taking 1 mL gas sample.

- The volume of methane produced per unit of time is calculated using Equation 3.1.

Table 3.2. Mineral Stock Solution for Methanogenic and Non-Methanogenic Activity Tests (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

3.4.3.Experimental Procedure for Non- Methanogenic Activity Test

Non-methanogenic activity test procedure developed by Soto et al. (1993), and modified by Hutnan et al., (1999) was used in this study.

-The volatile suspended solid content (VSS) of the sludge sample to be analyzed must be determined before the test is started (preferably 12 hour in advance).

- The concentration of volatile suspended solid (VSS) in the reactors is brought about 2000 mg/L by diluting sludge sample with a mineral stock solution given in Table 3.2.

-The pH of the reactors should be adjusted to 7.0.

- Reactors should be flushed with helium gas about a period of 10 minutes to maintain anaerobic conditions in the reactor. The taps of the reactors must be closed immediately after flushing.

-Temperature of the reactor content should be maintained 35±0.5 °C by heating water bath.

-Acclimatize the test sample for 12-16 hours. Gas production during the time can be neglected.

-Mixing system should be opened and data collection system should be reset.

-After the incubation period, substrate is added to the reactor.

-For COD measurements 20 mL sample is taken from the reactor for every three hours.

- The activity measurements are calculated using Equation 3.1.

3.4.4. Feed and Seed for Methanogenic And Non-Methanogenic Activity Tests

Acetate, propionate and butyrate were used as feeds during SMA tests. Approximately 72% of the methane formed during anaerobic digestion of complex substrate results from acetic acid (McCarty, 1964). Acetate concentrations in range of 1000-4000 mg/L were initially tested in order to find potential methane production (PMP) rate during the SMA tests. 2000 mg/L acetate concentration was found to be optimum. Secondly, propionate concentrations in a range of 1000-4000 mg/L were used to obtain PMP. 4000 mg/L propionate was detected to be optimum concentration. Similarly, different butyrate concentrations in a range of 1000-4000 mg/L were used to determine the optimum butyrate concentration and 4000 mg/L butyrate was found to be optimum. Finally, a VFA mixture of 2000 mg/L acetate, 500 mg/L propionate, and 500 mg/L butyrate was used to measure overall methanogenic activity (Soto et al., 1993).

Regarding to acidogenic activity measurements, glucose concentrations in a range of 1000-4000 mg/L were used during tests. Furthermore, different sucrose concentrations between 1000-4000 mg/L were used for determination of maximum hydrolytic activity.

Reactor sludges were collected from a full-scale anaerobic EGSB reactor at three different heights (bottom, mid and top) during April, May, and June 2007. Only bottom samples were used in both methanogenic and non-methanogenic activity tests. These samples were diluted to 2000 mg VSS/L for SMA tests as it was described in the laboratory routine.

3.4.5. Calculation of Specific Methanogenic and Non-Methanogenic Activities

The gas produced in the reactor was sent to a gas-washing flask. The methane content of the gas was measured by gas chromatograph. The potential methane production was calculated by the formula expressed below:

Specific Methanogenic activity was calculated as:

$$\text{SMA (mL } CH_4\text{/gVSS.d)} = (A \times B \times C \text{ x } 24) / (D \times E) \quad (3.1)$$

A: Biogas production per hour (mL/h)
B: Methane content of biogas produced (CH_4 %)
C: Valve factor
D: Active volume of the SMA test reactor (L)
E: Concentration of biomass in SMA test reactor (mgVSS/L)

Acidogenic and hydrolytic steps were analyzed via COD removal rate (Hutnan et al, 1999). Calculations and activity expressions were presented in the work Soto et al., (1993). The activity (Ac) is usually expressed as g COD per VSS per day and calculated from the substrate consumption rate (e. g. hydrolytic and acidogenetic phases).

Non-methanogenic Activity was calculated as:

$$Ac_s = - \frac{1}{\rho} \cdot \frac{d\rho(COD)}{dt} (g / g.d) \quad (3.2)$$

Ac_s = activity of the sludge (mg COD/ mg VSS.d)

t: time (d)

ρ: density of the sludge (mg/L)

3.5. Fluorescent *in situ* Hybridization (FISH)

3.5.1. Sampling and Short Term Fixation

As previously described in section 3.4.4, sludge samples were collected from three different levels of the EGSB reactor. Then, samples were transferred into sterile containers with the addition of absolute ethanol (1:1, v/v) on-site. Triplicate sludge samples from three different heights were used for FISH studies and samples were immediately transferred to the institute laboratory in cool-boxes maintained at 4°C or less. Upon arrival, samples were stored at -20°C and fixed within a week.

3.5.2. Standard Paraformaldehyde (PFA) Fixation

500 µL of granular sludge-ethanol mix (1:1, v/v) was washed once with phosphate-buffered saline (PBS) [130 mM NaCl, 10 mM sodium phosphate, pH 7.2]) and resuspended in 0.25 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) and stored at -20°C.

3.5.3. Hybridization

16S rRNA-targeted oligonucleotide probes used in this study and their target microbial groups nucleotide sequences are listed in Table 3.3. Optimal hybridization conditions for each probe are also given in Table 4.5. All probes were obtained commercially (Qiagen Corp.).

Table 3.3. 16S rRNA-Targeted Oligonucleotide Probes Used

Probe	Target Group	Prob dizilimi (5'-3')	Labelling (5')	Reference
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	CY3	Raskin et al., 1994
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994
MG1200	*Methanogenium* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994
MSMX860	*Methanosarcinaceae*	GGCTCGCTTCACGGCTTCCCT	CY3	Raskin et al., 1994
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994
MS821	*Methanosarcina*	CGCCATGCCTGACACCTAGGCCAGC	CY3	Raskin et al., 1994
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	TAMRA	Raskin et al., 1994
ARC915	*Archaea*	GTGCTCCCCCGCCAATTCCT	CY3	Stahl et al., 1988
UNIV1392	Virtualy all known organisms	ACGGGCGGTGTGTAC	TAMRA	Pace et al., 1986
NON338	Non sense probe	ACTCCTACGGCAGGCAGC	TAMRA	Manz et al., 1992
EUBMIX	*Bacteria*	GCTGCCTCCCGTAGGAGT	Fluorescein	Amann et al., 1990

Methanogenic- and *Archaea*-specific 16S rRNA probes with probe name, sequence, target site, and experimentally determined Td (wash solution consisting of 1%SDS x SSC). Probes MB314 and MS1242 did not perform satisfactorily, and, hence, no Td was determined (Raskin et al., 1994).

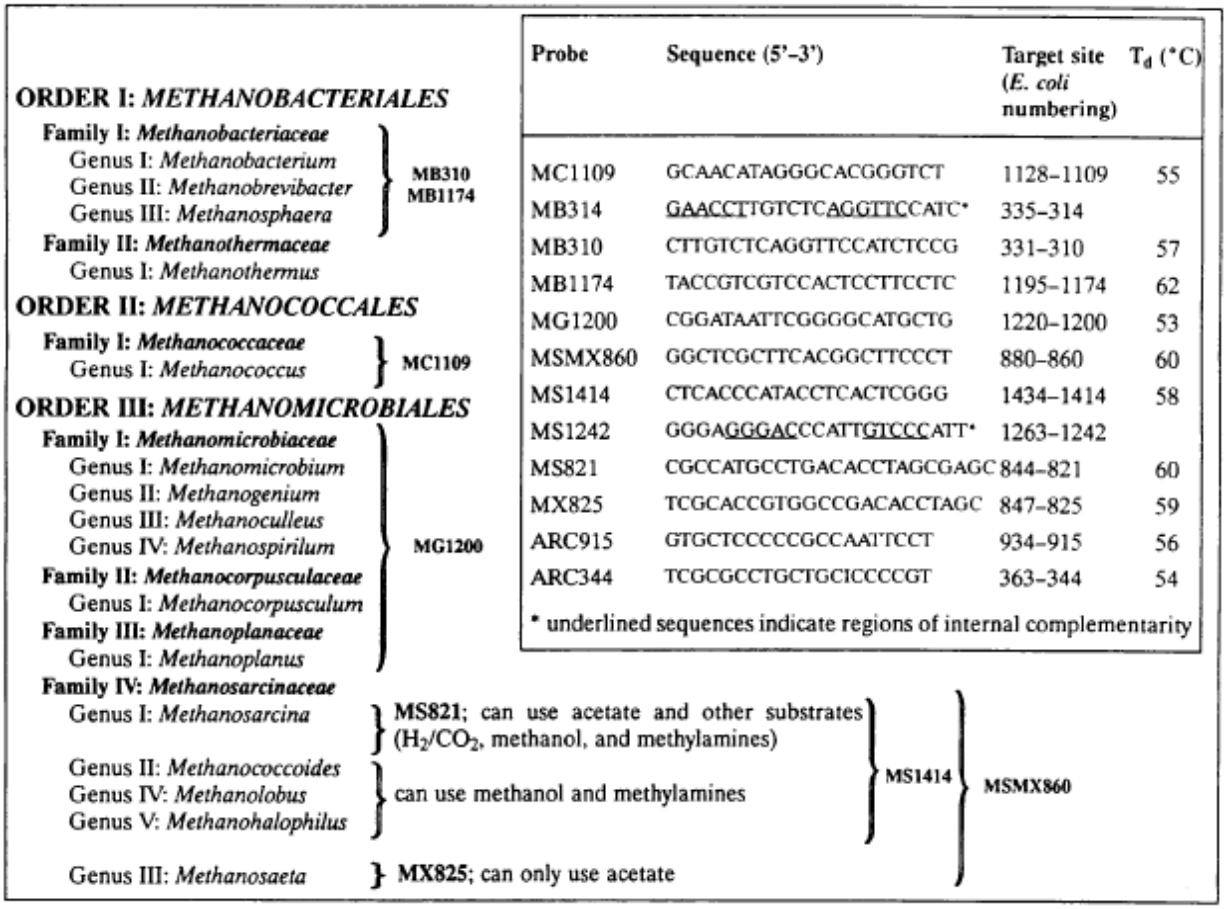


Figure 3.3. Classification of methanogens in relationship to the oligonucleotide probes characterized (Raskin et al., 1994)

For each sample hybridization, two negative controls were prepared; one of these controls was used to assess non-specific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was prepared to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the three EGSB sludge samples were also stained using DAPI staining to visualize intact cells in the samples.

Table 3.4. Optimum Hybridization Conditions for Oligonucleotide Probes Used

Probe	Formamide concentration	Hybridization temperature	Washing temperature	NaCl Concentration
MC1109	20%	46 °C	48 °C	225 mM
MB310	20%	46 °C	48 °C	225 mM
MG1200	30%	46 °C	48 °C	112 mM
MSMX860	30%	46 °C	48 °C	112 mM
MS1414	35%	46 °C	48 °C	84 mM
MS821	20%	46 °C	48 °C	225 mM
MX825	20%	46 °C	48 °C	225 mM
ARC915	35%	46 °C	48 °C	84 mM
EUBMIX	10%	46 °C	46 °C	450 mM
UNIV1392	10%	37 °C	37 °C	450 mM

200µL of the fixed samples were washed once with PBS and dehydrated at room temperature in increasing concentrations of ethanol (50%, 80%, and 100%). Dehydrated samples were resuspended in 40µL of hybridization buffer (0.9M NaCl, 2mg/mL Ficoll, 2mg/mL Bovine Serum Albumen, 2mg/mL polyvinyl pyrrolidone, 5mM EDTA, pH 8.0, 25 mM NaH_2PO_4, pH 7.0, 0.1% SDS, 5-35% deionised formamide) and prehybridized at the intended hybridisation temperature for 20 minutes. After prehybridisation, 2µl 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 a wash buffer containing 20 mM Tris-HCl (pH 7.2), 0.01% SDS, 0-5 mM EDTA and between 0.9 M and 56 mM NaCl according to the formula of Lathe (1985) for 15 min at the optimal washing temperature before a final wash in MilliQ water. The cells were resuspended in 200µl of MilliQ water, and a 10 µL aliquot was placed on a gelatin-coated slide and air dried. One drop of Citifluor antifadent (Citifluor Ltd.) was added to the sample, and a coverslip was applied to the preparation and sealed with nail polish before epifluorescence microscopy.

3.5.4. DAPI Staining

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

3.5.5. Visualization

Slides were examined under Olympus BX 50 Epifluorescence Microscope equipped with a 100 W high-pressure mercury lamp, U-MWIB and U-MWG filter cubes. Images were captured using a Spot RT charged coupled device (CCD) camera having special software supplied by the camera manufacturer (Diagnostic Instruments Ltd., UK). The images were processed and analyzed using Image-Pro Plus version 5.1 image analysis software (Media Cybernetics, U.S.A.).

Different fluorochromes are excited and emitted at different wavelengths. Optimum emission and excitation wavelengths and corresponding filter cubes for the fluorochrome used in this study are given in Table 3.5.

Table3.5. Optimum Emission and Excitation Wavelengths and Corresponding Filter Cubes for the Fluorochrome Used

Fluorochrome	Color of Fluorescence	Maximum excitation wavelength (nm)	Maximum emission wavelength (nm)	Filter cube used
FLUOS	Green	494	518	U-MWIB
TAMRA	Orange	555	580	U-MWG
CY3	Red	550	565	U-MWG
DAPI	Blue	350	470	U-MWG

3.5.6. Quantification

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

3.5.6.1. Quantification of Total Microorganisms. For each sample, firstly DAPI Stain was used to determine the average number of total microorganisms. For all times, triplicate samples were collected from three levels of the EGSB reactor (bottom, middle and top) at three different periods 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.

3.5.6.2. Quantification of Archaea and Methanogens. Quantification of methanogens involves application of FISH with oligonucleotide probes specific for different methanogenic groups given in Table. For all times, triplicate samples were collected from three levels of the EGSB reactor (bottom, middle and top) at three different periods and counts for 10 random fields of view were obtained for each sample, and the average cell count was calculated. Hence, a representative number of microorganisms in each group were found.

4. RESULTS AND DISCUSSION

4.1. Performance of the Full-scale Anaerobic EGSB Reactor

An anaerobic-aerobic two-stage biological treatment system has been used to treat a brewery wastewater. In this treatment system, an EGSB reactor has been operated for the anaerobic stage. Performance of the full-scale EGSB reactor in terms of COD removal efficiency for the April, May and June were 68%, 77%, and 88%; respectively, at an OLR of 15 kg COD/m^3.d. However, performances of EGSB reactors treating brewery wastewaters were as approximately 90-98% in terms of COD removal efficiency reported in literature (Zoutberg et al., 1997).

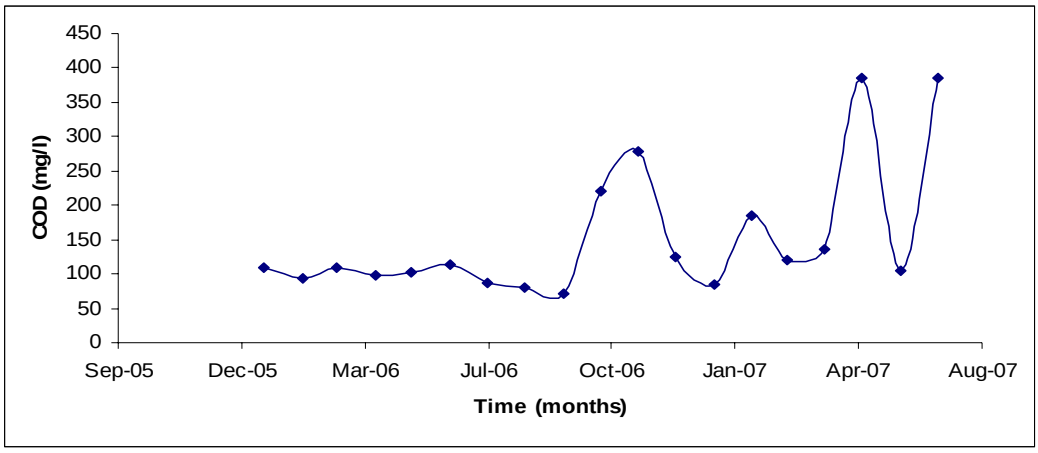


Figure 4.1.Effluent COD Concentrations from Full-scale Anaerobic EGSB Reactor (supplied by the brewery industry)

As seen in Figure 4.1 effluent COD concentrations were maintained approximately 100 mg/L until September 2006 after which those increased up to 400 mg/L. As clearly seen, the anaerobic EGSB reactor has encountered some problems in terms of maintaining performance and stability during the sampling period.

4.2. Activity Tests Results

Methanogenic and non-methanogenic activity tests had been carried out to determine the potential loading capacity and optimum operating conditions of the anaerobic EGSB reactor. Sludge activity measurements can be considered in two different ways: an overall measurement which gives information about the whole degradation activity and activity measurements of each biodegradation stage of the anaerobic process.

4.2.1. Methanogenic Activity Test Results

Four different substrates were used to determine specific methanogenic activity. Only bottom sludge samples were used for the methanogenic activity tests. The activity tests were repeated at least three times in order to determine precise and reproducible results. Firstly, acetate was used as substrate in order to measure the potential acetoclastic methanogenic activity. Then, propionate and butyrate were used as substrate for the respective trophic methanogenic activities. Finally, a VFA mixture (2000 mg/L acetate, 500 mg/L propionate and, 500 mg/L butyrate) was used as substrate for overall methanogenic activity (Soto et al, 1993).

In order to determine maximum acetoclastic methanogenic activity, acetate concentrations in a range of 1000-4000 mg/L were used for April, May and June samples as seen in Figure 4.2-4.4, respectively.

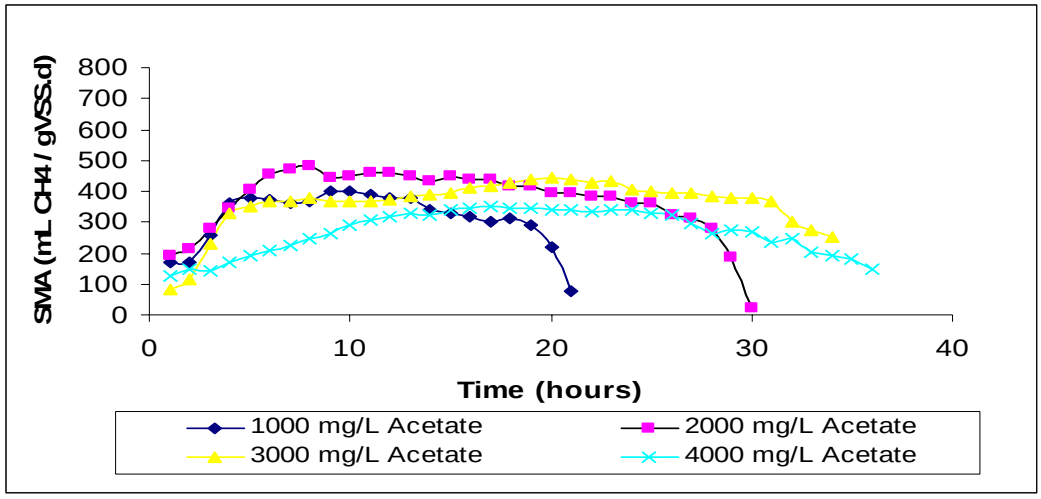


Figure 4.2. SMA Test Results of Anaerobic Reactor Sludge with Acetate (April 2007)

Maximum acetoclastic methanogenic activities of the April sample were found to be 457 and 445 mL CH_4/g VSS.d at 2000 mg/L and 3000 mg/L acetate concentrations, respectively. Both concentrations can be considered as optimum doses. When 4000 mg/L acetate was used, acetoclastic methanogenic activity decreased to 398 mL CH_4 /g VSS.d.

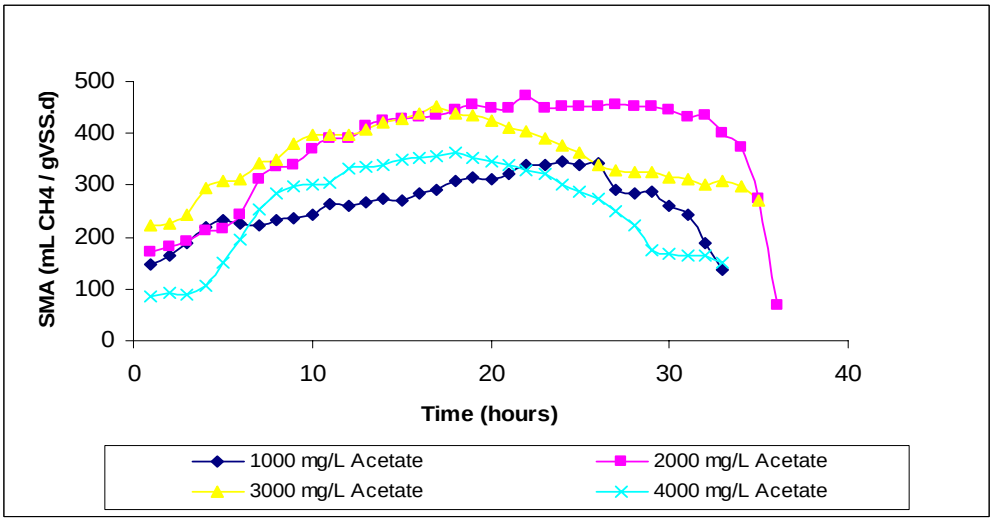


Figure 4.3. SMA Test Results of Anaerobic Reactor Sludge with Acetate (May 2007)

Maximum acetoclastic methanogenic activity for May sample was found to be 452 mL CH_4/g VSS.d and 440 mL CH_4/g VSS.d at 2000 mg/L and 3000 mg/L acetate, respectively as

shown in the Figure 4.3. May sample had quite similar results for 2000 and 3000 mg/L acetate concentrations. Acetoclastic methanogenic activity decreased to 360 mL CH_4/g VSS.d at 4000 mg/L acetate concentration. Similarly, approximate activity value was measured at 1000 mg/L acetate concentration.

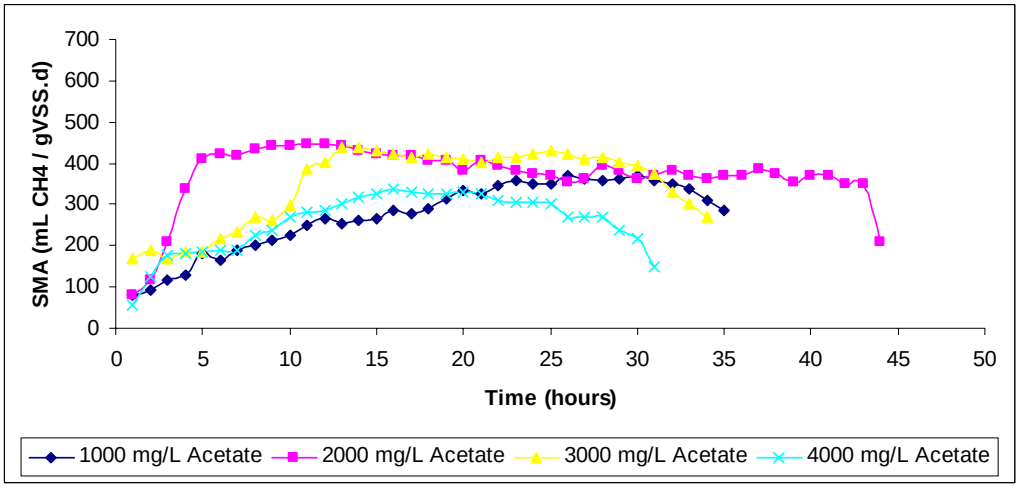


Figure 4.4. SMA Test Results of Anaerobic Reactor Sludge with Acetate (June 2007)

The maximum acetoclastic methanogenic activity result was found to be 447 mL CH_4/g VSS.d at 2000 mg/L acetate concentration for June sample. Acetoclastic methanogenic activity was 430 mL CH_4 / gVSS.d at 3000 mg/L acetate concentration. Like April and May samples, June sample had similar results for 2000 and 3000 mg/L acetate concentration. Finally, acetoclastic methanogenic activity of the sample decreased to 330 mL CH_4/g VSS.d at 4000 mg/L acetate concentration.

As clearly seen, acetoclastic methanogenic activity has decreased gradually during sampling periods. The acetoclastic methanogenic activity of April sample was 457 mL CH_4/g VSS.d, whereas the activity of June sample was 447 mL CH_4/g VSS.d. Approximately, 3% decrease was seen in activities of the samples.

Methane formation from acetate is known to be rate limiting step in methanogenic phase in anaerobic processes (Henze and Harremoes, 1982). During methanogenesis, acetate to

methane conversion has figured prominently in several investigations. It was found that acetate was the major VFA produced from which around 70% of methane was derived. The remaining approximately 30% was produced from the reduction of CO_2 (Bott and Thauer, 1989).

In the different reactors or compartments receive different substrate types and concentrations, which results in the development of different microbial communities because substrate type and concentration ultimately determine the biomass composition (Fox and Pohland, 1994; van Lier et al., 1996; van Lier et al., 1997). A number of physical and microbial ecology reasons for the observed structure are proposed, including the advantage of segregation for high-rate degradation of syntrophic substrates.

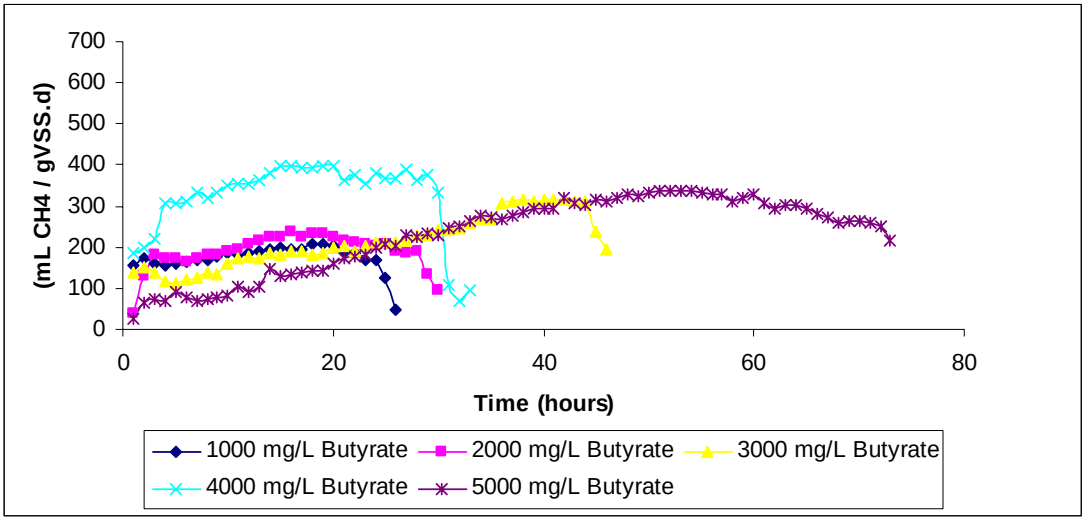


Figure 4.5. SMA Test Results of Anaerobic Reactor Sludge with Butyrate (April 2007)

Regarding the maximum methanogenic activity measurements, butyrate in a range of 1000-5000 mg/L concentrations were used to determine the maximum methanogenic activity. As shown in Figure 4.5, the activity was found to be 399 mL CH_4/g VSS.d when 4000 mg/L butyrate was used for the April sample. 5000 mg/L butyrate concentration had adverse effect on the activity of the sample and decreased to 335 mL CH_4/g VSS.d.

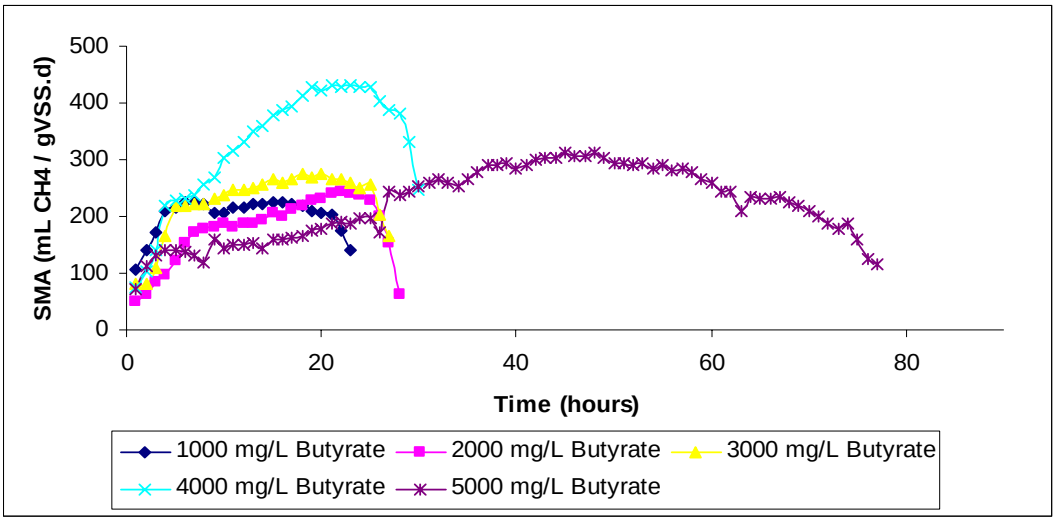


Figure 4.6. SMA Test Results of Anaerobic Reactor Sludge with Butyrate (May 2007)

As can be seen from Figure 4.6, the maximum activity for the May sample was 430 mL CH_4/g VSS.d at 4000 mg/L butyrate concentration. The activity decreased to 310 mL CH_4/g VSS.d at 5000 mg/L butyrate concentration.

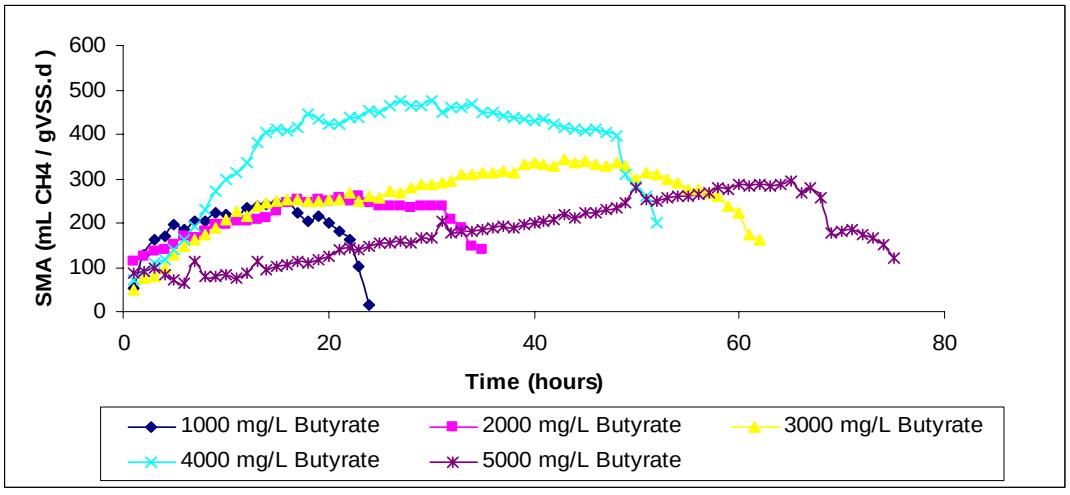


Figure 4.7. SMA Test Results of Anaerobic Reactor Sludge with Butyrate (June 2007)

Figure 4.7 shows; maximum methanogenic activity was measured as 460 mL CH_4/g VSS.d at 4000 mg/L butyrate for June sample. The activity decreased to 300 mL CH_4/g VSS.d at 5000 mg/L butyrate.

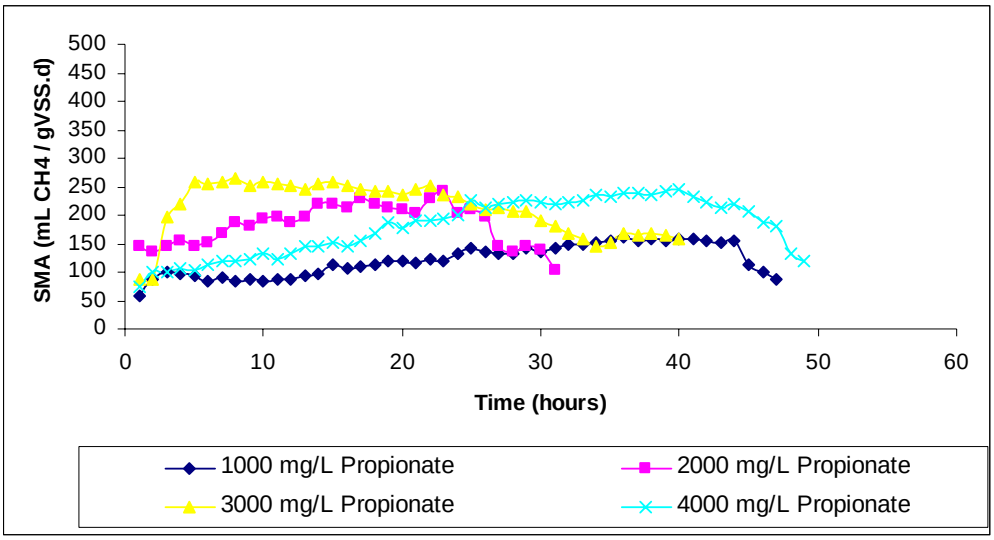


Figure 4.8. SMA Test Results of Anaerobic Reactor Sludge with Propionate (April 2007)

Figure 4.8 shows, the maximum specific methanogenic activity was found to be 250 mL CH_4/g VSS.d at 3000 mg/L propionate. Individual methanogenic activity was found to be 237 mL CH_4/g VSS.d at 4000 mg/L propionate.

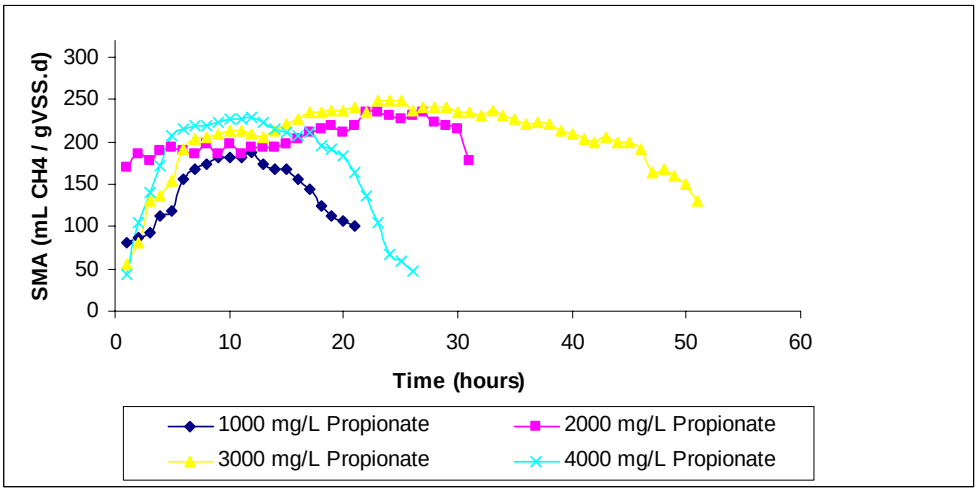


Figure 4.9. SMA Test Results of Anaerobic Reactor Sludge with Propionate (May 2007)

The individual methanogenic activity was found to be 235 mL CH_4/g VSS.d at 2000 mg/L propionate. In addition, individual methanogenic activity was measured as 248 mL CH_4/g VSS.d at 3000 mg/L propionate. As seen in Figure 4.9, activity results were

approximate to each other at 2000 and 3000 mg/L propionate concentration. On the other hand, the activity decreased to 226 mL CH_4/g VSS.d at 4000 mg/L propionate.

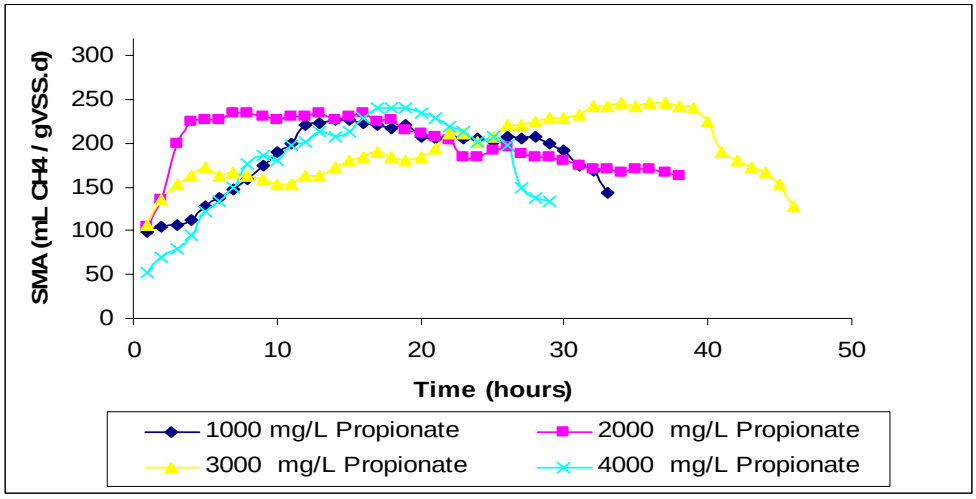


Figure 4.10. SMA Test Results of Anaerobic Reactor Sludge with Propionate (June 2007)

As illustrated in the Figure 4.10, specific methanogenic activity was measured as 230 mL CH_4/g VSS.d at 2000 mg/L propionate. Maximum methanogenic activity was found to be 254 mL CH_4/g VSS.d at 3000 mg/L propionate. Finally, the activity decreased to 240 mL CH_4/g VSS.d at 4000 mg/L propionate.

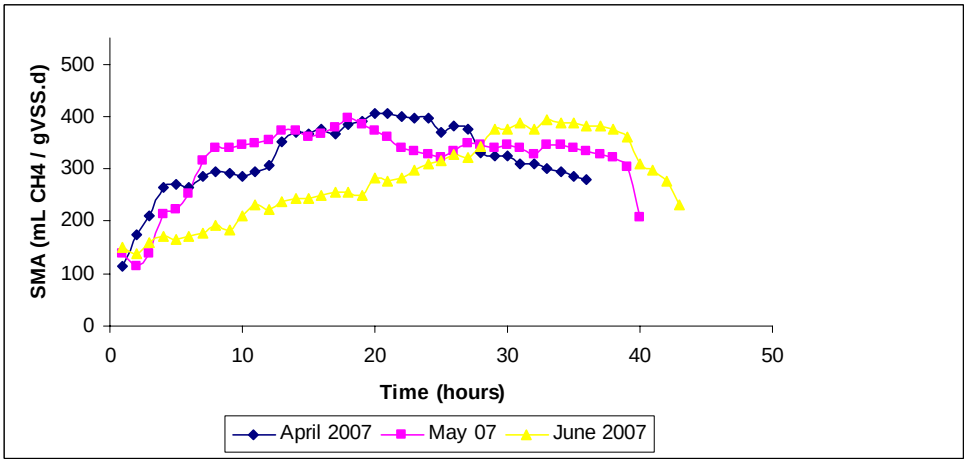


Figure 4.11. SMA Test Results of Anaerobic Reactor Sludge (2000 mg/L acetate, 500 mg/L butyrate and 500 mg/ l propionate)

A VFA mixture composed of 2000 mg/L acetate, 500 mg/L butyrate and 500 mg/ l propionate was used as substrate in order to determine overall methanogenic activity. The overall methanogenic activity of the April, May and June samples were found to be 400 mL CH_4/g VSS.d, 395 mL CH_4/g VSS.d and 390 mL CH_4/g VSS.d, respectively.

In order to compare results of this study with previous studies reported in literature, a method based on conversion of CH_4 to COD was used (see Appendix B). The overall methanogenic activity of the April sample was calculated as 1.01 g CH_4 COD/g VSS.d. In a previous study, the activity of VFA mixture (Acetate: propionate: butyrate = 2:1:1 as COD) was found as 1.03 g CH_4 COD/g VSS.d (Sun-Kee Han et al., 2004) which was quite similar to results of this study. In another study, the activity of VFA mixture (32:28:40 acetate/propionate/butyrate in a COD ratio) as the substrate was 2.3 g CH_4 (COD/VSS.d), which was comparably higher than those using acetate [1.6 g of CH_4 (COD/g VSS.d) and butyrate [1.8 g of CH_4 (COD/g VSS.d) as the substrate. On the other hand, the propionate substrate showed rather poor overall activity [0.5 g of CH_4 (COD/g VSS.d) (Biing-Teo Wong et al., 2007) as similarly obtained in this study. Table 4.1 shows a summary of maximum methanogenic activities in optimum concentrations.

Table 4.1. Maximum Methanogenic Activities of Anaerobic Reactor Sludge Samples

	Acetate (2000 mg/L)	**Butyrate (4000 mg/L)**	**Propionate (3000 mg/L)**	**VFA mixture***
April-07	457	399	250	400
May-07	452	430	248	395
June-07	447	460	246	390

*(2000 mg/L acetate, 500 mg/L butyrate and 500 mg/ l propionate) (Soto et al., 1993)

The maximum acetoclastic methanogenic activity was found to be 457 mL CH_4/g VSS.d in this study. Dogan (2001) found the maximum acetoclastic methanogenic activity 389 mL CH_4/g VSS.d for the sample obtained from an UASB reactor at an alcohol distillery. In a

previous study, a lab-scale anaerobic completely stirred tank reactor (CSTR) Acetoclastic methanogenic activity was found to be 336 mL CH_4/g TVS.d in a lab-scale anaerobic completely stirred tank reactor (CSTR)(Oz et al., 2004).

As a result of SMA tests conducted with butyrate, maximum specific methanogenic activity was found to be 460 mL CH_4/g VSS.d for three sampling periods. For example, in a previous study, maximum specific methanogenic activities measured approximately 400 mL CH_4/gVSS.d with different butyrate concentrations (Ianotti and Fischer,1983).They stated that 10000 mg/L butyrate concentration have significant inhibitory effect . In this study, 5000 mg/L butyrate concentration has slight inhibitory effect on the anaerobic biological sludge. Our result was approximately similar to those reported in literature. Specific methanogenic activity was found to be 385 mL CH_4/g VSS.d (1.01 g CH_4 COD/g VSS.d) at 2000 mg/L butyrate concentration, by Sun-Kee Han et al. (2004). Similarly, specific methanogenic activity with butyrate was found to be 300 mL CH_4/g VSS.d in this study.

Maximum specific methanogenic activity of propionate was found to be 250 mL CH_4/g VSS.d. In a previous study, Sun-Kee Han et al. (2004) found specific activity as 225 mL CH_4/g VSS.d (0.59 g CH_4 COD/g VSS.d) at 2000 mg/L propionate concentration. Reported studies in literature showed that over the 3000 mg/L propionate concentrations has vital effect on anaerobic sludges. According to a previous study, 100% specific methanogenic activity was obtained at 1000 mg/L propionate and contrary to literature the activity was approximately 73% at 3000 mg/L propionate concentration (Dogan, 2001).

4.2.2. Non-Methanogenic Activity Test Results

The biochemistry and microbiology of anaerobic digestion is a complex biogenic process involving a number of microbial populations, often linked by their individual substrate and product specifity. Non-methanogenic activity depends on substrate nature (complex or easy degradable). Although acidogenic step is not the limiting one, the evaluation acidogenic activity may offer important information about biomass development and dynamic behaviour

of anaerobic treatment systems (Soto et al., 1992). On the other hand, hydrolysis of some complex material is the limiting step of their anaerobic degradation of particulate organics. In this case the determination of the hydrolytic activity of anaerobic sludge on a specific substrate may be important in the selection of the most suitable reactor configurations or in the control of the process conditions.

In this study, both acidogenic and hydrolytic activity measurements have been carried out based on a method described by Soto et al. (1999).

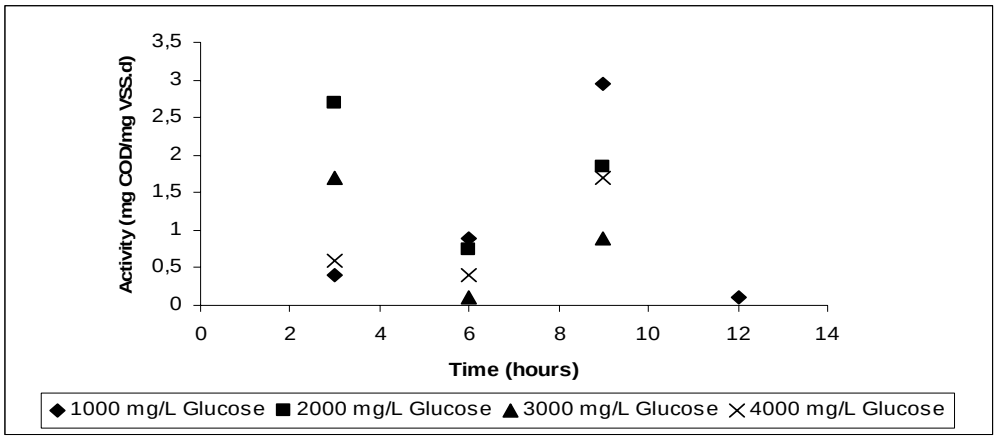


Figure 4.12. Acidogenic Activity Test Results of Anaerobic Reactor Sludge with Glucose (April 2007)

As illustrated in Figure 4.12, experiments have been carried out at 1000, 2000, 3000, 4000 mg/L Glucose concentrations to determine the maximum acidogenic activity for the three samples. The maximum acidogenic activity of the April sample was found as 2.9 mg COD/mg VSS.d at 1000 mg/L glucose concentration. The activity of the sample was slightly different as 2.7 mg COD/mg VSS.d at 2000 mg/L glucose concentration. When 3000 and 4000 mg/L glucose concentration were used, acidogenic activity test results decreased significantly. Therefore, the maximum acidogenic activity was 2.9 mg COD/mg VSS.d at 1000 mg/L glucose.

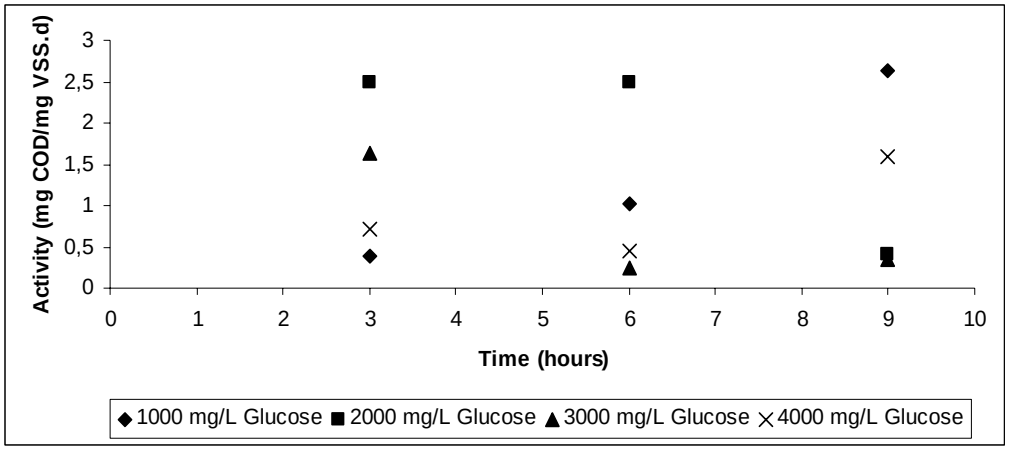


Figure 4.13. Acidogenic Activity Test Results of Anaerobic Reactor Sludge with Glucose (May 2007)

Glucose concentrations in a range of 1000 to 4000 mg/L were used to determine the maximum acidogenic activity for May sample. As shown in Figure 4.13, the maximum acidogenic activity was found to be 2.64 mg COD/mg VSS.d and 2.5 mg COD/mg VSS.d at 1000 mg/L glucose and 2000 mg/L glucose concentration, respectively.

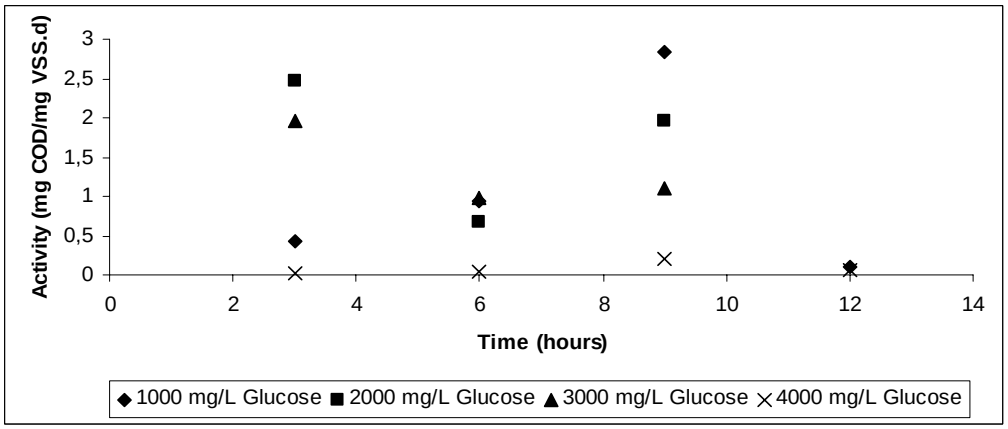


Figure 4.14. Acidogenic Activity Test Results of Anaerobic Reactor Sludge with Glucose (June 2007)

As seen in Figure 4.14, the maximum acidogenic activity of June sludge was 2.84 mg COD/mg VSS.d and 2.46 mg COD/mg VSS.d at 1000 and 2000 mg/L glucose concentrations.

During the anaerobic digestion of complex materials, the limiting step of the anaerobic process is often the hydrolytic step, where the polymers are split into smaller fragments or their monomers (Soto et al., 1993).

In addition to acidogenic activity measurements, hydrolytic activities were measured with four different sucrose concentrations in a range of 1000, 2000, 3000, 4000 mg/L.

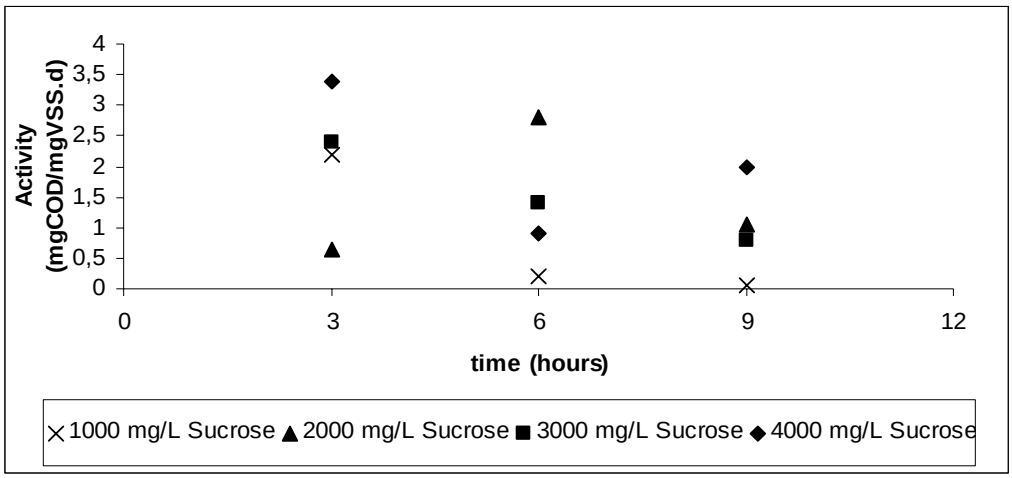


Figure 4.15. Hydrolytic Activity Test Results of Anaerobic Reactor Sludge with Sucrose (April 2007)

Figure 4.15 shows that the hydrolytic activity of the April sample was found to be 1.4, 3.2, 2.4, 3.0 mg COD/mg VSS.d at 1000, 2000, 3000 and 4000 mg/L sucrose concentration. The maximum hydrolytic activity was found at 2000 mg/L sucrose as 3.2 mg COD/mg VSS.d.

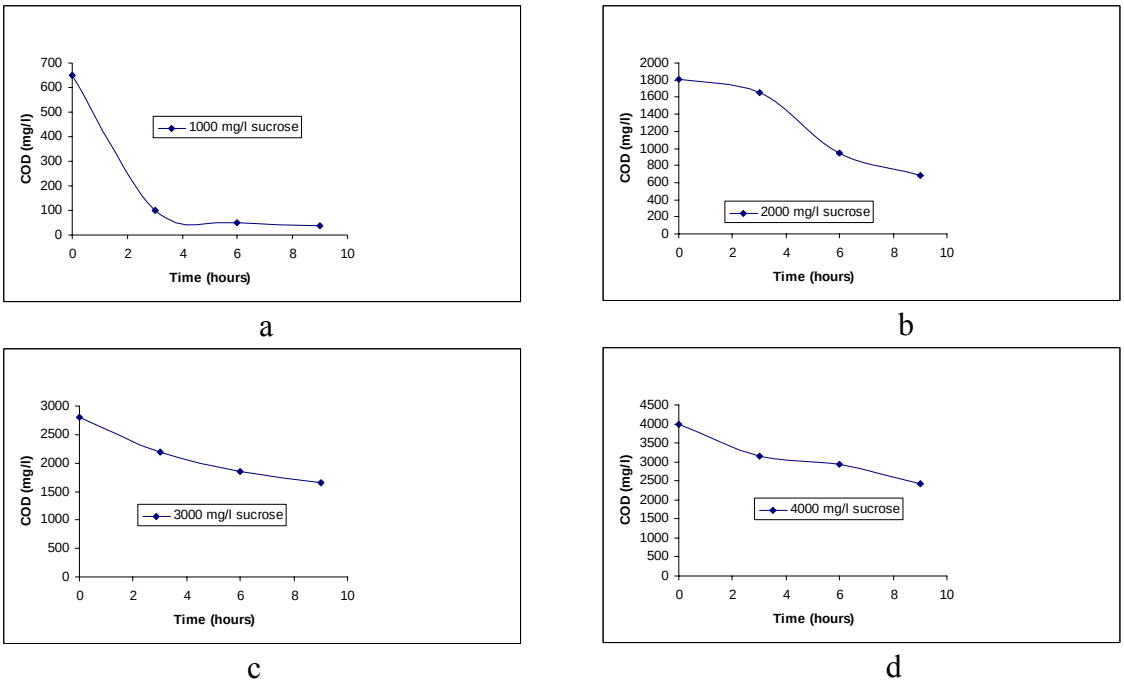


Figure 4.16. COD Measurements using Sucrose for Hydrolytic Activity (April 2007)

Figure 4.16 shows that COD values of the different sucrose concentrations has changed versus time. At 1000 and 2000 mg/L sucrose concentrations, there were sharp decreases but activity did not increase the maximum value. However, the hydrolytic activity increased at 3000 mg/L sucrose. Moreover, the maximum hydrolytic activity was obtained at 4000 mg/L sucrose concentration which was the high concentration.

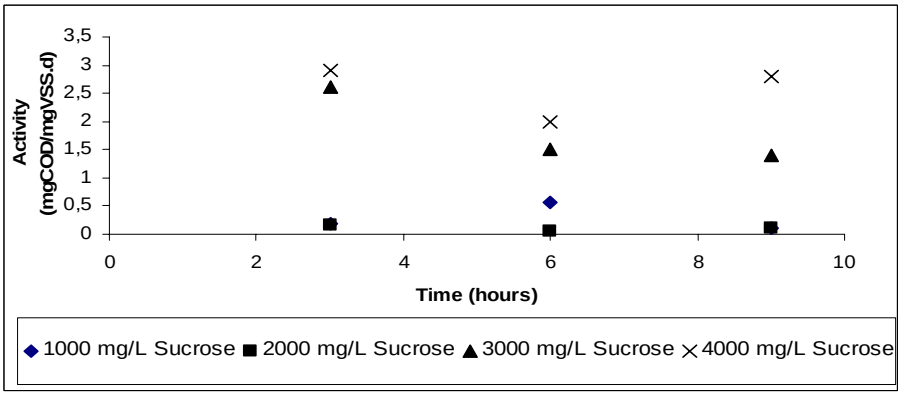


Figure 4.17. Hydrolytic Activity Test Results of Anaerobic Reactor Sludge with Sucrose (May 2007)

As seen in Figure 4.17, the hydrolytic activity of the May sample was found to be 0.55, 0.16, 2.6, 2.9 mg COD/mg VSS.d at 1000, 2000, 3000 and 4000 mg/L sucrose concentrations, respectively, indicating an increasing activity with increasing sucrose concentrations.

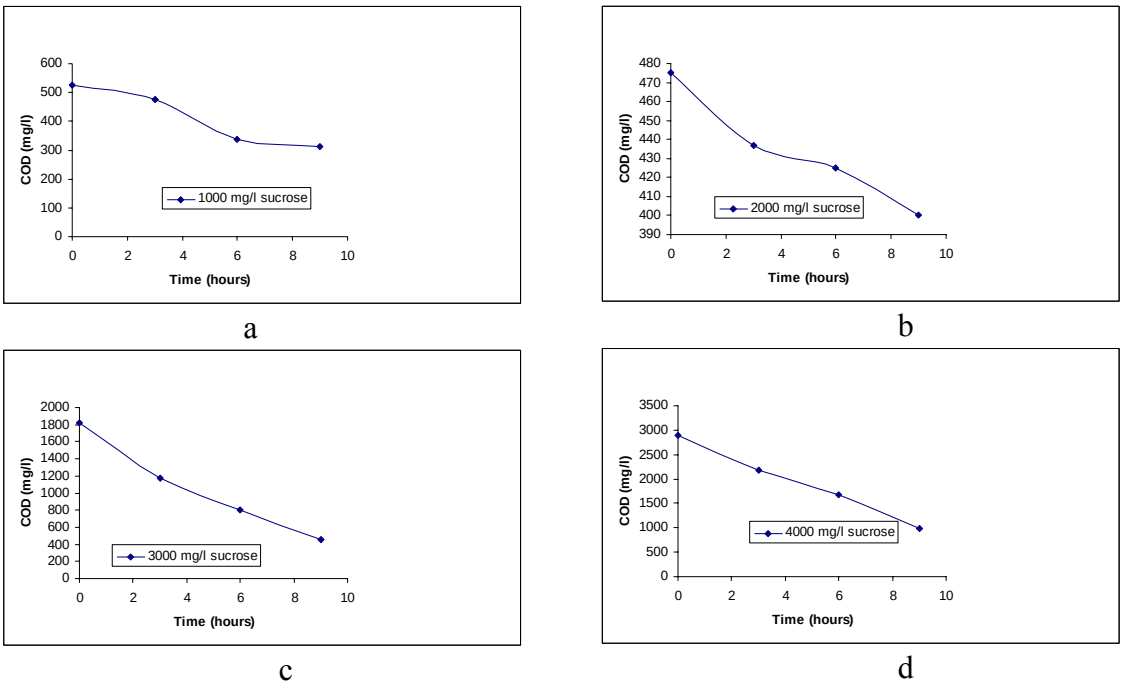


Figure 4.18. COD Measurements using Sucrose for Hydrolytic Activity (May 2007)

At 1000 and 2000 mg/L sucrose concentrations there was a sharp decrease in the hydrolytic activity as seen in the Figures 4.18a and 4.18b. The reason can be the substrate consumption by the acidogenic microorganisms. The hydrolytic activity period was not clearly detected because sampling intervals of three hours could be too long.

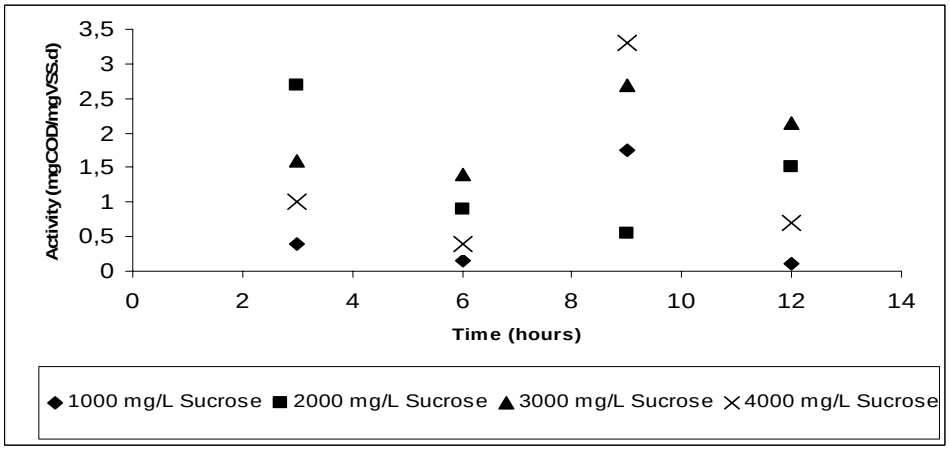


Figure 4.19. Hydrolytic Activity Test Results of Anaerobic Reactor Sludge with Sucrose (June 2007)

The hydrolytic activity test results showed that 1.7, 2.7, 2.7 and 3.3 mg COD/mg VSS.d were measured at 1000, 2000, 3000, 4000 mg/L sucrose as seen in Figure 4.19. Hutnan et al. (1999) calculated the specific hydrolytic activity of a UASB reactor as 3.52 mg COD/mg VSS.d at certain organic loading rate applied.

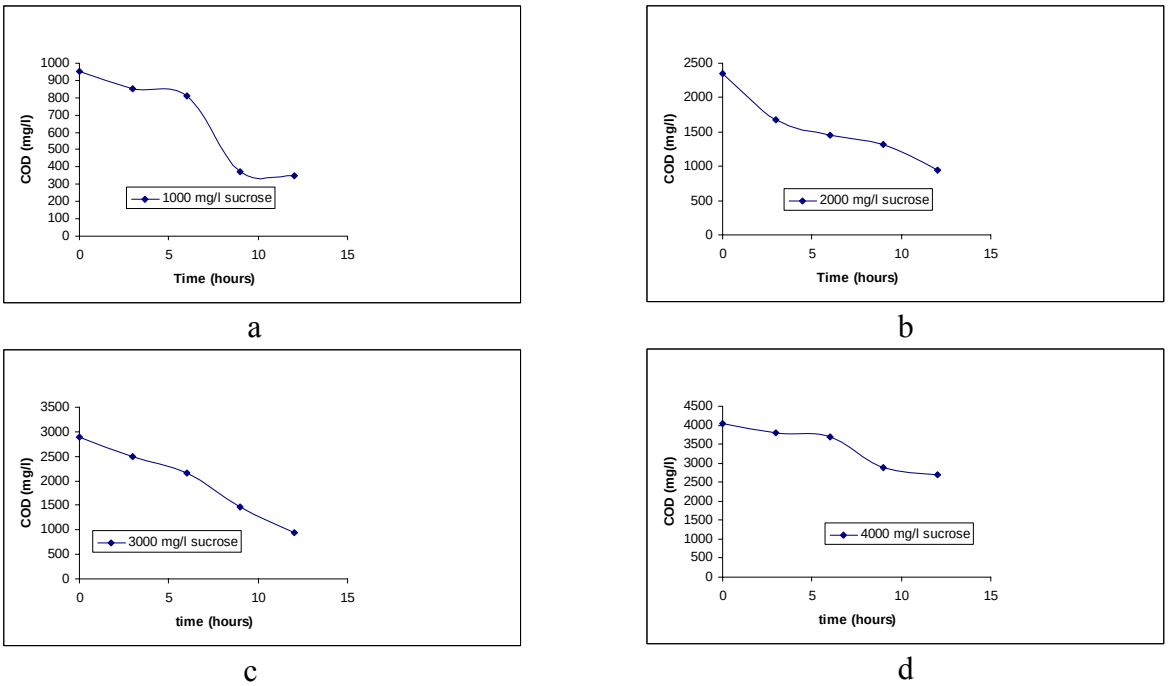


Figure 4.20. COD Measurements using Sucrose for Hydrolytic Activity (June 2007)

As mentioned above, the hydrolytic activities were not clearly observed at 1000 and 2000 mg/L due to speedy substrate consumption. On the other hand, at 3000 and 4000 mg/L sucrose concentrations the sharp decreases were not observed, but there was a slight decline in COD during the experimentation (Figure 4.20c and 4.20d).

Table 4.2. Maximum Non-Methanogenic Activities of Sludge Samples

Substrate	**Activity** (April 2007) mg COD/mg VSS.d	**Activity** (May 2007) mg COD/mg VSS.d	**Activity** (June 2007) mg COD/mg VSS.d
1000 mg/L Glucose	2.9	2.6	2.84
4000 mg/L Sucrose	3.4	3.56	3.3

Hydrolytic activity is increasing with increasing substrate concentrations as it is stated by Soto et al. (1993). Hydrolyses reaction was described as zero order kinetic reaction (Soto et al., 1993). The hydrolytic step is carried out by extracellular enzymes associated with acidogenic bacteria (Henze and Harremoes, 1983). In this study, significant COD decreases were not observed at 3000 mg/L and 4000 mg/L sucrose concentrations. It was indicated that the hydrolytic step was still dominant during the measurements, because complex substrate was not consumed in hydrolytic step but converted into monomers. This conversion did not cause COD decreases.

4.3. FISH Results

Samples were initially stained by DAPI before hybridization to observe intact cell concentration in the full-scale anaerobic EGSB reactor sludges. The microbial community structure of the sludges taken from bottom, mid and top levels of the EGSB reactor in April, May and June 2007 were characterized using fluorescent rRNA targeted oligonucleotide probes specific for Bacteria, *Archaea* and phylogenetically defined groups of Methanogens. For each sample hybridization, two negative controls were used; one of these controls was

used to assess nonspecific binding (with Non338 probe), and the other (lacking a probe) was used to monitor autofluorescence. In addition to negative controls, one positive control was used to assess success of cell permeabilization and rRNA content of the cells (with universal probe UNIV1392). Whole microbial community in the samples from three levels of the EGSB reactor and three sampling periods was also stained using DAPI stain to visualize intact cells in the samples. 10 random fields of views were used for each quantification study, namely bottom, mid and top.

Figure 4.21 shows that 41.3% ± 1.3% (mean ± standard deviation), 38.09% ± 1.3%, and 32.08% ± 0.5% of the cells belonged to archaeal domain in the bottom level of the EGSB reactor at sampling periods of April 2007, May 2007 and June 2007, respectively.

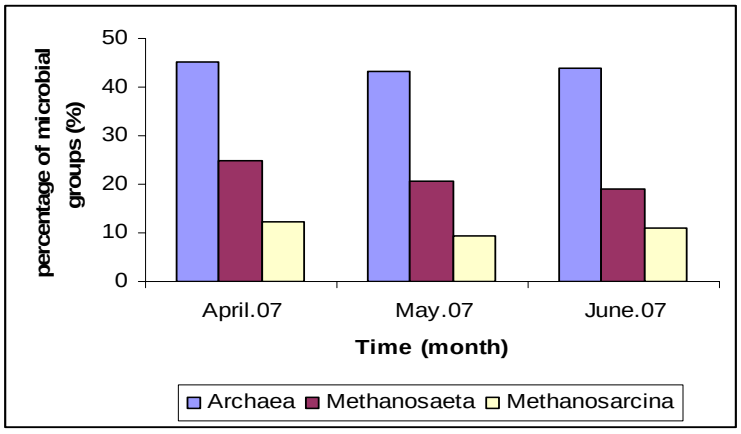


Figure 4.21. FISH Results of Anaerobic EGSB Reactor (Bottom Level)

75% total microorganisms produced positive signal, that is, 75% microorganisms were metabolically active and hybridized with UNIV1392 oligonucleotide probes. In addition, 35%±0.5% Eubacteria (with EUBMIX probe) was detected in the sludge sample taken from the bottom level in April sample. The archaeal subpopulation in April sample consisted of 41.3%±1.3% of members of the genus *Methanosaeta* (with MX825 probe), 20.03%±0.2 of *Methanosarcina* (with MS821 probe), 15.7%±0.5% of *Methanococcales* (with MC1109 probe), 10.5%±1.1% of *Methanobacteriales* (with MB310 probe), 12%±0.5% of

Methanogenium (with MG1200 probe) . In a previous study, 59%±2.6% of members of the genus *Methanosaeta* and 40%±1.3% *Methanobacteriales* were found in a full-scale UASB reactor sludge (Kolukirik, 2004).

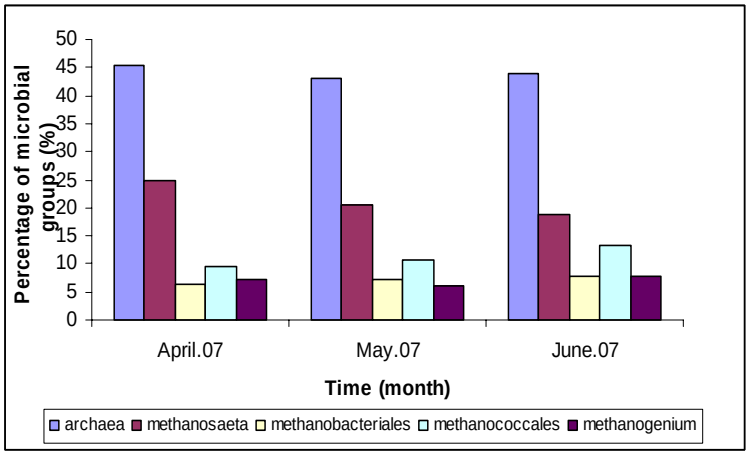
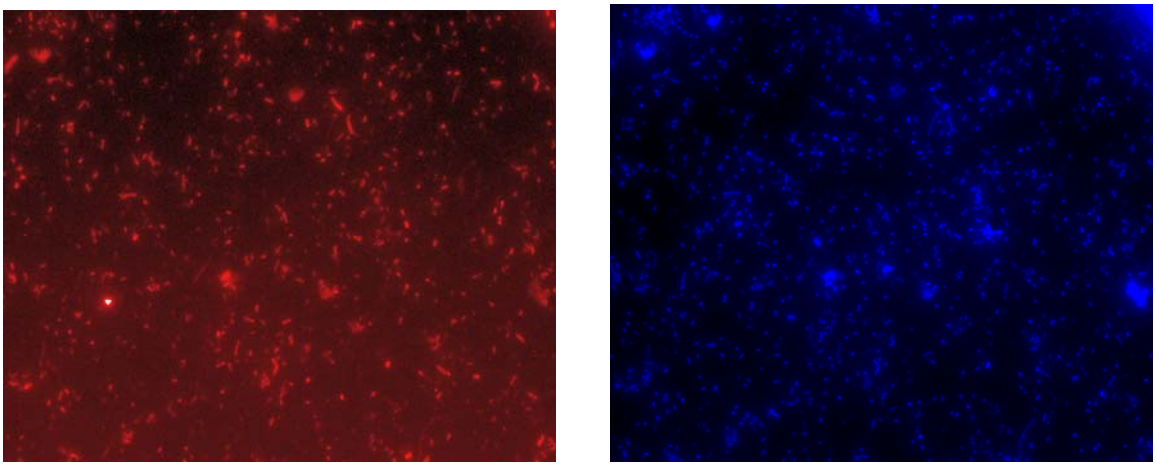
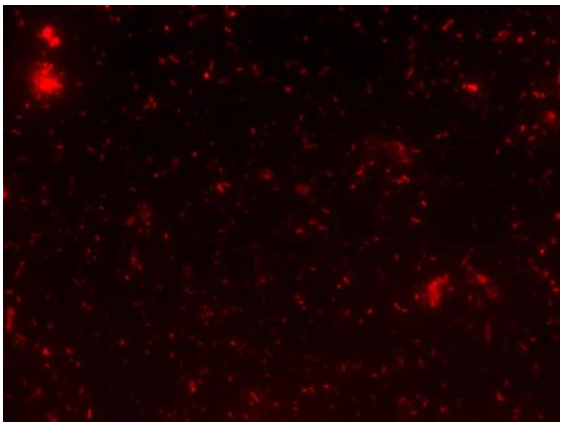


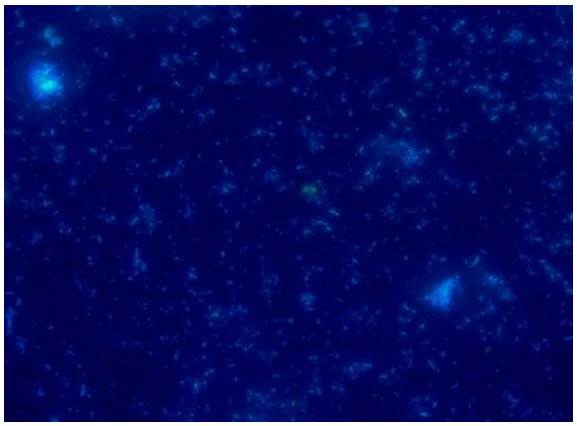
Figure 4.22. Distribution of Microbial Composition in Bottom Level of EGSB Reactor



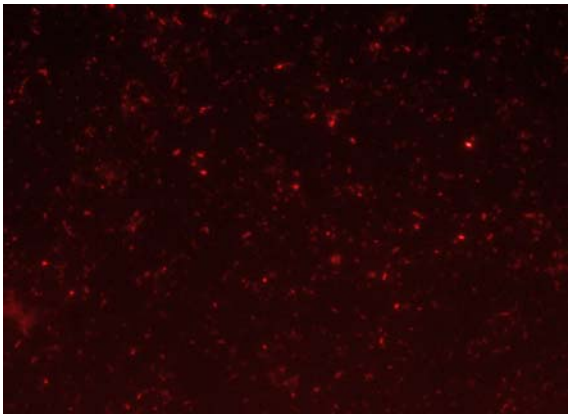
Archaea hybridized with ARC195 probe

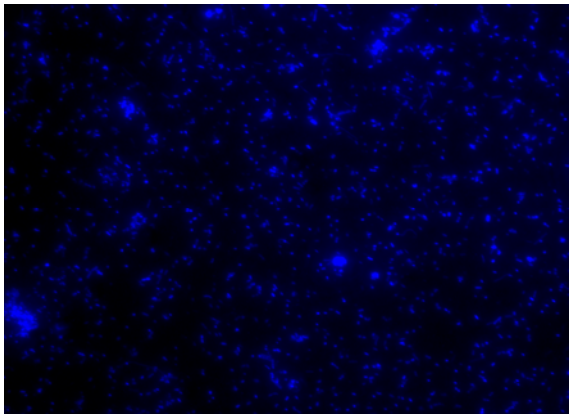
Figure 4.23. FISH Results of Anaerobic EGSB Reactor (Bottom Level, April Sample), (Fluorescent images on left, DAPI stained images on right)



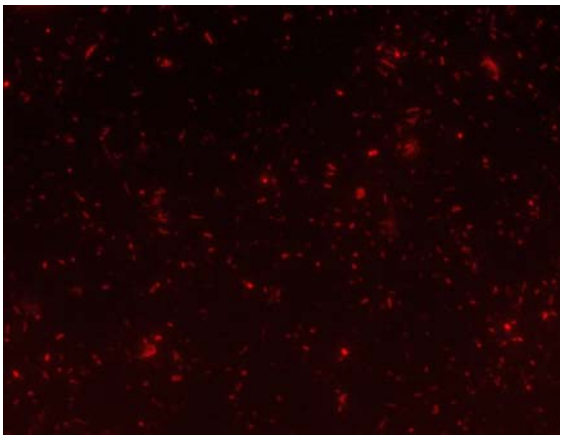


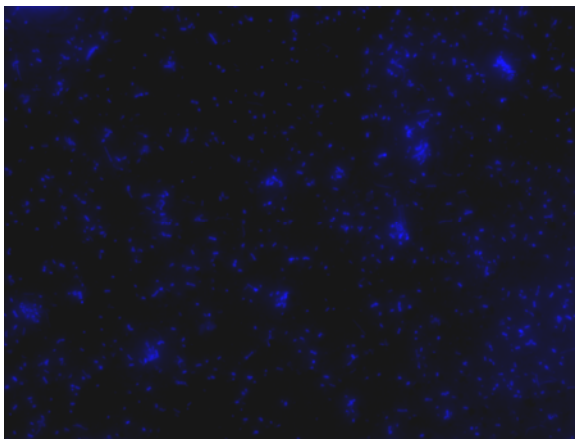
Methanobacteriales hybridized with MB310





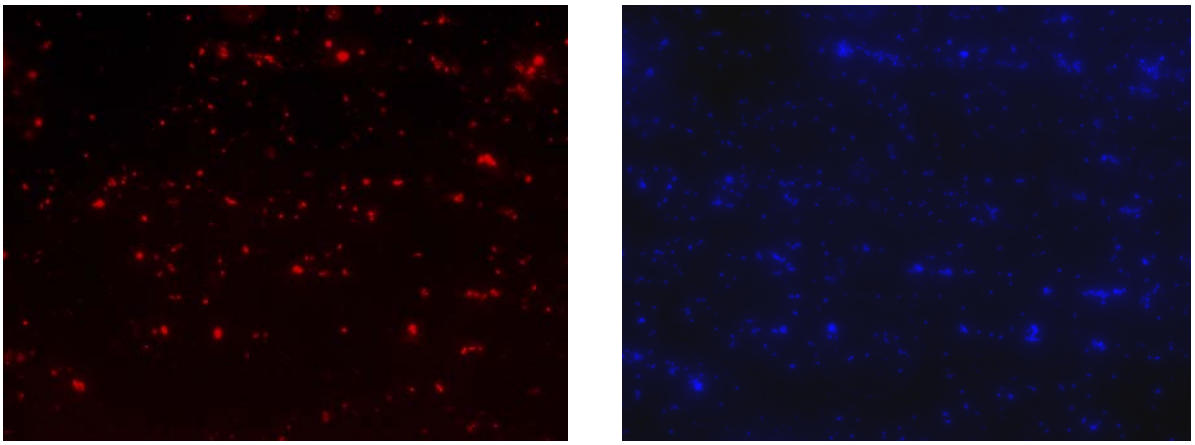
Methanococcales hybridized with MC1109



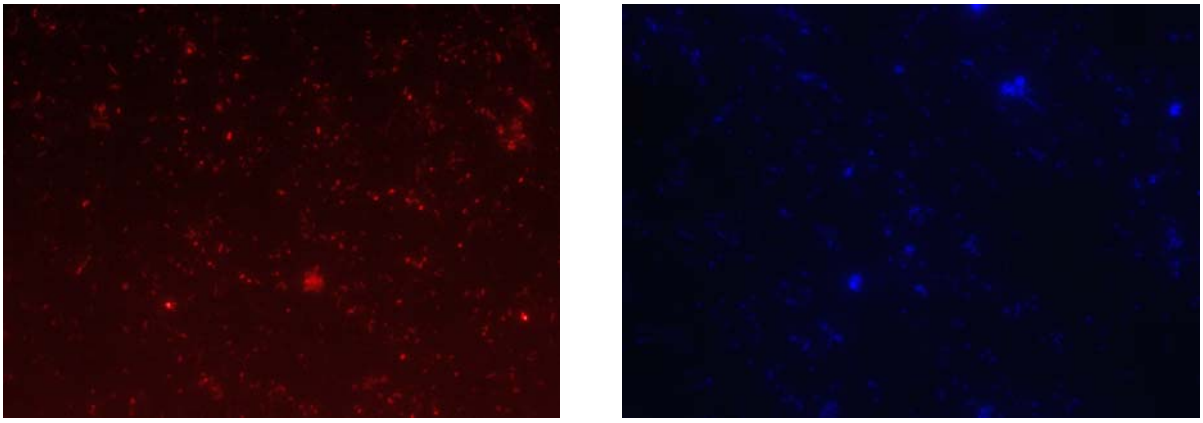


Active cells hybridized with UNIV1392

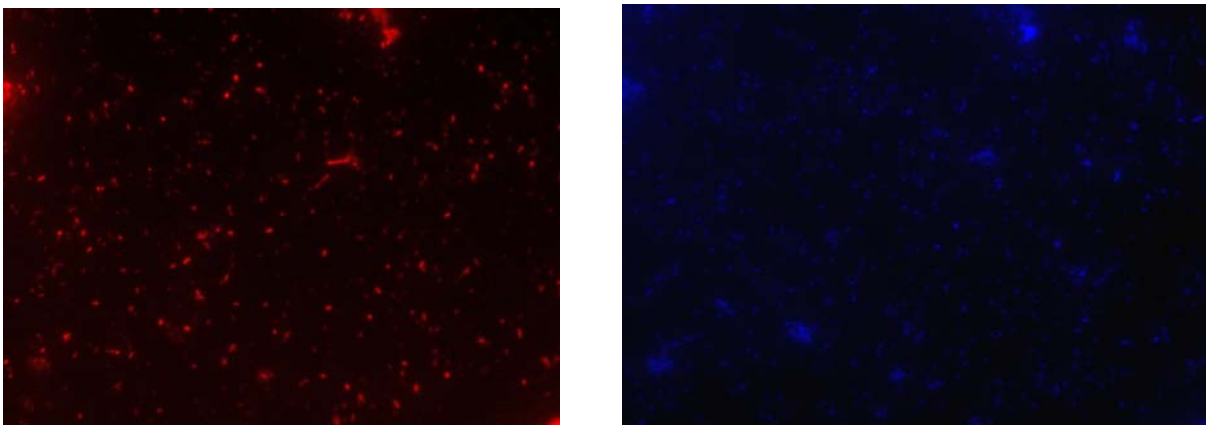
Figure 4.23. FISH Results of Anaerobic EGSB Reactor (Bottom Level, April Sample)(Continued)



Eubacteria hybridized with EUBMIX

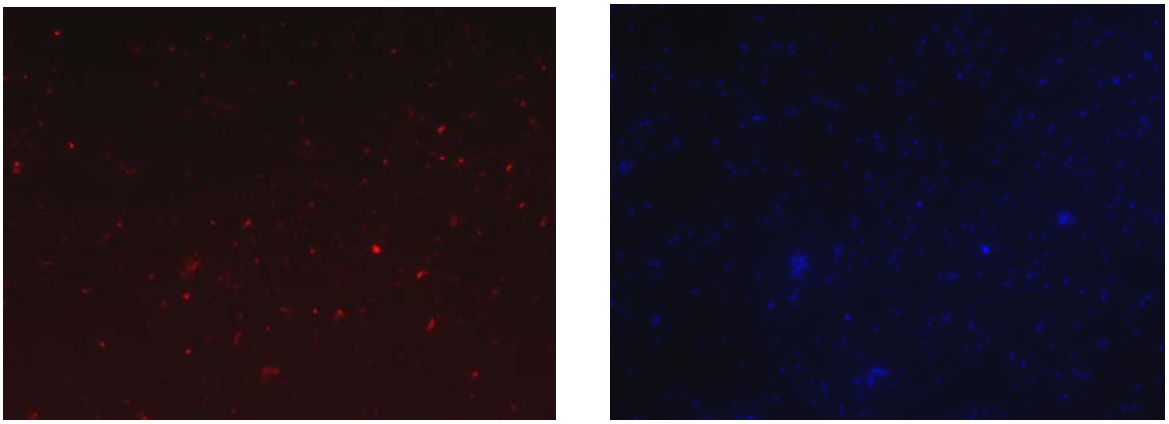


Methanosarcina hybridized with MS821



Methanosaeta hybridized with MX825

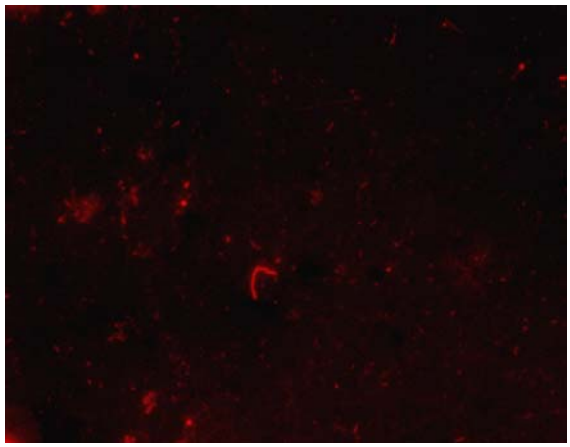
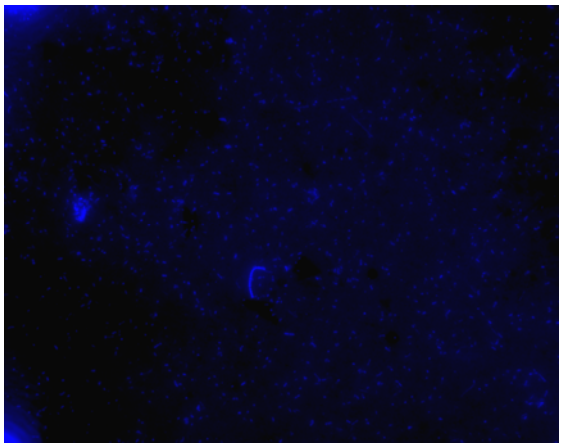
Figure 4.23. FISH Results of Anaerobic EGSB Reactor (Bottom Level, April Sample) (Continued)



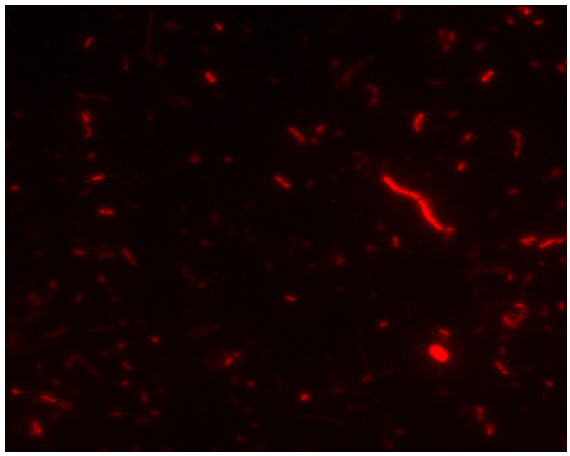
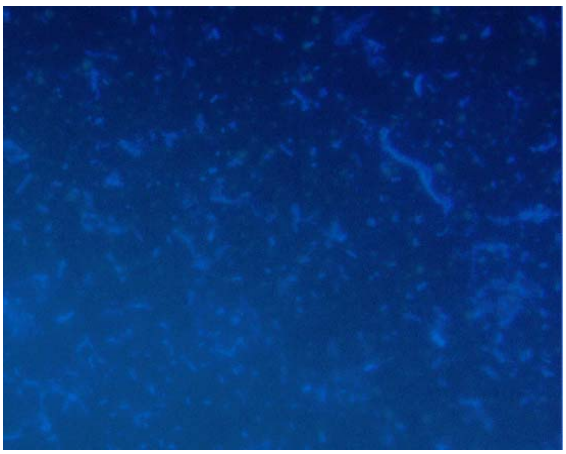
Methanogenium hybridized with MG1200

Figure 4.23. FISH Results of Anaerobic EGSB Reactor (Bottom Level, April Sample) (Continued)

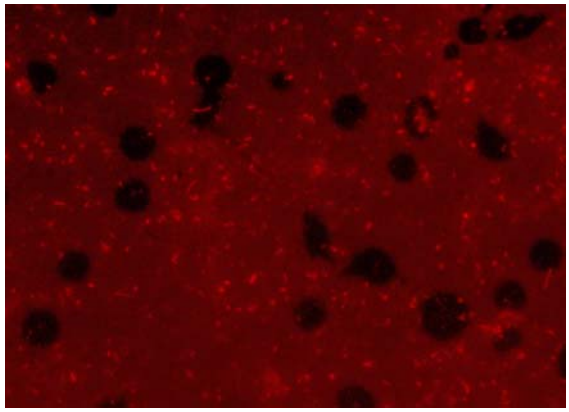
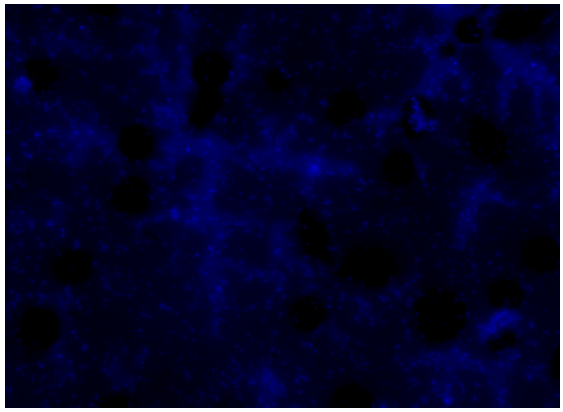
A higher percentage of active cells can be found in granular sludge from an EGSB treating brewery wastewater (Gonzales-Gil et al., 2001).In this study, total active microorganisms were found as 86%±1.6%. Bacterial population was detected as 29%±1.2%. Archaeal population decreased to 43.2%±1.1% in May sample. The subpopulation composed of 38.09%±1.3% of members of the genus *Methanosaeta*, 17.4%±0.2% *Methanosarcina,*19.8%±0.5% *Methanococcales,* 13.4%±1.1% *Methanobacteriales*, 11.9%±0.6% *Methanogenium*. In addition, the percentage of the *Methanosarcinaceae* family which composed of genus *Methanosaeta* and *Methanosarcina* was found 58.2%±1.1% correspondingly.

Active cells hybridized with UNIV1392

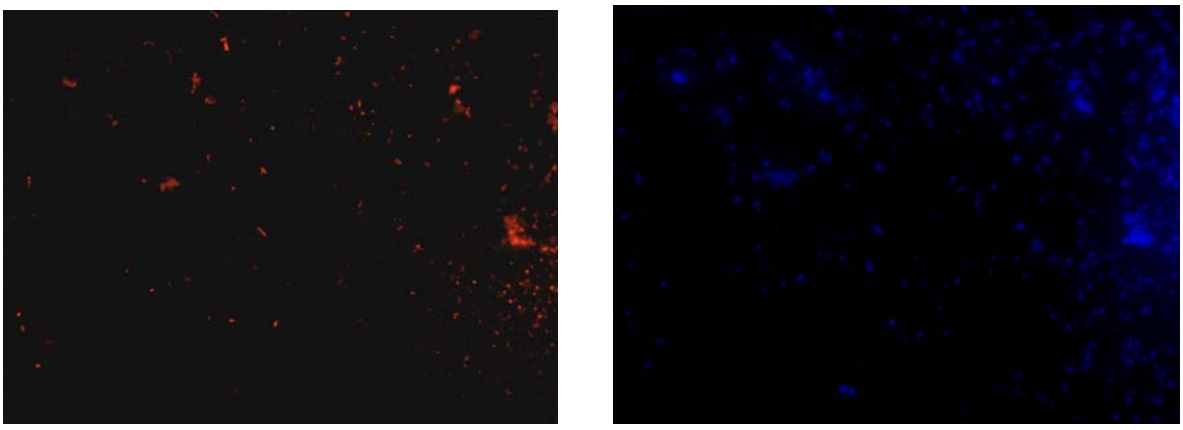



Eubacteria hybridized with EUBMIX

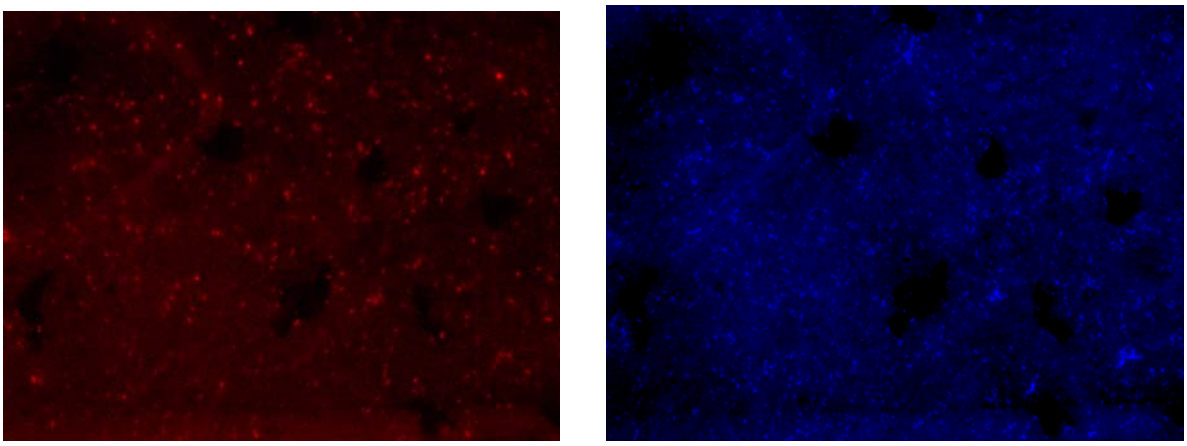



Archaea hybridized with ARC915

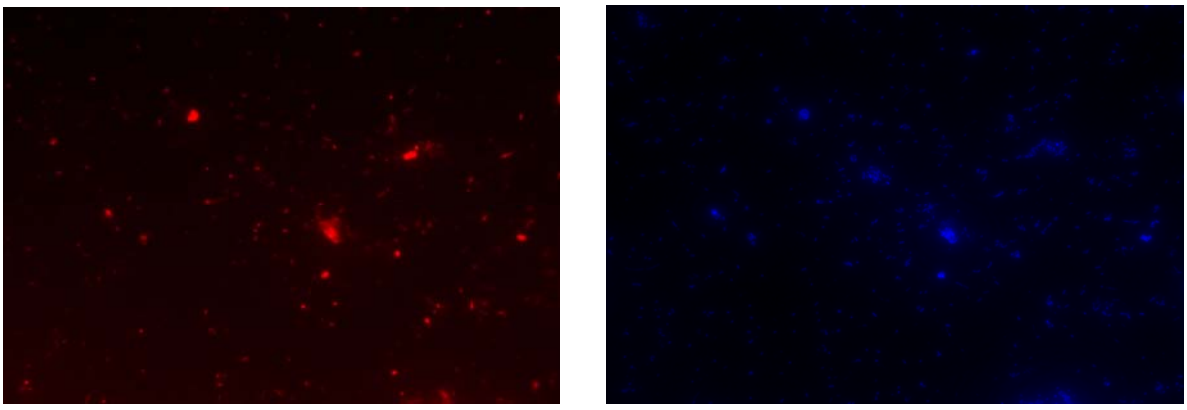
Figure 4.24. FISH Results of Anaerobic EGSB Reactor (Bottom Level, May Sample)
(Fluorescent images on left, DAPI stained images on right)



Methanobacteriales hybridized with MB310

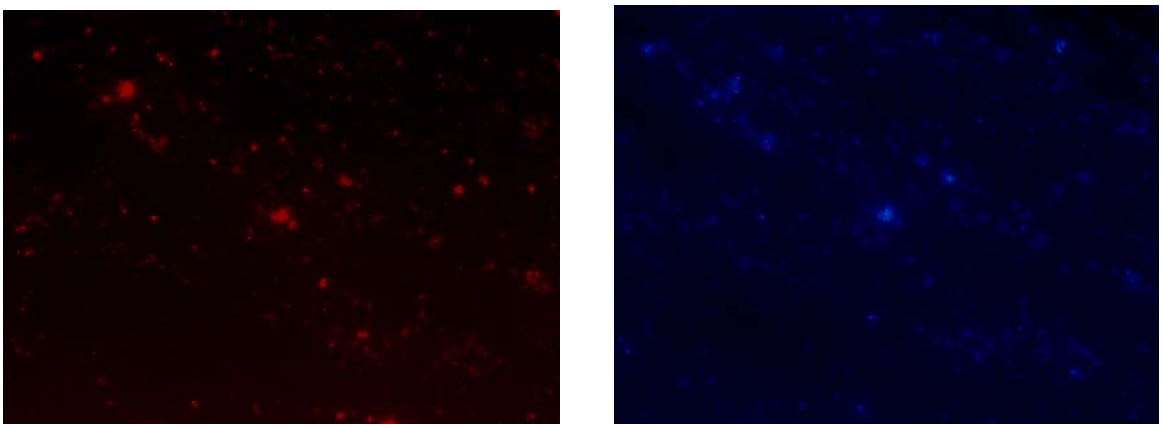


Methanococcales hybridized with MC1109

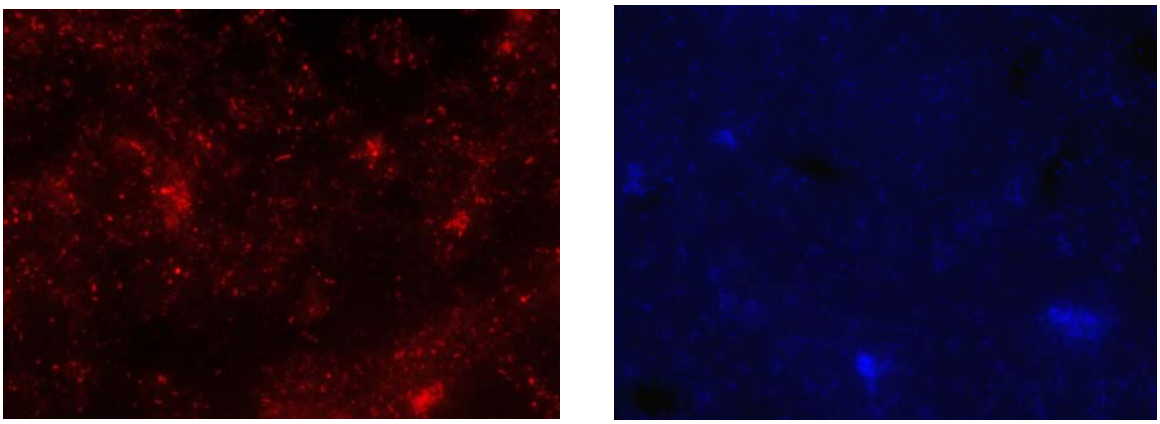


Methanogenium hybridized with MG1200

Figure 4.24. FISH Results of Anaerobic EGSB Reactor (Bottom Level, May Sample) (Continued)



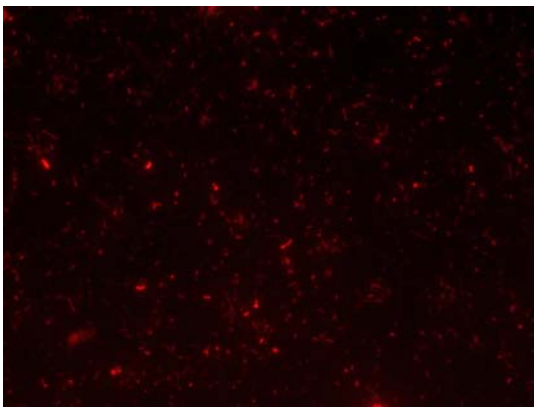
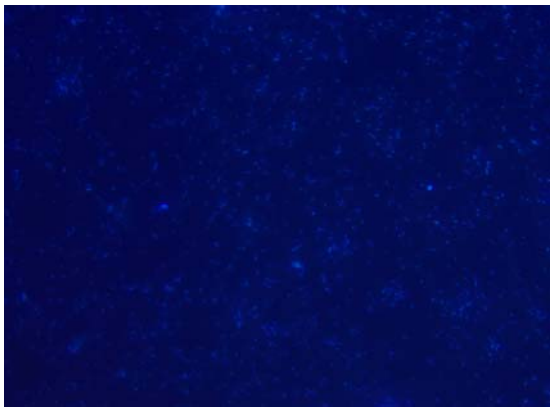
Methanosarcina hybridized with MS821



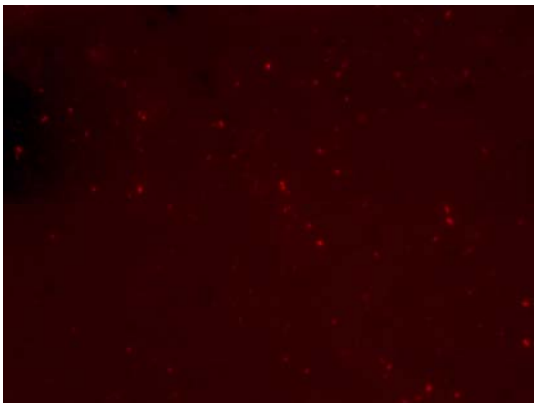
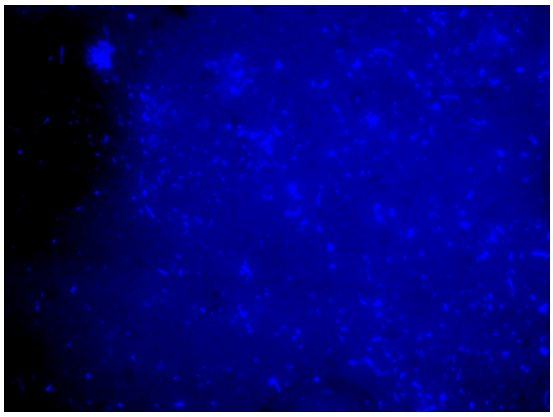
Methanosaeta hybridized with MX825

Figure 4.24. FISH Results of Anaerobic EGSB Reactor (Bottom Level, May Sample) (Continued)

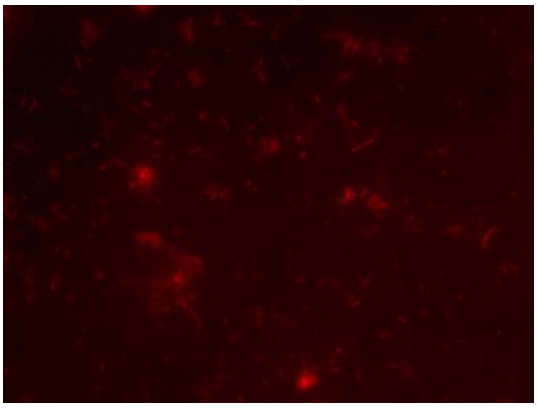
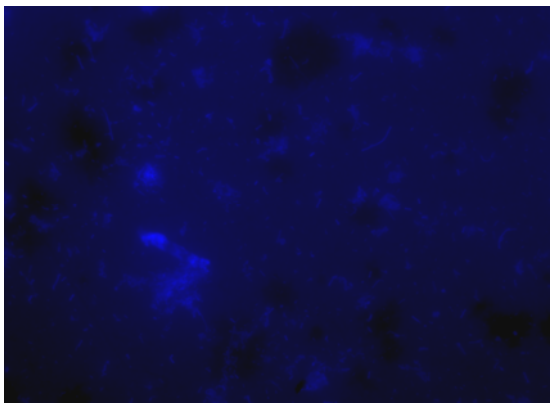
In June sample, active cells, Eubacteria and archaeal population was detected as 90.04%±1.3%, 24%±1.1% and 44%±0.6%, respectively. FISH results of the June sample shows that the percentage of the genuses of *Methanosaeta* and *Methanosarcina* were found as 32.08%±0.5% and 18.6%±0.3%, respectively. Subpopulation composed of 52.04%±0.3% of the members of *Methanosarcinaceae* family. In addition, archaeal subpopulation composed of 22.3%±0.7% *Methanococcales,* 13.04%±1.1% *Methanobacteriales*, 13.4%±0.8% *Methanogenium* were found in June sample. FISH images of June sample in bottom level were given in Figure 4.25.

Active cells hybridized with UNIV1392

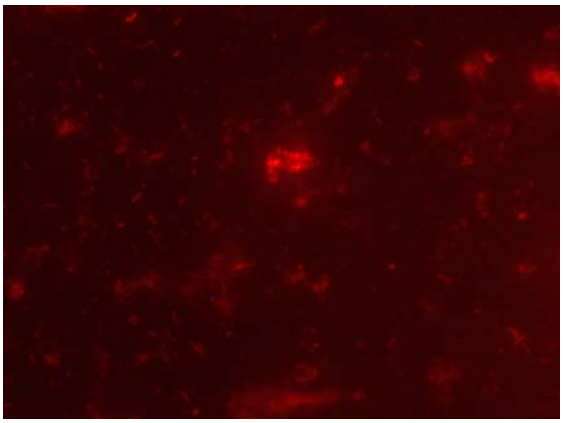



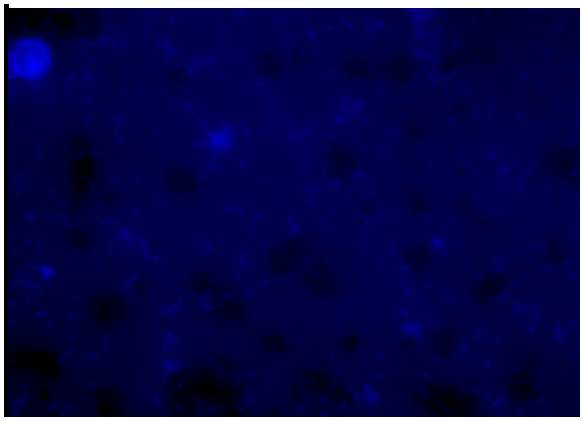
Eubacteria hybridized with EUBMIX

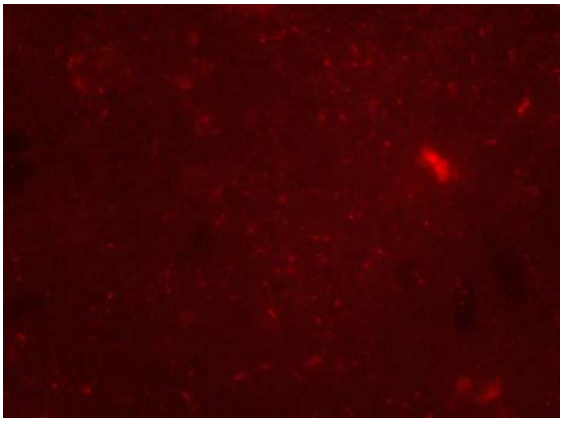
Archaea hybridized with ARC915

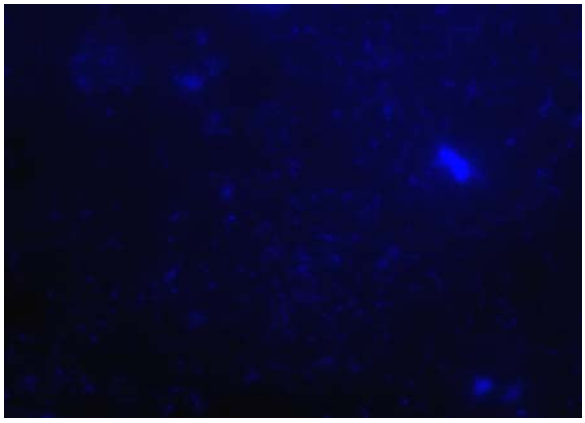
Figure 4.25. FISH Results of Anaerobic EGSB Reactor (Bottom Level, June Sample)
(Fluorescent images on left, DAPI stained images on right)



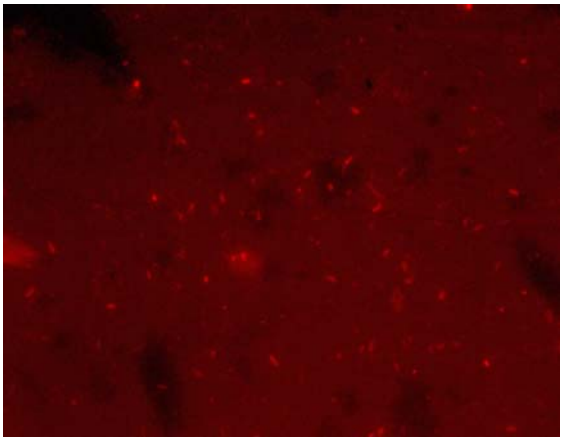


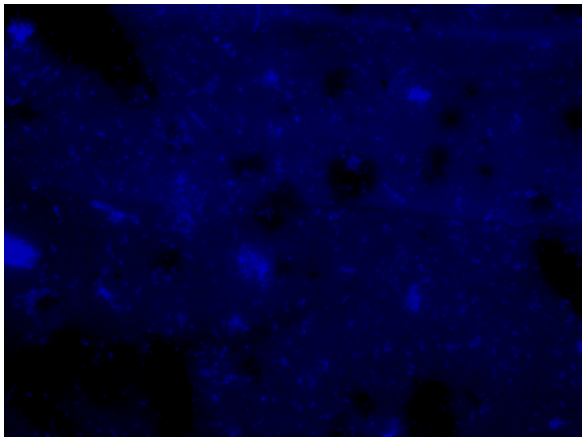
Methanosaeta hybridized with MX825





Methanosarcina hybridized with MS821

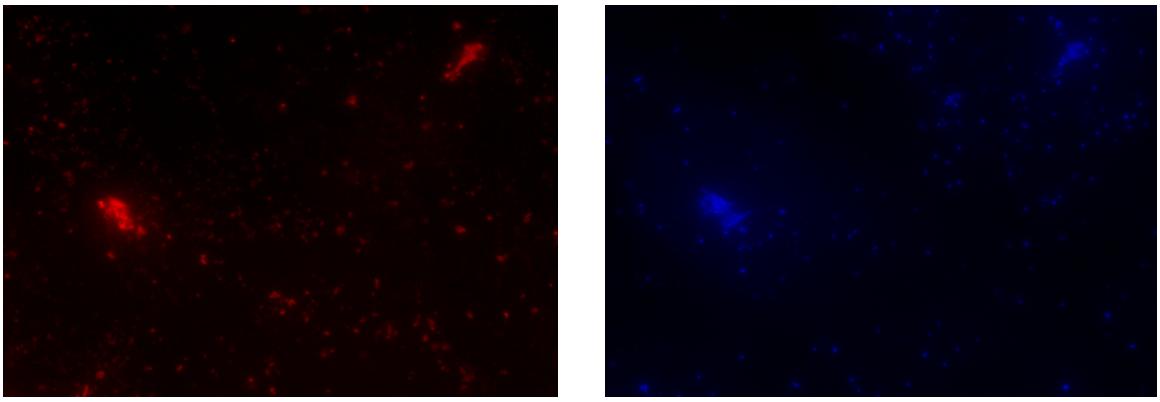




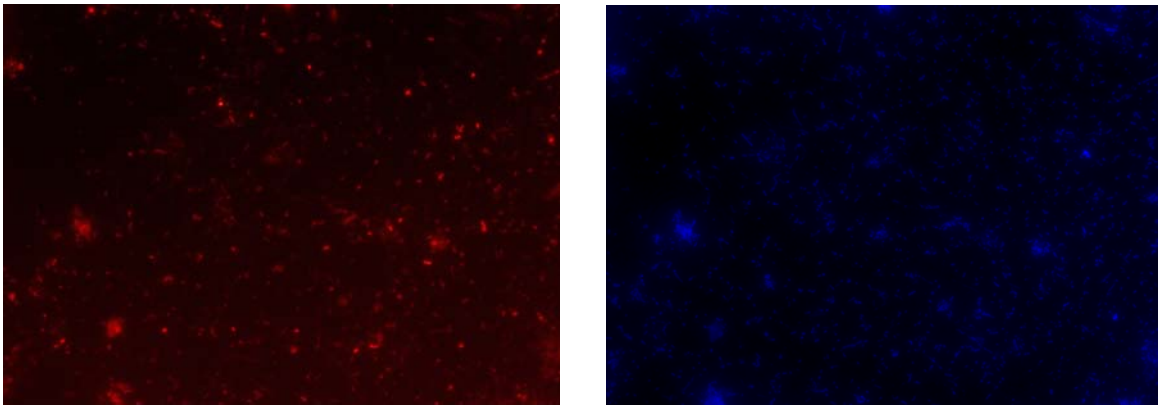
Methanogenium hybridized with MG1200

Figure 4.25. FISH Results of Anaerobic EGSB Reactor (Bottom Level, June Sample)

(Continued)



Methanobacteriales hybridized with MB310



Methanococcales hybridized with MC1109

Figure 4.25. FISH Results of Anaerobic EGSB Reactor (Bottom Level, June Sample) (Continued)

91%±1.7%, 89%±0.3%, and 87%±1.3% total microorganisms gave positive signal in the middle of the reactor for three samples. In addition, the number of Eubacteria decreased to 25%± 1.2%, 23%± 1.5%, 27%± 0.6% at April, May and June 2007, respectively. FISH images of this level are given in the Appendix B. The archaeal domain was found 24%±0.5% in April, 25%±1.2% in May, and 34%±0.7% in June 2007, respectively. The archaeal subpopulation in April sample consisted of 28%±0.8% *Methanosaeta*, 18%±0.5% *Methanosarcina,*21%±0.9% *Methanococcales*, 17%±0.4% *Methanobacteriales*, and 15%±1.4% *Methanogenium*. FISH images of the mid level are given in the Appendix C.

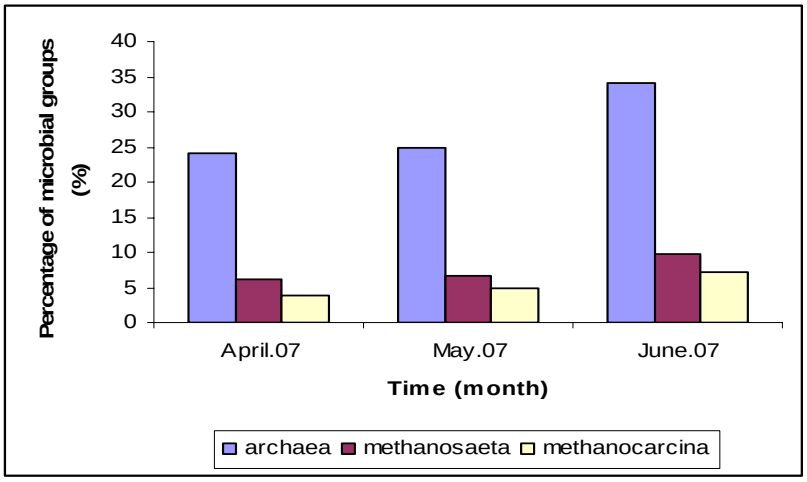


Figure 4.26. FISH Results of EGSB Reactor (Mid Level)

In addition, 26%±0.8% *Methanosaeta*, 13%±0.9% *Methanosarcina,* 27%±0.9% *Methanococcales*, and 23%±0.9% *Methanobacteriales* were found in the mid level of the reactor in May sample. Moreover, 17%±0.2% *Methanogenium* was detected. In June sample, Archaeal subpopulation consisted of 29%±1.2% *Methanosaeta*, 21%±0.4% *Methanosarcina,* 24%±0.9% *Methanococcales*, 27%±0.4% *Methanobacteriales*, and 19%±1.4% *Methanogenium*.

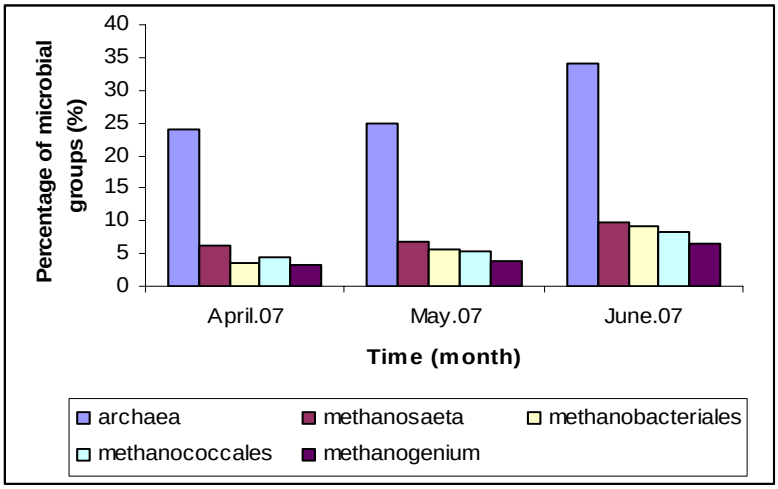


Figure 4.27. Distribution of Microbial Composition in Mid Level of EGSB Reactor

Finally, the microbial composition of the top level of the EGSB reactor was detected during three sampling periods. Total microorganisms number was detected as 93%± 0.25%, 67%±0.75% and 77%± 0.3%. Eubacteria was detected higher percentage at the top level than mid level of the reactor at all sampling periods. The quantification results of Eubacteria were found to be 37%±1.8%, 33%±1.5%, and 35%±1.7% at April, May and June 2007, respectively. In addition, Archaeal population was observed as 23%±1.25%, 22.5%±1.75, and 26%±1.6 in the top level of the reactor at April, May and June 2007, respectively. FISH images of the top level are given in the Appendix C.

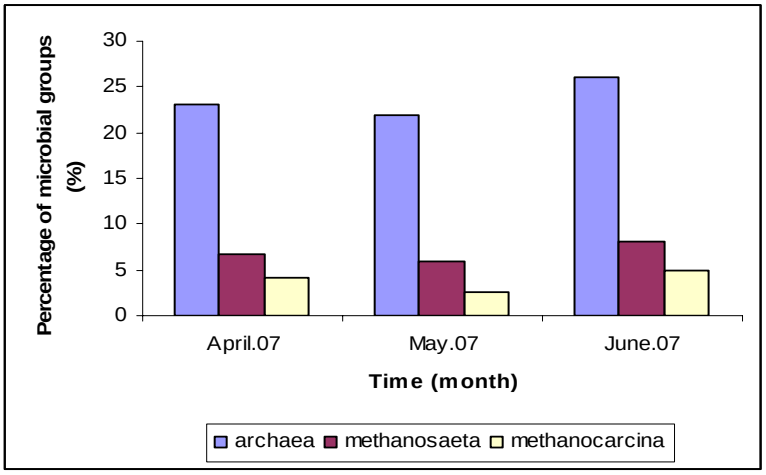


Figure 4.28. FISH Results of EGSB Reactor (Top Level)

17% ± 0.3% *Methanosaeta*, 8%±1.5% *Methanosarcina,* 27%±0.9% *Methanococcales*, and 13%± 0.7% *Methanobacteriales* were found in the top level of the reactor in April sample. In addition, 18% ± 1.2% *Methanogenium* was detected. Secondly, in May sample, 23%±1.8% *Methanosaeta*, 7.5%±1.1% *Methanosarcina,* 31%±1.9% *Methanococcales*, and 17%±1.4% *Methanobacteriales*, and 13%±0.9% *Methanogenium* were found in the top level of the reactor. In June sample, the archaeal subpopulation consisted of 19%±0.5% *Methanosaeta*, 5%±1.8% *Methanosarcina,* 28%±0.7% *Methanococcales*, and 23%±1.7% *Methanobacteriales*, and 16%±1.2% *Methanogenium* in the top level of the EGSB reactor.

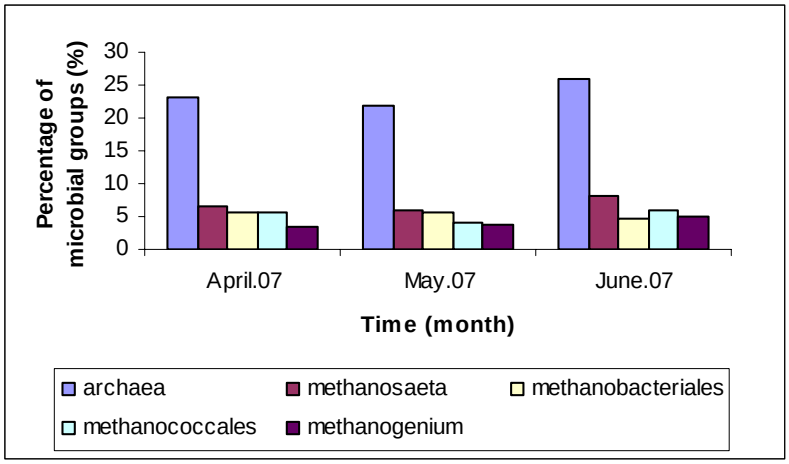


Figure 4.29. FISH results with top sample of the EGSB reactor

Table 4.3. Overall Quantification Results of Anaerobic EGSB Reactor Samples

		April 2007			May 2007			June 2007	
	bottom	**mid**	**top**	**bottom**	**mid**	**top**	**bottom**	**mid**	**top**
Active cells	75	91	93	86	89	67	90	87	77
Archaea	41	24	23	38	25	22	32	34	26
Eubacteria	35	25	37	29	23	33	24	27	35
Methanosaeta	41	28	17	38	26	23	32	29	19
Methanosarcina	20	18	8	17	13	7	18	21	5
Methanobacteriales	10	17	13	19	23	13	13	27	23
Methanococcales	15	21	27	13	27	31	22	24	28
Methanogenium	12	15	18	11	17	17	13	19	16

The results did not show any significant changes in the methane archaeal composition and all along the anaerobic reactor except decreasing in number of the microorganisms. On the other hand, slight changes occurred between archaeal subpopulation during sampling periods.

Two acetate-utilizing methanogenic genera, *Methanosarcina* and *Methanosaeta*, have been identified as important methanogens in granular sludge from upflow anaerobic sludge blanket (UASB) reactors (de Zeeuw, 1984; Grotenhuis, 1988; Hulshoff, 1989; Schmidth, 1996). *Methanosaeta spp.* are known to grow only on acetate (Jetten et al., 1992). Besides acetate, *Methanosarcina spp.* is also capable of growing on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide. *Methanosaeta spp*. have a lower growth rate at high acetate concentrations than do *Methanosarcina spp*., but their affinity for

acetate is 5 to 10 times higher (Jetten et al., 1992; Zinder, 1990). It is generally assumed that *Methanosaeta spp*. improves granulation and result in more stable reactor performance; consequently, *Methanosaeta spp.* should be favored over *Methanosarcina spp*. As it was also found in this study, *Methanosaeta spp*. was more numerous than *Methanosarcina spp*. at three sampling periods.

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). However, in this study, predominance of acetoclastic methanogens (*Methanosaeta spp*.) has tended to change to hydrogenotrophic methanogens (mainly *Methanococcales* and then *Methanobacteriales*) in the EGSB reactor. The tendency of acetoclastic methanogens to hydrogenotrophics can also be supported by deterioration in performance and stability of the anaerobic EGSB reactor.

In this study, the percentage of *Methanococcales* was higher than *Methanobacteriales.* Thus, predominance of *Methanococcales* and less abundance of *Methanobacteriales* in a subpopulation of hydrogenotophic methanogens are more difficult to explain, since the competition for common substrates among different hydrogenotophic methanogens has been studied less extensively than the competition for acetate among acetoclastic methanogens (Raskin et al., 1996).

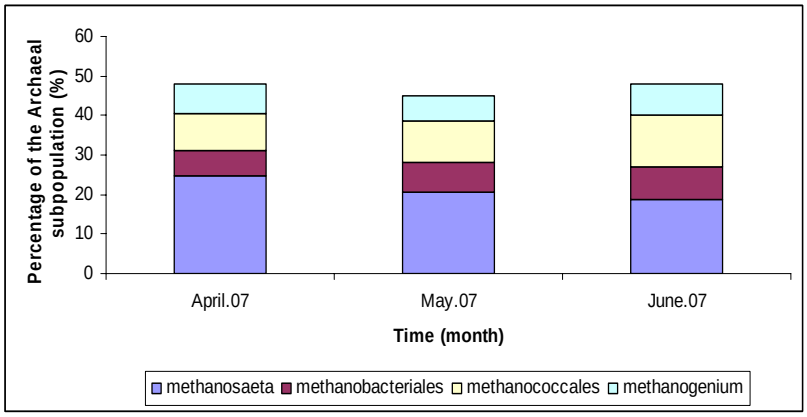


Figure 4.30. The percentage distribution of Archaeal subpopulation

As it was shown in Figure 4.30 the percentage of *Methanosaeta* decreased gradually during the three sampling periods in the bottom samples, whereas, the genera of *Methanococcales* increased slightly. It indicated that a syntrophic relationship between an acetate-oxidizing organism and a hydrogenotrophic methanogen has become an important route of acetate degradation in this reactor. As a result of that, the FISH results revealed that *Methanosaeta spp.* were the predominant methanogen in the EGSB reactor. However, predominance of *Methanosaeta spp.* had tended to change to hydrogenotrophic methanogens in the reactor during sampling periods. The relationship between this tendency and deterioration of anaerobic reactor performance was already explained of changing acetoclastic to hydrogenotrophic methanogens. There was a good correlation between SMA test and FISH results which revealed a decrease in the acetoclastic methanogenic activity of the EGSB sludge from 457 to 447 mL CH_4/g VSS.d mL coincided with a decrease in the relative abundance of acetoclastic *Methanosaeta* from 41.3%±1.3% to 32.08%±0.5% of the archaeal population. Relative abundance of hydrogenotrophic *Methanococcales* increased from 21%±0.5% to 30%±0.7% during the sampling periods.

Another study stated that The SMA test and (FISH) results revealed that a decrease in the acetoclastic methanogenic activity of the UASB sludge from 344 mL CH_4/gTVS d to 109 mL CH_4/g TVS.d coincided with a decrease in the relative abundance of acetoclastic Methanosaeta from 90%±1.2% to 79%±1.4% of the archaeal population, and an increase in the relative abundance of hydrogenotrophic Methanobacteriales from non-detectable levels to 24%±0.7% of the archaeal population during the 2-years operation of the UASB reactor. The relative abundance of archaeal cells within the UASB sludge was in the range of 15–17% (Kolukirik et al., 2007).

FISH has proven a useful method to characterize microbial community structures of anaerobic reactors treating various wastewaters. Merkel et al. (1999) studied the population dynamics in the membrane-coupled anaerobic pilot scale reactor, with population data provided by FISH technique. FISH results showed that the anaerobic population was dominated by *Archaea* belonging to the genus *Methanosaeta.* Gonzalez-Gil et al. (2001)

described ultrastructure of mesophilic aggregates from a full-scale expanded granular sludge bed reactor treating brewery wastewater. Fluorescent in situ hybridization using 16S rRNA probes of crushed granules showed that 70% and 30% of the cells belonged to the archaebacterial and eubacterial domains, respectively. Harmsen et al. (1996) used FISH to detect and localize microorganisms in the granules of two lab-scale upflow anaerobic sludge blanket reactors that had been fed for several months with either sucrose or a mixture of volatile fatty acids. The granules fed with volatile fatty acids showed an outer layer of mainly bacteria and then a thick layer of *Methanosaeta*-like methanogens mixed with a few bacteria and a layer of methanogens mixed with syntrophic propionate-oxidizing strains.

5. CONCLUSIONS

Anaerobic treatment has been widely used in different industries including brewery industry. Different wastewater composition leads to development of several reactor configurations. EGSB reactor is one of them which have effective treatment efficiency on the highly polluted, high and middle-strength wastewater. In this study, the sludge samples taken from the EGSB reactor treating brewery wastewater during a three month period (April, May and June 2007) were analyzed using SMA test and FISH.

In this study, activities of acetoclastic and the other trophic groups were determined. The maximum acetoclastic methanogenic activity was found to be 457 mL CH_4/g VSS.d for April sample. In addition, the maximum specific methanogenic activity was found as 460 mL CH_4/g VSS.d with butyrate for June sample. The maximum specific methanogenic activity was measured as 250 mL CH_4/g VSS.d with propionate in April sample. Finally, the VFA mixture (2000 mg/L acetate, 500 mg/L butyrate and 500 mg/ l propionate) produced a maximum overall methanogenic activity of 400 mL CH_4/g VSS.d in April sample.

Furthermore, non-methanogenic activity was measured according to substrate utilization rate. June sample had the maximum acidogenic activity of 2.84 mg COD/mg VSS. d with glucose. In addition, the hydrolytic step was dominant in 3000 and 4000 mg/L sucrose concentrations at three sampling periods.

Methanogenic and non-methanogenic activity test results showed that the anaerobic sludge has potential capacity to degrade organic pollutants, since all results of the activity measurements were found to be in parallel with previous studies reported in literature.

FISH results showed that *Methanosaeta spp*. were found to be the predominant methanogen all along the EGSB reactor in throughout the sampling period. However, predominance of acetoclastic methanogens (*Methanosaeta spp*.) has tended to change to

hydrogenotrophic methanogens (mainly *Methanococcales* and then *Methanobacteriales*) in the EGSB reactor during the sampling period. In addition, the numbers of the methanogens decreased apparently from bottom to top of the anaerobic EGSB reactor.

When overall performance of the full-scale anaerobic EGSB reactor, methanogenic and non-methanogenic activities and FISH results of the reactor sludge were analyzed, it can be said that the full-scale anaerobic EGSB reactor might have not been operated at optimum operating conditions particularly during the sampling period. This was reflected as poor performance and instability of the EGSB reactor.

6. RECOMMENDATIONS

In this study, the performance, numbers, compositions and activities of microbial populations in the full-scale anaerobic EGSB reactor were clearly evaluated.

Both methanogenic and non-methanogenic activities should be determined using different substrates such as formate, H_2-CO_2, glycogen, starch. In addition to this, various inhibitory substrates (solvents, chlorinated organics etc.) can be used for determination of their adverse effects.

In addition to determination of methanogenic archaeal composition, proteobacterial population should be identified and quantified to understand non-methanogenic steps clearly. Moreover, sulphate reducing bacteria which are mostly encountered in anaerobic reactors should be identified to observe syntrophic relationships.

Furthermore, PCR-based methods can be used to constitute 16S rRNA clone library. Hence, whole microbial composition can be explained.

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APPENDIX A: SMA TEST RESULTS

Table A.1. April Sample SMA Test Results

Acetate concentrations (mg/L)								
	1000	2000	3000	4000	1000	2000	3000	4000
Time (hours)	**Pulse**				**SMA (mL CH_4 / gVSS.d)**			
1	37	48	49	22	148	173	222	84
2	41	50	50	24	163	180	226	92
3	47	53	54	23	187	191	244	88
4	55	59	65	28	219	212	294	107
5	58	60	68	39	231	216	308	149
6	57	68	69	51	227	245	312	195
7	56	87	76	66	223	313	344	252
8	58	93	77	74	231	335	348	283
9	59	94	84	78	235	338	380	298
10	61	103	88	79	243	371	398	302
11	66	108	88	80	263	389	398	306
12	65	108	88	87	259	389	398	333
13	67	115	90	88	267	414	407	337
14	69	118	93	89	275	425	421	340
15	68	119	95	91	271	428	430	348
16	71	120	97	92	283	432	439	352
17	73	121	100	93	291	436	452	356
18	77	124	97	95	307	446	439	363
19	79	124	96	92	315	456	434	352
20	78	128	94	90	311	448	425	344
21	81	129	91	89	323	448	412	340
22	85	131	89	86	339	472	403	329
23	85	129	86	84	339	447	389	321
24	87	129	83	79	347	452	375	302
25	85	129	80	75	339	452	362	287
26	86	129	75	72	343	453	339	275
27	73	132	73	65	291	455	330	249
28	71	128	72	58	283	451	326	222
29	72	126	72	46	287	454	326	176
30	65	124	70	44	259	446	317	168
31	61	120	69	43	243	432	312	164
32	47	117	67	43	187	435	303	164
33	34	111	68	39	136	400	308	149
34		104	66	22		374	298	84
35		76	60			274		
36			49			68		

Table A.1. April Sample SMA Test Results (Continued)

Butyrate Concentrations (mg/L)										
	1000	2000	3000	4000	5000	1000	2000	3000	4000	5000
Time (hours)	**Pulse**					**SMA (mL CH_4 / gVSS.d)**				
1	68	8	55	30	5	154	40	137	187	25
2	76	26	60	32	13	172	131	150	200	64
3	71	36	55	35	22	160	181	137	219	74
4	68	34	47	49	25	154	171	117	306	69
5	70	34	45	49	21	158	171	112	306	69
6	72	33	48	50	18	163	166	120	312	89
7	74	34	51	53	16	167	171	127	331	79
8	75	36	55	51	14	169	181	137	318	69
9	79	36	53	53	15	178	181	132	331	74
10	82	38	64	56	16	185	191	160	350	79
11	81	39	70	57	17	183	196	175	356	84
12	82	41	71	57	17	185	207	177	356	84
13	85	43	70	58	18	192	217	175	362	89
14	87	45	75	61	20	196	227	187	381	99
15	88	45	73	64	20	199	227	182	400	99
16	86	47	77	64	21	194	237	192	400	104
17	87	45	76	63	20	196	227	190	393	99
18	92	46	73	63	21	208	232	182	393	104
19	92	46	74	64	30	208	232	185	400	148
20	89	45	80	64	24	201	227	200	400	119
21	85	43	81	58	26	192	217	202	362	129
22	82	42	76	60	24	185	212	190	375	119
23	75	41	82	57	26	169	207	205	356	129
24	74	40	85	61	27	167	202	212	381	134
25	56	41	85	59	28	126	207	212	368	139
26	21	38	84	59	29	47	191	210	368	143
27		37	87	62	30		186	217	387	148
28		38	92	58	32		191	230	362	158
29		27	91	60	35		136	227	375	173
30		19	97	53	35		96	242	331	173
31		8	97	17	36		40	242	106	178
32			99	11	37			247	69	183
33			103		39			257		193
34			107		39			267		193
35			107		40			267		198
36			123		40			307		198
37			124		42			310		208
38			126		42			315		208
39			124		45			310		223
40			127		46			317		228
41			126		45			315		223
42			127		45			317		223
43			125		47			312		233
44			124		49			310		242

Table A.1. April Sample SMA Test Results (Continued)

45			96		48			240		237
46			78		50			195		247
47					51					252
48					51					252
49					52					257
50					53					262
51					55					272
52					55					272
53					54					267
54					56					277
55					58					287
56					58					287
57					59					292
58					59					292
59					75					322
60					62					307
61					71					302
62					64					317
63					63					312
64					65					322
65					66					326
66					67					331
67					67					331
68					68					336
69					68					336
70					68					336
71					68					336
72					67					331
73					66					326
74					63					326
75					65					312
76					63					322
77					63					326
78					61					307
79					62					292
80					62					302
81					57					302
82					64					292
83					64					282
84					55					272
85					52					257
86					53					262
87					51					252
88					51					252
89					44					262
90					42					257
91					33					218

Table A.1. April Sample SMA Test Results (Continued)

Propionate Concentrations (mg/L)								
	1000	2000	3000	4000	1000	2000	3000	4000
Time (hours)	**Pulse**				**SMA (mL CH_4 / gVSS.d)**			
1	18	27	19	25	59	145	87	75
2	27	25	19	31	88	134	87	101
3	31	27	43	33	101	145	196	108
4	30	29	48	32	98	155	219	104
5	29	27	57	35	95	145	260	114
6	26	28	56	37	85	150	255	121
7	28	31	57	37	91	166	260	121
8	26	35	58	38	85	188	264	124
9	27	34	55	41	88	182	251	134
10	26	36	57	38	85	193	260	124
11	27	37	56	41	88	198	255	134
12	27	35	55	45	88	188	251	147
13	29	37	54	45	95	198	246	147
14	30	41	56	47	98	220	255	153
15	35	41	57	45	114	220	260	147
16	33	40	55	48	108	214	251	156
17	34	43	54	51	111	230	246	166
18	35	41	53	57	114	220	241	186
19	37	40	53	54	121	214	241	176
20	37	39	52	58	121	209	237	189
21	36	38	54	58	118	204	246	189
22	38	43	55	59	124	230	251	192
23	37	45	52	61	121	241	237	199
24	41	38	51	69	134	204	232	225
25	43	39	48	65	140	209	219	212
26	42	37	46	67	137	198	210	218
27	41	27	47	68	134	145	214	222
28	41	25	45	69	134	134	205	225
29	43	27	45	68	140	145	205	222
30	42	26	42	67	137	139	191	218
31	43	19	40	68	140	102	182	222
32	45		37	69	147		169	225
33	45		35	72	147		159	235
34	46		32	71	150		146	231
35	47		33	73	153		150	238
36	48		37	73	160		169	238
37	47		36	72	153		164	235
38	48		37	74	157		169	241
39	48		36	75	153		164	244
40	47		35	71	157		159	231
41	46		19	68	157		87	222
42	47			65	153			212
43	35			67	150			218
44	31			63	153			205
45	27			57	114			186
46	25			55	101			179
47	18			41	88			134
48				37				121

Table A.2. May Sample SMA Test Results

Acetate concentrations (mg/L)								
	1000	2000	3000	4000	1000	2000	3000	4000
Time (hours)	**Pulse**				**SMA (mL CH_4/gVSS.d)**			
1	37	48	49	22	148	173	222	84
2	41	50	50	24	163	180	226	92
3	47	53	54	23	187	191	244	88
4	55	59	65	28	219	212	294	107
5	58	60	68	39	231	216	308	149
6	57	68	69	51	227	245	312	195
7	56	87	76	66	223	313	344	252
8	58	93	77	74	231	335	348	283
9	59	94	84	78	235	338	380	298
10	61	103	88	79	243	371	398	302
11	66	108	88	80	263	389	398	306
12	65	108	88	87	259	389	398	333
13	67	115	90	88	267	414	407	337
14	69	118	93	89	275	425	421	340
15	68	119	95	91	271	428	430	348
16	71	120	97	92	283	432	439	352
17	73	121	100	93	291	436	452	356
18	77	124	97	95	307	446	439	363
19	79	124	96	92	315	456	434	352
20	78	128	94	90	311	448	425	344
21	81	129	91	89	323	448	412	340
22	85	131	89	86	339	472	403	329
23	85	129	86	84	339	447	389	321
24	87	129	83	79	347	452	375	302
25	85	129	80	75	339	452	362	287
26	86	129	75	72	343	453	339	275
27	73	132	73	65	291	455	330	249
28	71	128	72	58	283	451	326	222
29	72	126	72	46	287	454	326	176
30	65	124	70	44	259	446	317	168
31	61	120	69	43	243	432	312	164
32	47	117	67	43	187	435	303	164
33	34	111	68	39	136	400	308	149
34		104	66			374	298	
35		76	60			274	271	
36		19				68		

Table A.2. May Sample SMA Test Results (Continued)

Butyrate Concentrations (mg/L)										
	1000	2000	3000	4000	5000	1000	2000	3000	4000	5000
Time (hours)	**Pulse**					**SMA (mL CH_4/gVSS.d)**				
1	27	11	17	16	17	105	49	80	74	73
2	36	15	17	22	26	140	62	80	102	111
3	44	20	23	30	31	171	83	109	140	132
4	54	23	35	47	33	210	96	166	219	141
5	55	29	46	49	33	214	120	218	228	141
6	58	37	46	50	32	226	154	218	233	137
7	58	41	47	51	31	226	170	223	237	132
8	57	43	47	55	28	222	179	223	256	119
9	53	44	49	58	37	206	183	232	270	158
10	53	45	50	65	34	206	187	237	302	145
11	55	44	52	68	35	214	183	246	316	149
12	55	45	52	71	35	214	187	246	330	149
13	57	45	53	75	36	222	187	251	349	154
14	57	47	54	77	34	222	195	256	358	145
15	58	50	56	81	37	226	208	265	377	158
16	58	48	55	83	37	226	199	260	386	158
17	57	51	56	85	37	222	212	265	395	162
18	56	53	58	89	37	218	220	275	414	166
19	54	55	57	92	38	210	228	270	428	175
20	53	56	58	91	39	206	233	275	423	179
21	52	58	56	93	41	203	241	265	432	188
22	45	59	56	92	41	175	245	265	428	192
23	36	58	55	93	42	140	241	260	432	188
24		57	53	92	44		237	251	428	196
25		55	54	92	45		228	256	428	196
26		48	43	87	44		199	204	405	171
27		37	35	83	46		154	166	386	243
28		15		82	46		62		381	239
29				71	40				330	243
30				53	57				246	252
31					56					260
32					57					265
33					59					260
34					61					252
35					62					265
36					61					277
37					59					290
38					62					290
39					65					294
40					68					286
41					68					290
42					69					299

Table A.2. May Sample SMA Test Results (Continued)

43					67					303
44					68					303
45					70					311
46					71					307
47					71					307
48					73					311
49					72					303
50					72					294
51					73					294
52					73					290
53					71					294
54					69					286
55					69					290
56					68					282
57					69					286
58					67					277
59					68					265
60					66					260
61					67					243
62					65					243
63					62					209
64					61					235
65					57					230
66					57					230
67					49					235
68					55					226
69					54					218
70					54					209
71					55					201
72					53					188
73					51					179
74					49					188
75					47					158
76					44					124
77					42					115

Table A.2. May Sample SMA Test Results (Continued)

Propionate Concentrations (mg/L)								
	1000	2000	3000	4000	1000	2000	3000	4000
Time (hours)	**Pulse**				**SMA (mL CH_4/gVSS.d)**			
1	13	14	16	11	81	170	56	43
2	14	20	23	27	87	186	80	105
3	15	33	37	36	94	178	129	140
4	18	41	39	44	112	190	136	172
5	19	45	44	53	119	194	154	207
6	25	43	55	55	156	190	192	215
7	27	46	58	56	168	186	203	218
8	28	47	59	56	175	198	206	218
9	29	46	60	57	181	186	210	222
10	29	45	61	58	181	198	213	226
11	29	48	61	58	181	186	213	226
12	30	45	60	59	187	194	210	230
13	28	48	59	57	175	194	206	222
14	27	45	61	55	168	194	213	215
15	27	47	63	54	168	198	220	211
16	25	47	65	53	156	203	227	207
17	23	47	67	54	144	211	234	211
18	20	48	67	50	125	215	234	195
19	18	49	68	49	112	219	238	191
20	17	51	68	47	106	211	238	183
21	16	52	69	42	100	219	241	164
22		53	67	35		236	234	137
23		51	71	27		236	248	105
24		53	71	17		232	248	66
25		57	71	15		227	248	59
26		57	68	12		232	238	47
27		56	69			236	241	
28		55	69			223	241	
29		56	69			219	241	
30		57	67			215	234	
31		54	67			178	234	
32			66				231	
33			68				238	
34			66				231	
35			65				227	
36			63				220	
37			64				224	
38			63				220	
39			61				213	
40			60				210	

Table A.2. May Sample SMA Test Results (Continued)

41			58				203	
42			57				199	
43			59				206	
44			57				199	
45			57				199	
46			55				192	
47			47				164	
48			48				168	
49			46				161	
50			43				150	
51			37				129	

Table A.3. June Sample SMA Test Results

Acetate concentrations (mg/L)								
	1000	2000	3000	4000	1000	2000	3000	4000
Time (hours)	**Pulse**				**SMA (mL CH_4 / gVSS.d)**			
1	13	13	24	11	79	80	169	55
2	15	19	27	25	91	117	190	125
3	19	34	24	35	115	209	169	176
4	21	55	26	36	127	338	183	181
5	30	67	26	37	181	411	183	186
6	27	69	31	38	163	423	219	191
7	31	68	33	38	187	417	233	191
8	33	71	38	45	199	436	268	226
9	35	72	37	47	212	442	261	236
10	37	72	42	54	224	442	296	271
11	41	73	55	56	248	448	388	281
12	44	73	57	57	266	448	402	286
13	42	72	62	60	254	442	437	301
14	43	70	62	63	260	430	437	316
15	44	69	61	65	266	423	430	326
16	47	68	60	67	284	417	423	336
17	46	68	59	66	278	417	416	331
18	48	66	60	65	290	405	423	326
19	52	66	59	65	314	405	416	326
20	55	62	58	66	332	380	409	331
21	54	66	57	65	326	405	402	326
22	57	64	59	62	344	393	416	311
23	59	62	59	61	357	380	416	306
24	58	61	60	61	350	374	423	306
25	58	60	61	60	350	368	430	301
26	61	58	60	54	369	356	423	271
27	60	59	58	54	363	362	409	271
28	59	64	59	54	357	393	416	271
29	60	62	57	47	363	380	402	236
30	61	59	56	43	369	362	395	216
31	59	60	53	30	357	368	374	150
32	58	62	47		350	380	331	
33	56	60	43		338	368	303	
34	51	59	38		308	362	268	
35	47	60			284	368		
36		60				368		
37		63				350		
38		61				350		
39		58				209		

Table A.3. June Sample SMA Test Results (Continued)

Butyrate Concentrations (mg/L)										
	1000	2000	3000	4000	5000	1000	2000	3000	4000	5000
Time (hours)	**Pulse**					**SMA (mL CH_4 / gVSS.d)**				
1	13	35	11	16	22	52	115	51	70	87
2	32	38	16	21	23	129	125	74	92	91
3	40	41	17	25	25	161	134	78	109	99
4	42	43	21	27	21	169	141	97	118	83
5	49	46	28	32	18	197	151	129	140	71
6	46	52	32	37	16	185	171	147	162	63
7	51	51	35	44	29	206	167	161	192	114
8	51	55	38	53	20	206	180	175	232	79
9	55	60	41	62	20	222	197	189	271	79
10	54	60	45	68	21	218	197	207	297	83
11	53	62	49	72	19	214	203	225	315	75
12	58	62	47	77	22	234	203	216	337	87
13	59	63	52	87	29	238	207	239	380	114
14	60	65	53	92	24	242	213	244	402	95
15	61	69	54	94	26	246	226	248	411	103
16	62	75	55	87	27	250	246	253	409	107
17	55	77	55	95	29	222	253	253	415	114
18	51	75	54	102	28	206	246	248	446	110
19	53	77	54	99	29	214	253	248	433	118
20	50	76	55	97	29	202	249	253	424	126
21	45	78	55	97	30	181	256	253	424	138
22	40	76	58	100	32	161	249	267	437	142
23	25	79	54	100	32	101	259	248	437	138
24	4	75	57	104	35	16	246	262	455	146
25	5	73	56	103	35		239	258	450	154
26		72	59	106	36		236	271	463	154
27		72	58	109	35		236	267	476	158
28		71	61	106	37		233	281	463	154
29		73	62	106	39		239	285	463	166
30		72	62	109	39		236	285	476	166
31		72	63	103	40		236	290	450	205
32		63	64	105	40		207	294	459	178
33		57	67	105	39		187	308	459	181
34		45	67	107	42		148	308	468	181
35		42	68	103	42		138	313	450	185
36			68	103	52			313	450	189
37			69	101	45			317	441	193
38			68	100	46			313	437	189
39			72	99	46			331	433	197
40			73	98	47			336	428	201
41			72	99	48			331	433	205
42			71	97	49			327	424	209
43			75	95	48			345	415	217
44			73	94	50			336	411	213

Table A.3. June Sample SMA Test Results (Continued)

45			74	93	51			340	407	221
46			72	94	51			331	411	221
47			71	92	52			327	402	229
48			73	91	53			336	398	233
49			71	71	55			327	310	245
50			65	65	54			299	284	280
51			68	60	56			313	262	253
52			67	46	56			308	201	249
53			65		58			299		256
54			63		58			290		260
55			60		59			276		260
56			60		59			276		264
57			58		62			267		268
58			57		71			262		280
59			52		64			239		276
60			48		63			221		288
61			38		65			175		284
62			35		66			161		288
63					66					284
64					67					288
65					68					296
66					68					268
67					68					280
68					68					256
69					71					178
70					70					181
71					73					185
72					72					174
73					73					166
74					72					150
75					73					122

Table A.3. June Sample SMA Test Results (Continued)

Propionate Concentrations (mg/L)								
	1000	2000	3000	4000	1000	2000	3000	4000
Time (hours)	**Pulse**				**SMA (mL CH_4 / gVSS.d)**			
1	32	27	24	10	98	105	106	53
2	34	35	31	13	104	137	137	69
3	35	51	35	15	107	199	154	80
4	37	59	37	18	113	225	163	96
5	42	58	39	23	129	226	172	123
6	45	58	37	25	138	226	163	133
7	48	60	38	28	147	234	167	149
8	52	60	37	33	159	234	163	176
9	57	59	36	35	175	230	159	187
10	62	58	35	34	190	226	154	181
11	65	59	35	37	199	230	154	197
12	72	59	37	38	221	230	163	203
13	73	60	37	40	224	234	163	213
14	74	58	39	39	227	226	172	208
15	74	59	41	40	227	230	181	213
16	73	60	42	43	224	234	185	229
17	72	59	43	45	221	225	189	240
18	71	58	42	45	217	226	185	240
19	72	55	41	45	221	215	181	240
20	68	54	42	44	208	211	185	235
21	67	53	44	43	205	207	194	229
22	68	52	48	41	208	203	211	219
23	67	47	48	40	205	183	211	213
24	67	47	46	38	205	183	203	203
25	66	49	47	39	202	191	207	208
26	68	50	50	37	208	195	220	197
27	67	48	50	28	205	187	220	149
28	68	47	51	26	208	183	225	139
29	65	47	52	25	199	183	229	133
30	63	46	52		193	179	229	
31	57	45	53		175	176	233	
32	55	44	55		168	172	242	
33	47	44	55		144	172	242	
34		43	56			168	247	
35		44	55			172	242	
36		44	56			172	247	
37		43	56			168	247	
38			49			164	243	
39			47				240	
40			45				225	
41			43				189	
42			41				181	
43			39				172	
44			38				167	
45			35				154	
46			29				128	

APPENDIX B: PERFORMANCE OF THE ANAEROBIC EGSB REACTOR IN TERMS OF COD REMOVAL RATE

Table B.1. Performance of Anaerobic EGSB Reactor in Terms of COD Removal Rate

April 2007			May 2007			June 2007		
Parameters	**Influent**	**Effluent**	**Parameters**	**Influent**	**Effluent**	**Parameters**	**Influent**	**Effluent**
Total COD	10325 mg/L	3450 mg/L	Total COD	8275 mg/L	2350 mg/L	Total COD	5625 mg/L	1100 mg/L

CONVERSIONS

mL CH_4 / gVSS.d conversion to g CH_4 COD / gVSS.d by following two equations:

$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$ (B.1)

1 g of CH_4 is equal to 4 g O_2 (COD)

$P \times V = M/ MW \times R \times T$ (B.2)

P: air pressure (101.325 kPa)

V: volume (L)

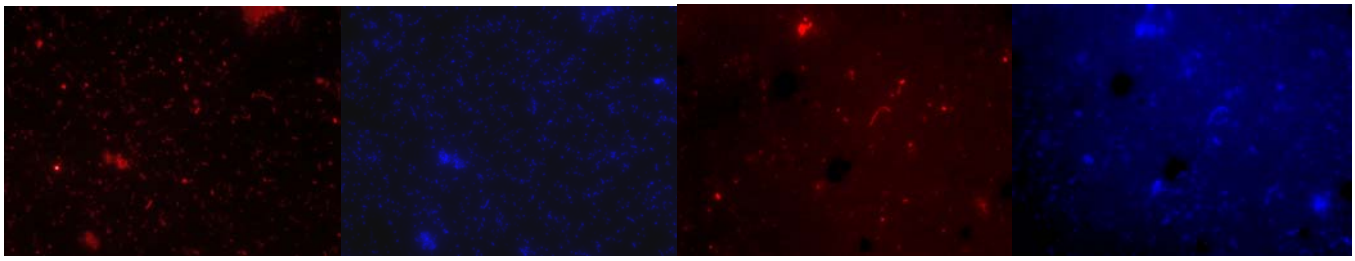
M: mass (g)

MW: molecular weight (g/mol)

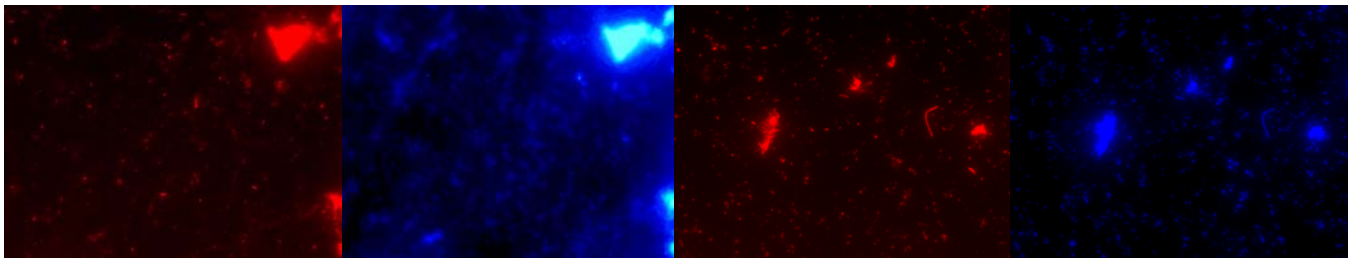
R: universal gas constant (8, 3144 L.kPa / mol. K)

T: temperature (K= 273+ T ° C)

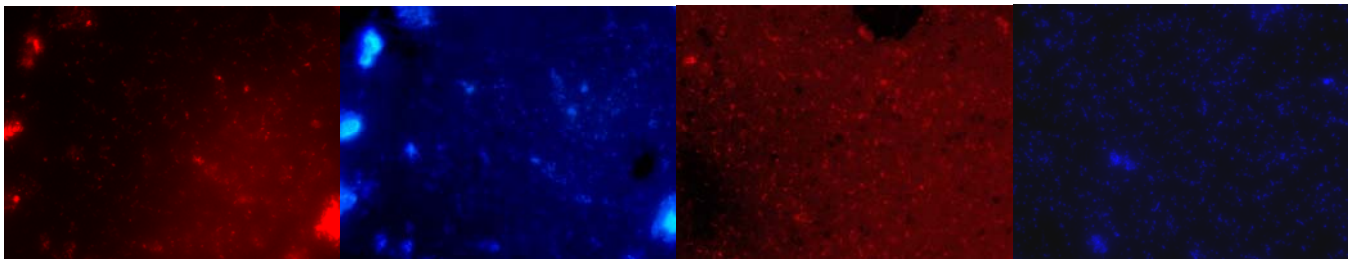
APPENDIX C: FISH RESULTS OF THE ANAEROBIC EGSB REACTOR SAMPLES



Archaea Methanobacteriales

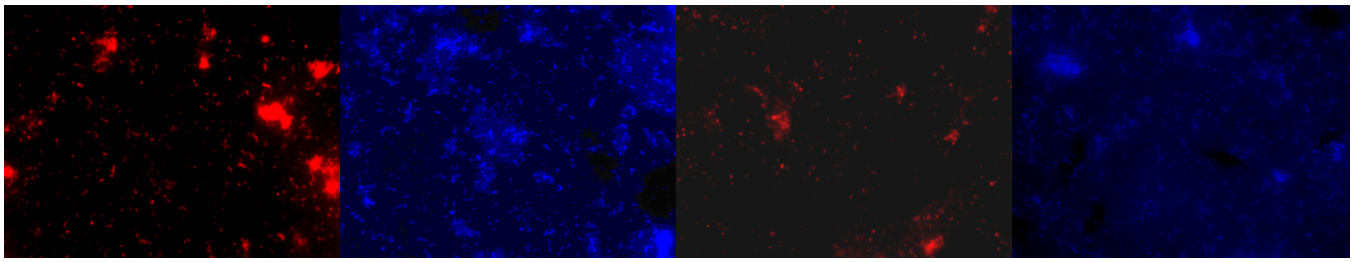


Methanosaeta Active cells

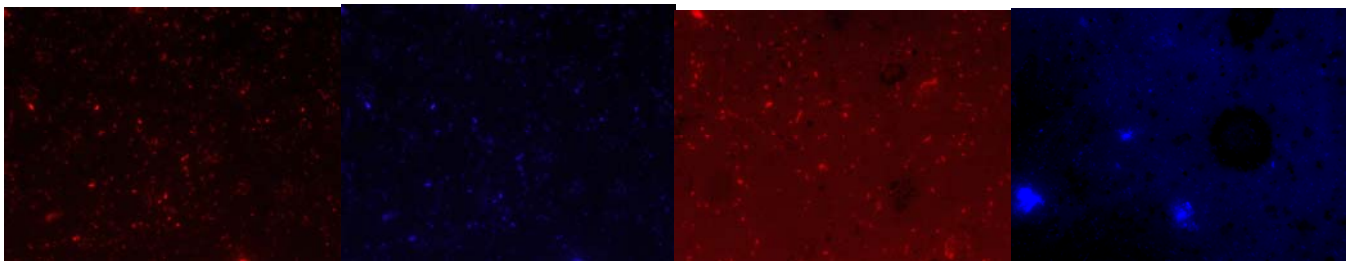


Methanosarcina Methanococcales

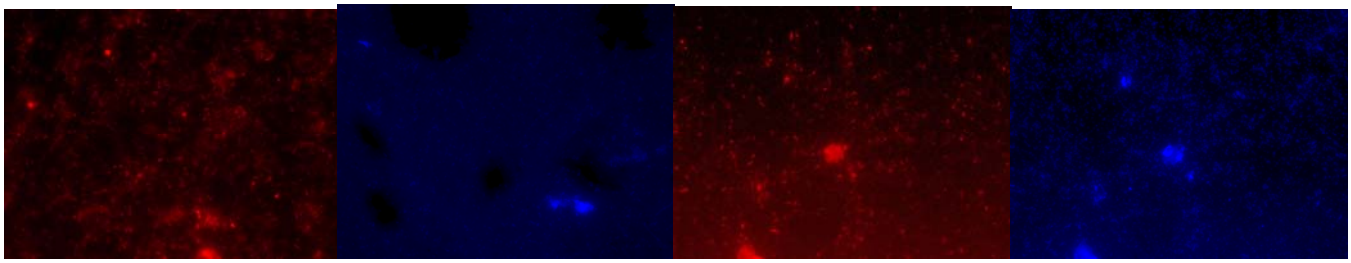
Figure C.1. FISH Results of the Mid Level of Anaerobic EGSB Reactor (April Sample)
(Fluorescent images on left, DAPI stained images on right)



Archaea Eubacteria

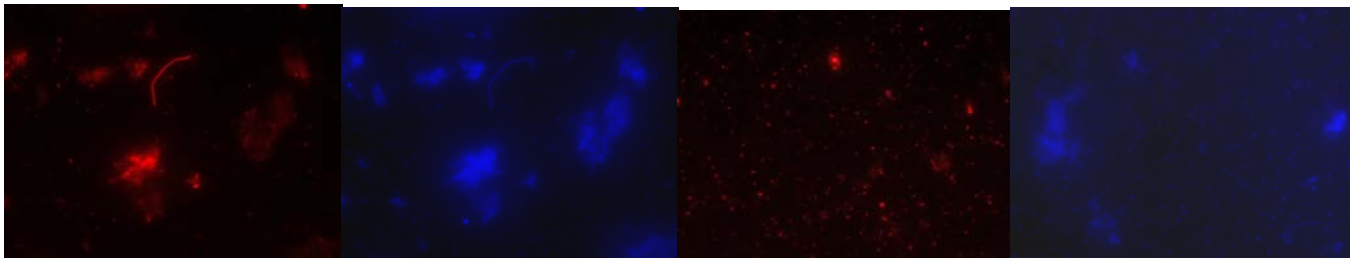


Methanobacteriales Methanosaeta



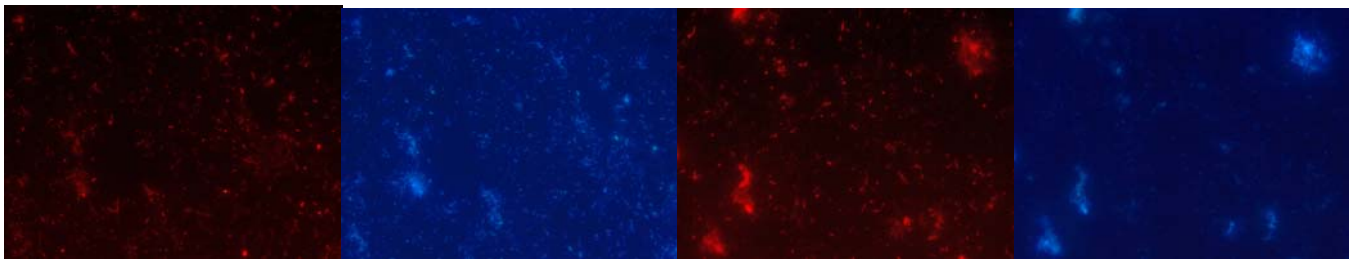
Methanogenium Methanococcales

Figure C.2. FISH Results of the Mid Level of Anaerobic EGSB Reactor (May Sample)
(Fluorescent images on left, DAPI stained images on right)

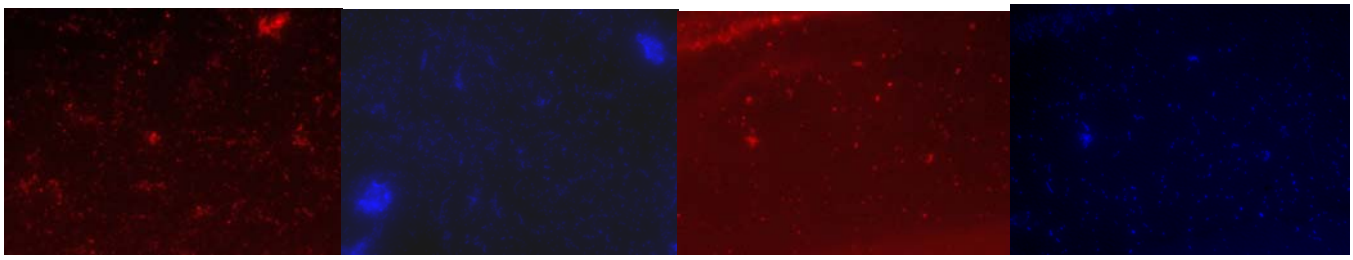


Archaea Eubacteria

Figure C.3. FISH Results of the Mid Level of Anaerobic EGSB Reactor (June Sample)

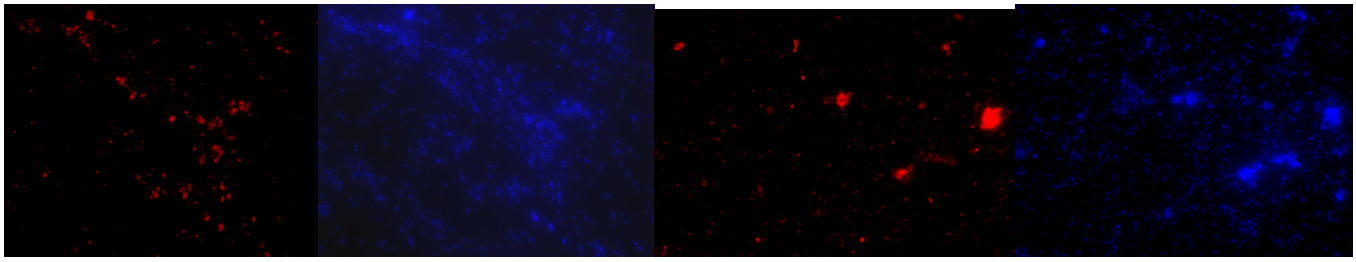


Methanogenium Methanobacteriales

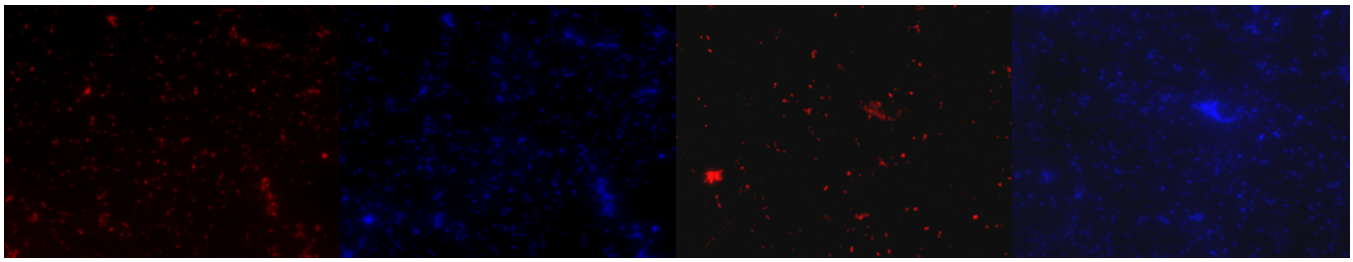


Methanosaeta Methanococcales

Figure C.3. FISH Results of the Mid Level of Anaerobic EGSB Reactor (June Sample) (Continued)

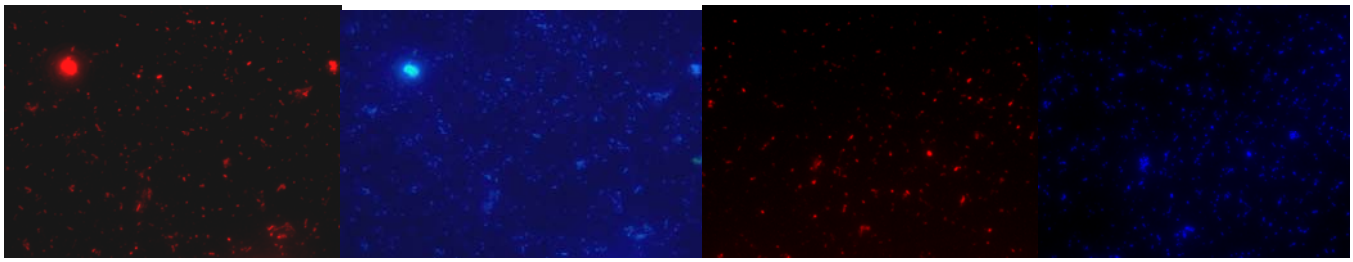


Methanosarcina Methanosaeta



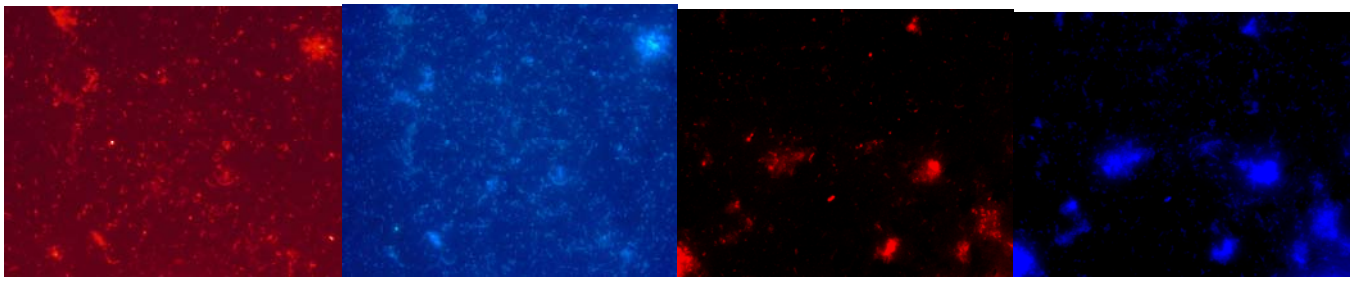
Active cells Methanococcales

Figure C.4. FISH Results of the Top Level of Anaerobic EGSB Reactor (April Sample)

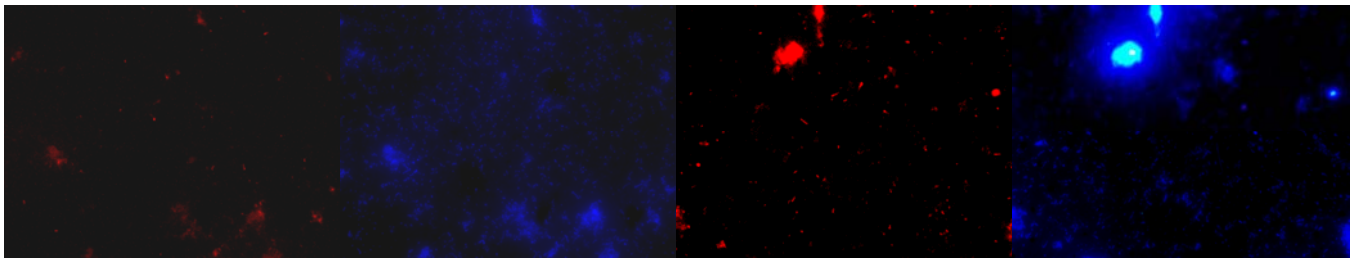


Eubacteria Methanogenium

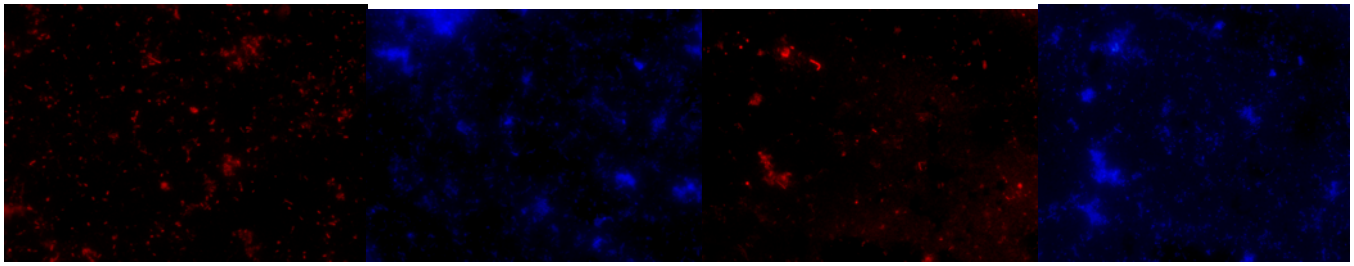
Figure C.4. FISH Results of the Top Level of Anaerobic EGSB Reactor (April Sample) (Continued)



Archaea Methanobacteriales

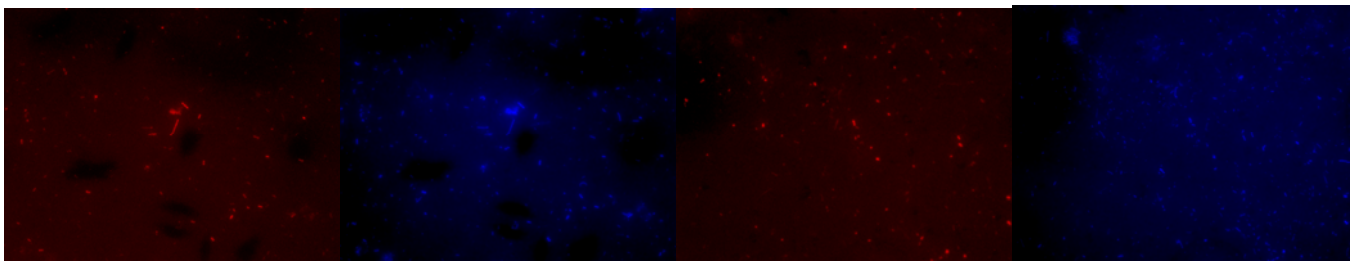


Methanococcales Methanogenium

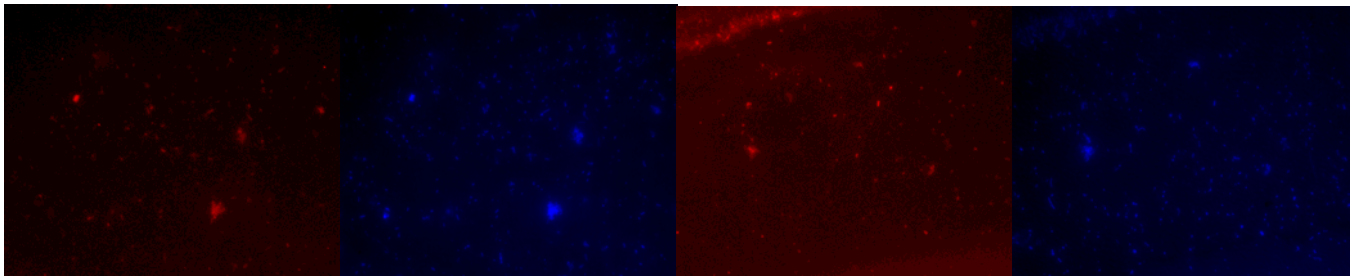


Methanosaeta Active cells

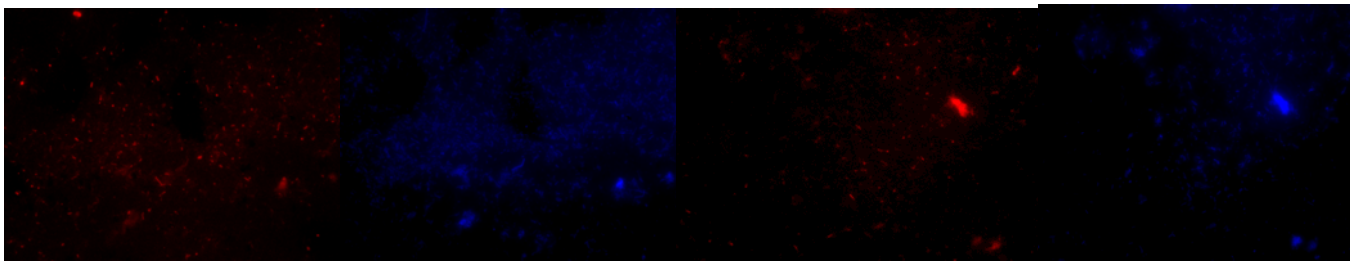
Figure C.5. FISH Results of the Top Level of Anaerobic EGSB Reactor (May Sample)



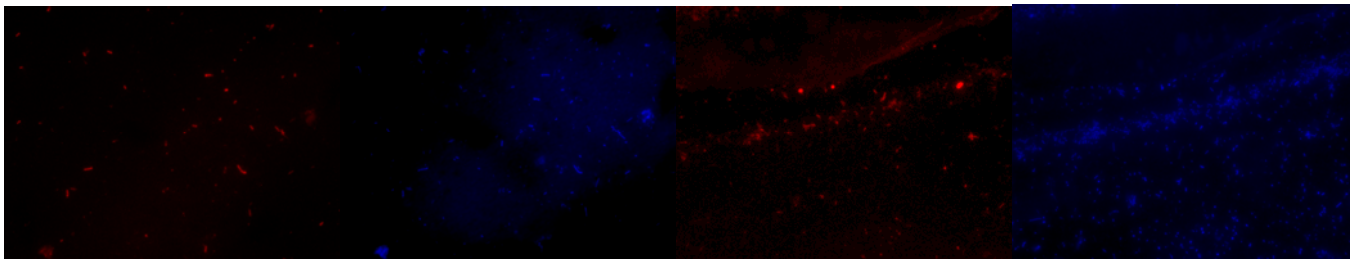
Archaea Eubacteria



Methanobacteriales Methanococcales



Methanogenium Methanosaeta



Methanosarcina Active cells

Figure C.6. FISH Results of the Top Level of Anaerobic EGSB Reactor (June Sample)