ISTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

DISTRIBUTION, DYNAMICS AND ACTIVITY OF ARCHAEAL METHANOGENIC POPULATION IN A FULL-SCALE ANAEROBIC CONTACT REACTOR TREATING PULP AND PAPER MILL EFFLUENTS

M.Sc. Thesis by
Sevgi ULUGÖL

Department: **Environmental Engineering**
Programme: **Environmental Biotechnology**

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

KAĞIT ENDÜSTRİSİ ATIKSULARINI ARITAN GERÇEK ÖLÇEKLİ TAM KARIŞIMLI HAVASIZ TEMAS REAKTÖRÜNDEKİ METAN ARKELERİ POPULASYONUNUN DAĞILIMI, DİNAMİĞİ VE AKTİVİTESİ

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ABBREVIATIONS

AAFEB	: Anaerobic Attached-Film Expanded-Bed Reactor
AFB	: Anaerobic Fluidized Bed
AMP	: Actual Methane Production
EGSB	: Expanded Granular Sludge Bed
FISH	: Fluorescence *in situ* Hybridization
OLR	: Organic Loading Rate
PMP	: Potential Methane Production
ssrRNA	: Small Subunit Ribosomal RNA
SMA	: Specific Methanogenic Activity
SRB	: Sulphate Reducing Bacteria
SS	: Suspended Solids
UASB	: Upflow Anaerobic Sludge Blanket
VFA	: Volatile Fatty Acid
VSS	: Volatile Suspended Solids

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DISTRIBUTION, DYNAMICS AND ACTIVITY OF ARCHAEAL METHANOGENIC POPULATION IN A FULL-SCALE ANAEROBIC CONTACT REACTOR TREATING PULP AND PAPER MILL EFFLUENTS

SUMMARY

Pulp and paper mill effluents are highly polluted. The upflow anaerobic sludge blanket (UASB) and contact processes are the most widely applied anaerobic systems. From a practical standpoint, given the importance of methanogens in anaerobic treatment processes, an understanding of their ecology is essential to make effective control of the start-up and operation of anaeorobic bioreactors possible. In addition, a sufficient quantity of active methanogenic populations should be maintained within an anaerobic reactor so that required COD removal efficiency can be obtained. It is, therefore, necessary to monitor any changes in the numbers or activities of methanogenic species in anaerobic reactors using available techniques such as fluorescence *in situ* hybridization (FISH) and specific methanogenic activity (SMA) tests under controlled conditions.

In the present study, a full-scale anaerobic contact reactor at wastewater treatment plant of Modern Karton-Pulp and Paper Industry effluents were investigated in terms of performance, acetoclastic methanogenic capacity and microbial community structure during steady-state conditions on July 2005 and during start-up and operation conditions after 15 days maintenance. Changes in the potential methane production (PMP) rates of the anaerobic sludge were determined using specific methanogenic activity (SMA) test. Microbial community structures of the anaerobic sludge were also characterized using fluorescent rRNA targeted oligonucleotide probes specific for phylogenetically defined groups of methanogens. The principal aim of this study is to compare the steady-state conditions with the start up and operation of the system after maintenance.

The applied hydraulic retention time (HRT) (4 days) was in a range of stated in literature for the successful applications treating pulp and paper mill effluents (0.5-5 days). The applied temperature (34-37 ^{0}C) and pH (6.4-7.5) in the anaerobic contact reactor were optimum values.

During the monitoring period of the anaerobic contact reactor, observed COD removal efficiency (47-55%) values stayed in the lower limit. But methane yield (0.19±0.01 m^3CH_4/kg $COD_{removed}$) values of the anaerobic contact reactor were higher than the methane yield (0.08-0.16 m^3CH_4/kg $COD_{removed}$) values of similar anaerobic reactors treating pulp and paper mill effluents in literature. In such a case,

it can be said that the performance of the anaerobic contact reactor has showed a good performance during the monitoring period of 5 months.

Nutrients are not added into the anaerobic contact reactor and COD/N/P ratio in the anaerobic contact reactor is not known. The anaerobic reactors treating pulp and paper mill effluents are operated successfully with 176:5:1 ratio (COD/N/P) and generally extra addition of nutrient isn't necessary for maintaining this proportion.

Organic loading rate (OLR) values of similar anaerobic reactors, treating pulp and paper mill effluents, are reported 0.5-5 kg COD m^{-3} day^{-1} in literature. But during the monitoring period of 5 months, observed OLRs (1.6-1.8 kg COD m^{-3} day^{-1}) of anaerobic contact reactor stayed in the lower limit. It is previously reported in literature that increasing OLRs (from 0.75 to 3 kg COD m^{-3} day^{-1}) of completely stirred anaerobic reactors have useful effects on methane activity and system performance.

Applied F/M (food/biomass) ratio (0.15-0.17 g COD/g VSS.day) was quite lower than the typical F/M ratios of similar anaerobic reactors (0.5-1.0 g COD/g VSS.day). It is previously reported in literature that increasing F/M ratios of operated anaerobic reactors with low F/M ratio have positive effects on methane activity and system performance.

Specific methanogenic activity (SMA) test results revealed that 62%, 38% and 22% losses in the PMP rates were occurred in the anaerobic contact reactor sludge taken from 4, 8 and 12 meters height during the operation between July and August 2005. When the PMP rates were compared with actual methane production (AMP) rates obtained from the anaerobic contact reactor, AMP/PMP ratios were evaluated to be in a range of 0.2-0.4 showing that the anaerobic sludge had been using only 20-40% of its potential acetoclastic methanogenic capacity during their observed operation period. When anaerobic reactors were run at an AMP/PMP ratio of 0.6-0.7, high operating stability and excellent COD removal could be achieved. The SMA test results and operational data of the anaerobic contact reactor showed that the most important adverse effect might have caused by the excess sludge retention in the reactor resulting in a significant decrease in potential activity of acetoclastic methanogens since all other operational parameters such as pH, temperature, alkalinity, organic loading, hydraulic retention time etc. have been maintained within their desired ranges.

The all-operational parameters (such as pH, temperature, organic loading, hydraulic retention time etc.) except F/M ratio were maintained within desired ranges. The reason behind of this could be due to retaining high amount of sludge within anaerobic contact reactor resulting in an F/M ratio, which is relatively much lower than the typical values reported for similar reactors. Pulling out more sludge from anaerobic contact reactor or increasing applied OLRs can increase applied F/M ratio. But when applying these suggestions, the system potential should be taken into consideration and OLRs should be increased while AMP/PMP ratio is remained 0.6-0.7.

FISH results seem to be compatible with SMA test results. The relative amount of acetoclastic methanogens (*Methanosaeta* spp., and *Methanosarcina* spp.) has

decreased in the ratio of 47 % from July 2005 to August 2005. At the same time acetoclastic methanogenic activity has decreased in the ratio of 44 %. In parallel with acetoclastic methanogenic activity loss, the relative amount of hydrogenotrophic methanogens has decreased in the ratio of 67 %.

Even though the reactor is completely stirred tank, SMA and FISH results, the concentration of methanogenic community at 4, 8 and 12 meters height of the anaerobic contact reactor, the relative quantities of specific species and acetoclastic methane activities have been showed important differences. Because of that there was not a homogenous dispersion in the anaerobic contact reactor. The cause of this problem could be the completely stirring problem of the anaerobic contact reactor. For testing this problem, a trace matter can be poured into the anaerobic contact reactor and the changes of the concentration of a trace matter can be observed at the anaerobic contact reactor effluent.

KAĞIT ENDÜSTRİSİ ATIKSULARINI ARITAN GERÇEK ÖLÇEKLİ TAM KARIŞIMLI HAVASIZ TEMAS REAKTÖRÜNDEKİ METAN ARKELERİ POPULASYONUNUN DAĞILIMI, DİNAMİĞİ VE AKTİVİTESİ

ÖZET

Kağıt endüstrisi atıksuları oldukça kirlidir. Bu tür atıksuların arıtımında yüksek biyokütle içeriğine ve zengin mikrobiyal çeşitliliğe sahip olan yukarı akışlı havasız çamur yatağı (UASB) reaktörü ve temas prosesleri yaygın olarak kullanılmaktadır. Havasız arıtım sistemlerinde istenilen KOİ giderim verimini elde edebilmek ve sistem stabilitesinin sürekliliğini sağlayabilmek için, reaktörde yeterli miktarda aktif metan arke popülasyonları tutulmalıdır. Bu nedenle, havasız reaktörlerin işletilmesi sırasında metan arkelerinin sayısında, türlerinde ve aktivitelerinde meydana gelebilecek değişimleri floresanlı *yerinde* hibritleşme (FISH) ve spesifik metan aktivite (SMA) testi gibi mevcut yöntemleri kullanarak belirlemek önemlidir.

Bu çalışmada kağıt endüstrisi atıksularını arıtan Modern Karton fabrikasında bulunan gerçek ölçekli tam karışımlı havasız temas reaktörünün son beş aylık süreç içerisindeki işletim performansları ile reaktörlerden bu süreç içerisinde alınan biyolojik çamur örneklerinin mikrobiyal komunite yapıları ve potansiyel metan üretim (PMÜ) hızları tartışılmıştır. Temmuz ayındaki normal işletme koşullarıyla Ağustos ayındaki 15 günlük bakım sonrası tesis tekrar işletmeye alındığı durumdaki koşullar etkin olan türlerin dağılımındaki değişiklikler, potansiyel aktivitelerindeki kayıplar ve buna karşılık işletme koşulları göz önünde bulundurularak karşılaştırılmıştır. Potansiyel metan üretim hızlarını belirleyebilmek için Spesifik Metan Aktivite (SMA) test düzeneği kullanılmıştır. Biyolojik çamur örneklerinin mikrobiyal komünite yapısı filogenetik olarak tanımlanmış metan arkelerine spesifik problar kullanılarak yapılan floresanlı yerinde hibritleşme (FISH) tekniği kullanılarak belirlenmiştir. Temmuz ayındaki normal işletme koşullarıyla Ağustos ayındaki 15 günlük bakım sonrası tesis tekrar işletmeye alındığı durumdaki koşullar, etkin olan türlerin dağılımındaki değişiklikler, potansiyel aktivitelerindeki kayıplar ve buna karşılık işletme koşulları göz önünde bulundurularak karşılaştırılmıştır.

Havasız temas reaktörünün çalıştırıldığı hidrolik bekletme süresi (HRT) (4 gün), literatürdeki başarılı uygulamalar için verilen değer aralığındadır (0.5-5 gün). Reaktördeki sıcaklık (34-37 ^{0}C) ve pH (6.4-7.5) optimum değer aralıklarında tutulmaktadır.

Bu çalışmanın konusu olan havasız temas reaktörünün KOİ giderim verimi (%47-55), literatürde kağıt endüstrisi atıksularını arıtan havasız temas reaktörleri için

verilen değer aralığının (%40-80) alt sınırı içinde kalmaktadır. Fakat reaktörden elde edilen metan verimi (0.18-0.20 $m^3CH_4/gKOİ_{giderilen}$), kağıt endüstrisi atıksularını arıtan havasız reaktörler için literatürde verilen değerlerin (0.08-0.16 $m^3CH_4/gKOİ_{giderilen}$) üstündedir. Bu durumda, reaktörün izlendiği 5 aylık süreç içerisinde performansının iyi olduğu söylenebilir.

Havasız temas reaktörüne nütrient ilavesi yapılmamakta ve reaktörün çalıştırıldığı KOİ/N/P oranı bilinmemektedir. Kağıt endüstrisi atıksularını arıtan havasız reaktörler 176:5:1 KOİ/N/P oranlarında başarıyla çalıştırılmakta ve bu oranın sağlanması için genellikle ekstra nütrient ilavesi gerekmemektedir.

Bu çalışmanın konusu olan havasız temas reaktörüne uygulanan organik yükleme hızı (OYH) (1.6-1.8 kg KOİ $m^{-3}gün^{-1}$), literatürde kağıt endüstrisi atıksularını arıtan havasız temas reaktörleri için verilen değer aralığının (0.5-5 kg KOİ m^{-3} $gün^{-1}$) alt sınırı içinde kalmaktadır. Tam karışımlı havasız reaktörlerde OYH'nin arttırılmasının (0.75'ten 3 kg KOİ m^{-3} $gün^{-1}$'e) metan aktivitesi ve sistem performansı üzerindeki olumlu etkileri daha önce rapor edilmiştir.

Reaktöre uygulanan F/M (besin/biokütle) oranı dışındaki bütün işletme parametreleri (pH, sıcaklık, organik yükleme hızı, hidrolik bekletme süresi vb.) optimum aralıklarında tutuldukları için, reaktör çamurunda meydana gelen aktivite kayıplarının nedeni reaktöre uygulanan F/M oranının havasız reaktörlere uygulanan tipik F/M oranlarının bir hayli altında kalması olabilir (0.15-0.17<0.5-1 gKOİ/g.UAKM.gün). Bu çalışmanın konusu olan havasız temas reaktörüne uygulanan F/M oranı reaktörden daha fazla çamur çekilerek veya OYH artırılarak daha yüksek seviyelere çekilebilir. Fakat bunu yaparken sistemin potansiyeli göz önünde tutulmalı, GMÜ/PMÜ oranı 0.6-0.7 aralığında kalacak şekilde yüklemeler arttırılmalıdır.

Temmuz 2005 SMA test sonuçları Ağustos 2005 sonuçlarıyla karşılaştırıldığında reaktörün 4.m, 8.m ve 12.m'lerindeki potansiyel metan üretim (PMÜ) hızlarında sırasıyla %62, %38 ve %22'lik kayıplar meydana geldiği görülmektedir. SMA testi sonucunda bulunan PMÜ hızları reaktörün gerçek metan üretim (GMÜ) hızları ile oranlandığında, Temmuz ve Ağustos'ta GMÜ/PMÜ oranları yaklaşık olarak sırasıyla 0.2 ve 0.4 olarak bulunmuştur. Bu oranlar bize bu çalışmanın konusu olan havasız temas reaktörünün maksimum kapasitesinin çok altında yüklendiğini göstermektedir. Literatürde havasız reaktörlerde stabilitenin sürekliliğinin sağlanması ve istenilen sistem performansının elde edilmesi için GMÜ/PMÜ oranının 0.6-0.7 aralığında tutulması gerektiği belirtilmiştir.

FISH sonuçları SMA sonuçlarıyla uyum göstermektedir. Asetoklastik metanojenlerin (*Methanosaeta* spp. ve *Methanosarcina* spp.) toplam komünite içerisindeki rölatif miktarı Temmuz 2005'ten Ağustos 2005'e %32 azalırken, aynı süreç içerisinde asetoklastik metan aktivitesi %44 azalmıştır. Bu çalışmada SMA test düzeneği ile sadece asetoklastik metan aktivitesi ölçülmüş, hidrogenotrofik metan aktivitesi ölçülmemiştir. Fakat asetoklastik metan aktivitesinde yaşanan %44'lük düşüşe pararel olarak hidrogenotrofik metanojenlerin toplam komünite içerisindeki rölatif çokluğunda %67'lik bir düşüş gözlenmiştir.

FISH ve SMA testi sonuçları; reaktör tam karışımlı olmasına rağmen, reaktörün 4.m, 8.m ve 12.m'lerinde metanojenlerin kompozisyonu, spesifik türlerin miktarları ve asetoklastik metan aktivitelerinde önemli farklılıklar bulunduğunu göstermiştir. Reaktörde, homojen olmayan bu dağılım, tam karşımın gerçekleşmesinde meydana gelen bir problemden kaynaklanıyor olabilir. Bunu test etmek için, reaktöre verilen inert bir iz maddesinin konsantrasyonunun değişimi reaktör çıkışında izlenebilir.

CHAPTER 1 INTRODUCTION

Biological treatment systems are widely used to achieve high quality effluent for environmental disposal. Performance of biological treatment systems is related to the composition and activity of microbial populations they contain. The types of organisms present and their relative population levels in reactor biomass depend on wastewater characteristics as well as operational conditions maintained in anaerobic reactor. Improvements in the understanding of both the microbial communities and processes in anaerobic reactors are essential to design and control anaerobic systems effectively (Akarsubasi et al., 2005a).

The anaerobic treatment is an energy generating process rather than one, which demands a regular high, input of energy, as in an aerobic biological system. The amount of energy resulting from production of biogas in any anaerobic treatment system depends upon retention of adequate level of active biomass in the anaerobic reactor. Various methods have, therefore, been developed for a range of reactor configurations with the anaerobic contact process being the one of advanced anaerobic digestion technologies (Ince et al., 2001a). The anaerobic treatment is a technically simple, relatively inexpensive technology and consumes little energy. It also requires less space and produces less amount of sludge (Savant et al., 2005).

The pulp and paper industry is a water intensive industry and ranks only third in the world, after the primary metals and the chemical industries, in terms of freshwater withdrawal. Even with the most modern and efficient operational techniques, about 60 m^3 of water is required to produce a ton of paper resulting in the generation of large volumes of wastewaters. It is the sixth largest polluter discharging a variety of gaseous, liquid and solid wastes. The use of anaerobic treatment in the pulp and paper industry began in 1970s. The first system introduced was anaerobic lagoon. Contact reactor and UASB process were used in the early 1980s. Today, about 50 full-scale anaerobic

treatment systems treating pulp mill wastewaters are operating around the world. Anaerobic microorganisms can be preserved unfed for long periods of time without any serious deterioration of their activity. The nutrient requirement for anaerobic treatment is low. It is less sensitive to toxic substances. Hence, it is proving to be a viable technology for pulp and paper wastewater treatment. It can remove about 75-85% of the BOD and 55-65% COD in pulp mill effluents. The major treatment methods include anaerobic lagoon, anaerobic contact processes, UASB, fluidized bed, anaerobic filters and hybrid processes. The comparison of the published data is difficult due to differences in the bleaching processes, nature of the bleach effluents and the proportion of the chlorination and extraction stage effluents are mixed. However it can be said that the efficiency of anaerobic lagoons is very low. The UASB and contact processes are the most widely applied anaerobic systems (Savant et al., 2005).

Anaerobic treatment systems are microbial processes requiring careful design and control. In practice, engineers and plant operators base their design generally on loading rate, expresses in terms of reactor volume without reference to the quality or quantity of either the seed sludge or the active biomass developed within the reactor during operation. 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. During start-up and steady-state operation of anaerobic treatment systems, a sufficient quantity of active methanogenic populations should be maintained within an anaerobic reactor so that required COD removal efficiency can be obtained. It is, therefore, necessary to monitor any changes in the numbers or activities of methanogenic species in anaerobic reactors using available techniques such as fluorescence *in situ* hybridization (FISH), microscopic counts, most probable number (MPN), adenosine three phosphate (ATP), coenzyme F_{420}, dehydrogenic activity (DA) and specific methanogenic activity (SMA) tests under controlled conditions. The specific methanogenic activity (SMA) test, therefore, was developed to determine the

maximum acetoclastic methanogenic activity (Ince et al, 1995a; Monteggia, 1991; Valcke and Verstraete, 1983). The SMA test has been reported to be a control parameter and a means of determining the optimum operating conditions of anaerobic treatment systems (Ince et al., 1994b, 2001a; Monteggia, 1991).

Several anaerobic process variants having specific biomass retention mechanisms are available for field application. Laboratory-, pilot- and full-scale studies have made varied claims regarding applicability and performance of these variants. Maintenance of sufficient methanogenic populations in the system is critical for stable performance. Methanogenic species types and their relative population levels in reactor biomass depend on wastewater characteristics as well as operational/environmental conditions maintained. Any imposed stress (intentional or otherwise) may lead to a change in species types and their relative population levels which is ultimately reflected in the reactor performance. However, little effort has been made to assess reactor biomass in terms of relative population levels of methanogenic species under varied operational/environmental conditions (Jawed et al., 1999). Several investigators have made counts of methanogens and non-methanogens in reactor biomass. These efforts led to the development of well-established laboratory techniques. However, these techniques require a high level of skill, advanced equipment, and costly and specific growth media, which restrict its application at the plant site. SMA tests on anaerobic sludge (biomass) have been gaining importance. Initially, these tests were mainly used to select an adapted sludge as inoculums but now these tests can also be used for many other purposes such as to:

- Evaluate the behavior of sludge under the effect of potentially inhibitory compounds,
- Establish the degree of degradability of various substances,
- Follow the changes in sludge activities due to a possible build-up of inert materials,
- Estimate maximum applicable loading rate to certain sludge,
- Evaluate batch kinetic parameters, etc.

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 (Amann et al., 1990a; Head et al., 1998; Hugenholtz et al., 1998). Research into anaerobic digestion using rRNA-based molecular techniques has provided detailed descriptions of the complex bacterial and archaeal populations present, obviating the need for anaerobic culture techniques (Harmsen et al., 1996; Godon et al., 1997; Merkel et al., 1999). 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 obtained (Poulsen et al., 1993). It also allows identify and quantify methanogens at different levels of phylogenetic depth and localize individual community members in their natural spatial positions and provide a basis to estimate the *in situ* growth rates of methanogens in natural populations.

Since methanogenic Archaea affect the efficiency of the whole anaerobic process, the occurrence and status of methanogenic Archaea are very important. Furthermore, methane gas, produced during anaerobic treatment process, is an important energy source. Thus, any decrease in its amount is economically significant. Therefore methanogenic Archaea should be kept under control.

In this study, therefore, a fully computerized Specific Methanogenic Activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince et al. (1995a) was used to determine acetoclastic methanogenic activity. Archaeal methanogenic population dynamics in a full-scale anaerobic contact reactor treating pulp and paper mill effluents was also determined using fluorescence *in situ* hybridization (FISH) technique. Finally, performance and methanogenic activity of the anaerobic contact reactor were discussed concerning their archaeal methanogenic compositions during the period of study.

CHAPTER 2 RESEARCH AIM AND OBJECTIVES

Wastewaters from the food and beverages industry, pulp and paper mills and from the chemical industry are highly loaded with organic pollutants and can be treated advantageously by means of anaerobic processes.

Anaerobic treatment involves a complex interaction of several groups of bacteria, methanogens being the terminal group. They convert acetate and carbon dioxide into methane. Methanogens play a key role in the system by keeping the hydrogen partial pressure low, a condition necessary for the growth of many acetogenic bacteria. Stability of anaerobic treatment requires a balanced activity of the mixed population of bacteria: this stability can be easily disturbed by different factors causing a rapid increase in the concentration of volatile fatty acids with a concurrent decrease in methane production. Anaerobic treatment failure can occur occasionally and it has been stated that methanogens may be the most sensitive members of the bacterial consortium (Codina et al., 1998).

Anaerobic treatment systems are microbial processes requiring careful design and control. The reactor performance is usually evaluated in terms of process efficiency and stability through estimation of organic matter, VFA levels, quantity and composition of biogas produced etc. In recent years, identification of active methanogenic species as a control parameter of an anaerobic treatment is becoming increasingly attractive. For instance, any deterioration in the performance of an anaerobic reactor may have been due to the change in the dominant species or a decrease in the quantity of active methanogens. While early recognition of problems using improved instrumentation will undoubtedly be of value, it would be clearly more suitable to utilize a population able to respond quickly so that problems do not occur.

As it seen in previous paragraphs two major points are getting more important: defining which species are main workers in which numbers in the process and their activity.

Consequently, an understanding of both the microbial ecology and their activity are essential to operate the anaerobic reactors effectively. It is therefore, necessary to determine the amount of active methanogenic populations in anaerobic reactors. In this respect the specific methanogenic activity (SMA) test gives information about activity of acetoclastic methanogens and also provides information on potential loading capacity and optimum operating conditions of anaerobic reactors (Akarsubasi et al., 2005b).

The principal aim of this study is to compare the steady-state conditions with the start up and operation of the system after maintenance. Therefore, the system was investigated under two conditions:

(1) During the steady-state conditions on July 2005:

By this study, (a) investigation of changes in composition and amount of methanogens during operation of a full-scale anaerobic contact reactor at wastewater treatment plant of Modern Karton-Pulp and Paper Industry, (b) determination the interaction between Archaeal methanogenic population dynamics and wastewater composition, operating conditions and system performance are intended.

(2) During start up and operation of the system after 15 days maintenance on August:

(a) investigation of changes in composition and amount of methanogens during operation of the system after 15 days maintenance, (2) comparison the interaction between Archaeal methanogenic population dynamics and wastewater composition, operating conditions and system performance with the steady-state conditions.

In this study, therefore, a fully computerized Specific Methanogenic Activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince et al. (1995a) was used to determine acetoclastic methanogenic activity. Acetate was used as feed during SMA tests, since approximately 72% of the methane formed during anaerobic digestion of complex substrate results from acetic acid. Different acetate concentrations were initially tested in order to reach maximum potential methane production (PMP) rate during the SMA tests. Archaeal methanogenic population dynamics in a full-scale anaerobic contact reactor treating pulp and paper mill effluents was also determined using fluorescence *in situ* hybridization (FISH) technique. Several oligonucleotide probes specific for methanogenic Archaea were determined from literature and applied to these purposes. Firstly, optimum hybridization and washing conditions were found

out for each probe. Then the FISH results were correlated with the SMA test results. Finally, performance and methanogenic activity of the anaerobic contact reactor were discussed concerning their archaeal methanogenic compositions during the period of study.

With the realization of this study: (1) Application of FISH technique for characterization of methanogens will be improved, (2) Production of methane gas as an energy source will be optimized, (3) Characterization of active methanogenic populations under changing environmental conditions inherent to a full- scale anaerobic contact reactor treating pulp and paper mill effluents will help to understand problems encountered in that system.

CHAPTER 3 FUNDAMENTALS OF ANAEROBIC TREATMENT

3.1 Anaerobic Treatment Process

Anaerobic treatment is a microbial process of degradation and stabilization of complex organics in the absence of oxygen by the action of anaerobic bacteria to produce biogas comprised of a mixture of CO_2 and CH_4.

The history of Anaerobic Treatment Technology:

- Originally : Slurry Digestion (Manures, Sludges)
- 60 to 80's : Agroindustrial Effluents (Brewery, Distillery, Food Processing)
- 80 to 90's : Pulp/Paper Effluents

  (Condensates, (Chemi) Thermo-mechanical pulping (TMP), Bleachery)
- 90's : Chemical/Petrochem. Effluents (Terephthalate, Phenols)
- 90 to 00's : Anaerobic Bioremediation (PCE (tetrachloroethene)),

  BTEX (benzene, toluene, ethylbenzene, and the xylene isomers)

In the wastewater engineering field organic pollution is measured by the weight of oxygen it takes to oxidize it chemically. This weight of oxygen is referred to as the "chemical oxygen demand" (COD). COD is basically a measure of organic matter content or concentration. The best way to appreciate anaerobic wastewater treatment is to compare its COD balance with that of aerobic wastewater treatment, as shown in Figure 3.1 below.

Anaerobic Treatment: The COD in wastewater is highly converted to methane, which is a valuable fuel. Very little COD is converted to sludge. No major inputs are required to operate the system.

Aerobic Treatment: The COD in wastewater is highly converted to sludge, a bulky waste product, which costs lots of money to get rid of. An aerobic wastewater treatment facility is in essence a "waste sludge factory". Elemental oxygen has to be continuously supplied by aerating the wastewater at a great expense in kilowatt-hours to operate the aerators.

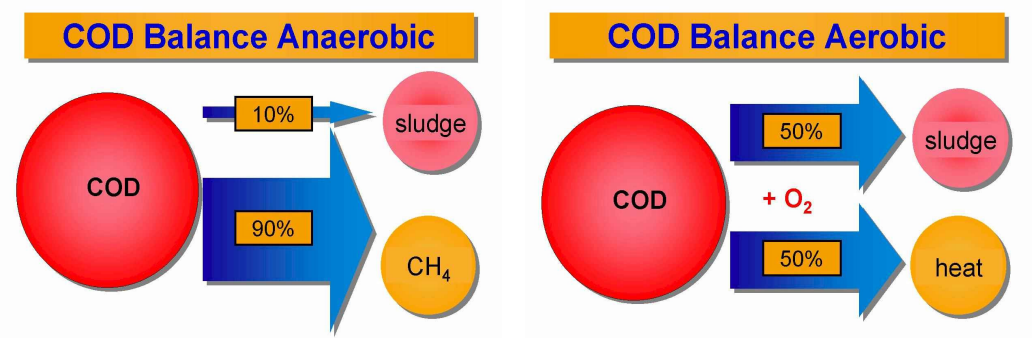


Figure 3.1: Comparison of the COD balance during anaerobic and aerobic treatment of wastewater containing organic pollution

You can see advantages and disadvantages of anaerobic and aerobic treatment processes in Table 3.1.

Table 3.1: Anaerobic treatment as compared to aerobic treatment

	Aerobic systems	**Anaerobic systems**
Energy consumption	high	low
Energy production	no	yes
Biosolids production	high	low
COD removal	90-98 %	70-85 %
Nutrients (N/P) removal	high	low
Space requirement	high	low
Discontinuous operation	difficult	easy

The amount of energy released during methanogenesis is relatively low compared to other terminal electron accepting processes. Thus, the amount of biomass produced per unit of substrate degraded is much less than that of other terminal electron accepting

processes. For this reason, methanogenesis has been used as the treatment of choice for sewage and other complex wastes since 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 the low cell yields associated with anaerobic treatment make it attractive 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, great advances have been achieved in the past 20 years in our understanding of biochemistry and energetic on anaerobic metabolism. 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).

3.1.1 Biochemistry of Anaerobic Treatment

The biological degradation of complex organic compounds takes place in several consecutive biochemical steps (chain reaction), each performed by different groups of specialized bacteria. These steps can take place simultaneously in one bioreactor (one phase systems), or partially separated in two consecutive tanks (two phase systems). Several intermediate products are continuously generated and immediately processed further. In practise it is important to realize that all steps have to occur at matching rates, in order to avoid a build-up of intermediate products. Without good 'teamwork' of all the microbial communities involved, no complete degradation is possible (Gavrilescu, 2002).

Anaerobic treatment consists of complicated pathways of serial and parallel reactions and processes, involving numerous microbial populations, in which interact in many ways (Rozzi and Remigi, 2004).

Four different phases can be distinguished in the overall conversion process; these are Hydrolysis, Acidogenesis, Acetogenesis and Methanogenesis. The mechanisms of these different processes are as follows (Figure 3.2 and Figure 3.3):

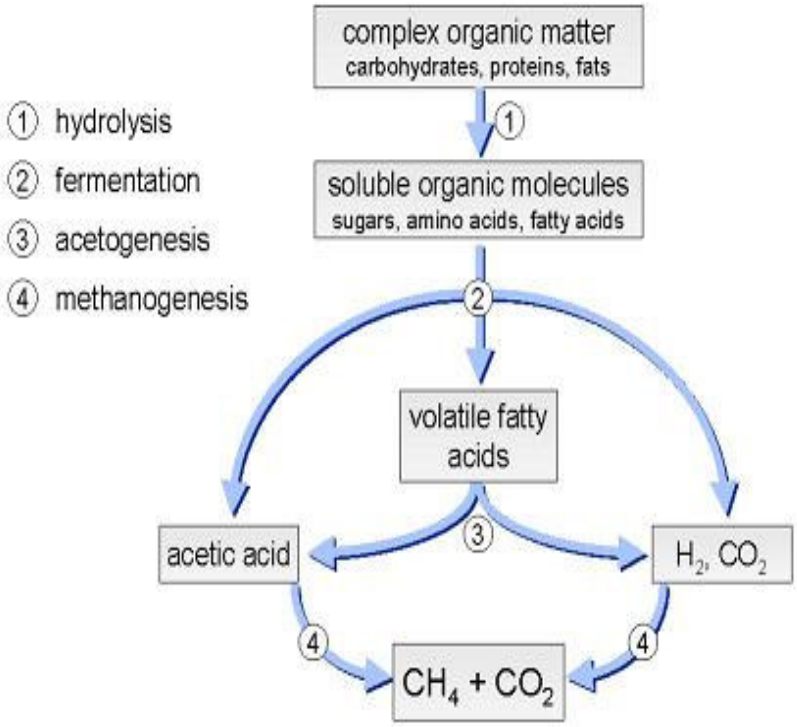


Figure 3.2: Schematic diagram of the patterns of the carbon cycle in anaerobic processes

Hydrolysis:

First, complex polymeric materials such as polysaccharides, proteins and lipids (fats and grease) are hydrolysed by extracellular enzymes to soluble products of a size small enough to allow their transport across cell membrane.

Acidogenesis:

These relatively simple, soluble compounds are fermented or anaerobically oxidised to short - chain fatty acids, alcohol, carbon dioxide, hydrogen and ammonia.

Acetogenesis:

The short-chain fatty acids (other than acetate) are converted to acetate, hydrogen gas and carbon dioxide.

Methanogenesis:

Methanogenesis occurs from carbon dioxide reduction by hydrogen and from acetate to produce methane.

The biogas produced in anaerobic treatment consists mainly of methane (CH_4, 60-70 %), carbon dioxide (CO_2, 30-40 %), and traces of hydrogen sulphide (H_2S, 0.5-1 %).

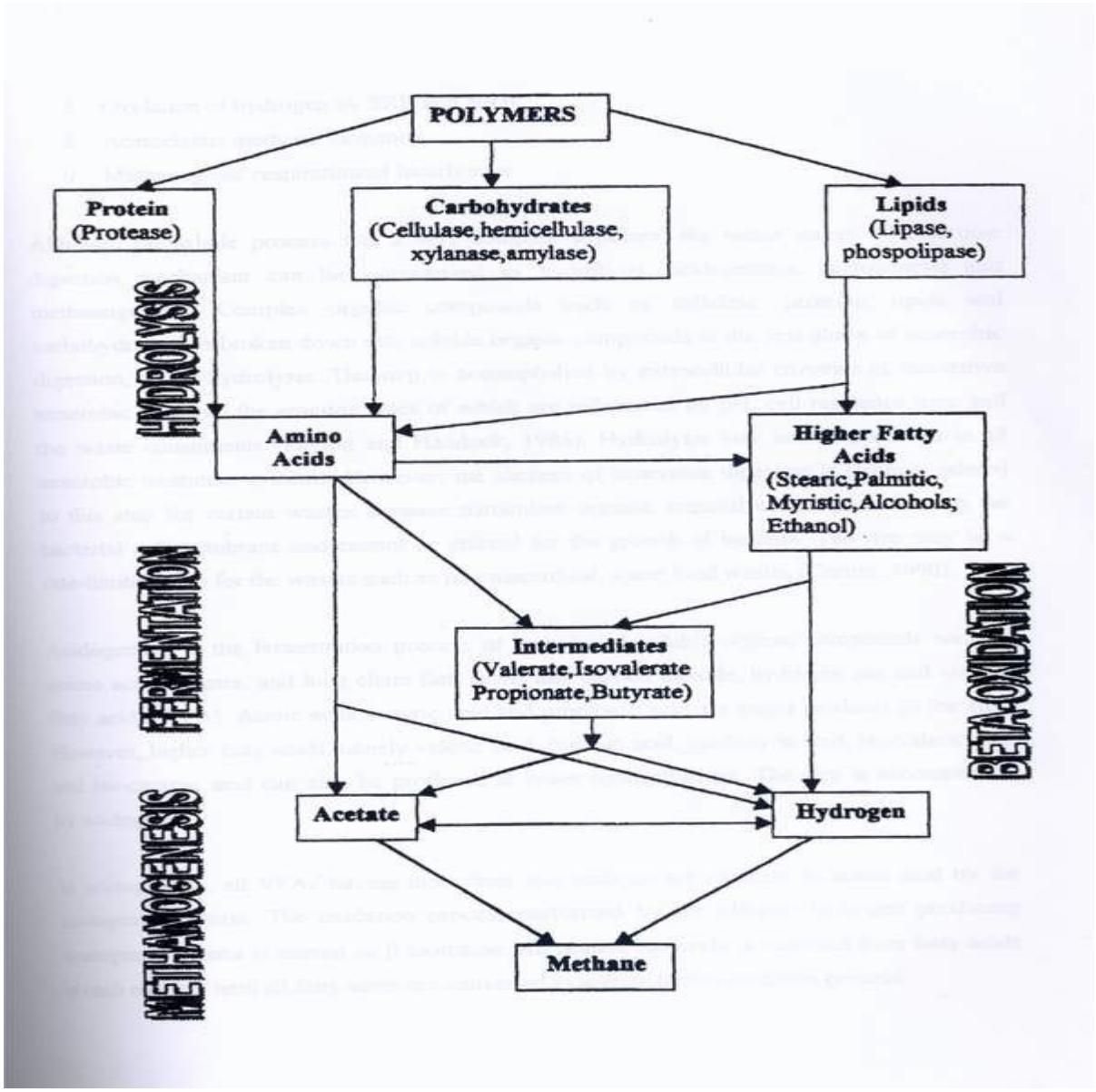


Figure 3.3: The breakdown of organic polymers (Stronach et al., 1986)

3.1.2 Process Microbiology

In anaerobic treatment process the production of methane from the degradation of organic matter depends on the complex interaction of different groups of bacteria. The major groups of bacteria and the reactions taking place in anaerobic digestion are as follows (Figure 3.4):

a) Hydrolytic fermentative bacteria

b) Acidogenic (acid forming) bacteria

c) Hydrogen-producing acetogenic bacteria

d) Hydrogen-utilizing acetogenic bacteria

e) Carbondioxide-reducing methanogens

f) Acetoclastic methanogens

g) Sulphate-reducing bacteria

The consortium of anaerobic organisms that work together to bring about the conversion of organic sludge and wastes can be grouped as follows (Gavrilescu, 2002):

- Organisms responsible for hydrolyzing organic polymers and lipids to basic structural building blocks, such as monosaccharides, amino-acids, and related compounds. This step is carried out by extracellular enzymes of facultative or obligate anaerobic bacteria, e.g. *Clostridium* (degrading compounds which contain cellulose and starch) and *Bacillus* (degrading proteins and fats).

- Anaerobic bacteria, which ferment the breakdown products to simple organic acids, the most common of which in an anaerobic reactor is acetic acid (acidogens or acid formers). These bacteria are described as non-methanogenic and can be *Clostridium spp., Peptoccoccus anaerobus, Bifidobacterium spp., Desulphovibrio spp., Corynebacterium spp., Lactobacillus, Actinomyces, Staphylococcus,* and *Escherichia coli.*

- Organisms, which convert the hydrogen and acetic acid, formed by the acid formers to methane gas and carbon dioxide designated as methanogens or methane formers.

The most important microorganisms that have been identified include the rods (*Methanobacterium, Methanobacillus*) and spheres (*Methanococcus, Methanosarcina*).

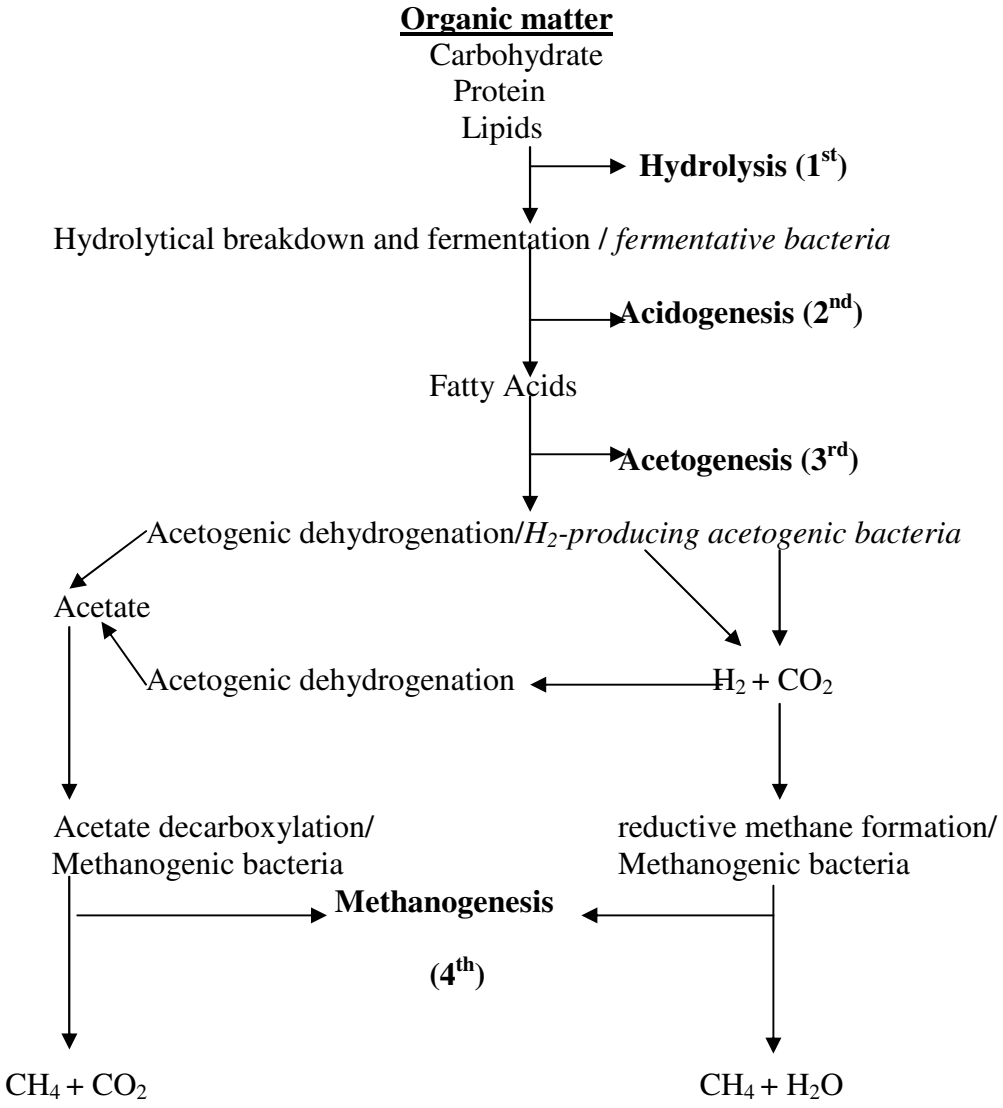


Figure 3.4: Anaerobic degradation processes

3.1.2.1 Phylogeny of Archaea

Modern views on evolution are largely formed by molecular pylogenetics; on the basis of phylogenetic information derived from comparative 16S rRNA analysis. RNA is a single-stranded molecule, which are used for protein synthesis. Therefore, all cells must have rRNA. Similarities and differences in primary structures of 16S rRNAs show

phylogenetic relationship between different organisms. Furthermore, they have 1500 base pairs, which provide enough genetic information to classify organisms. rRNAs have conserved and variable regions which are very useful to distinguish the organisms.

Woese et al. (1990) proposed a new classification for living organisms, dividing life on earth into three major domains: Bacteria, Archaea and Eucarya (Figure 3.5.).

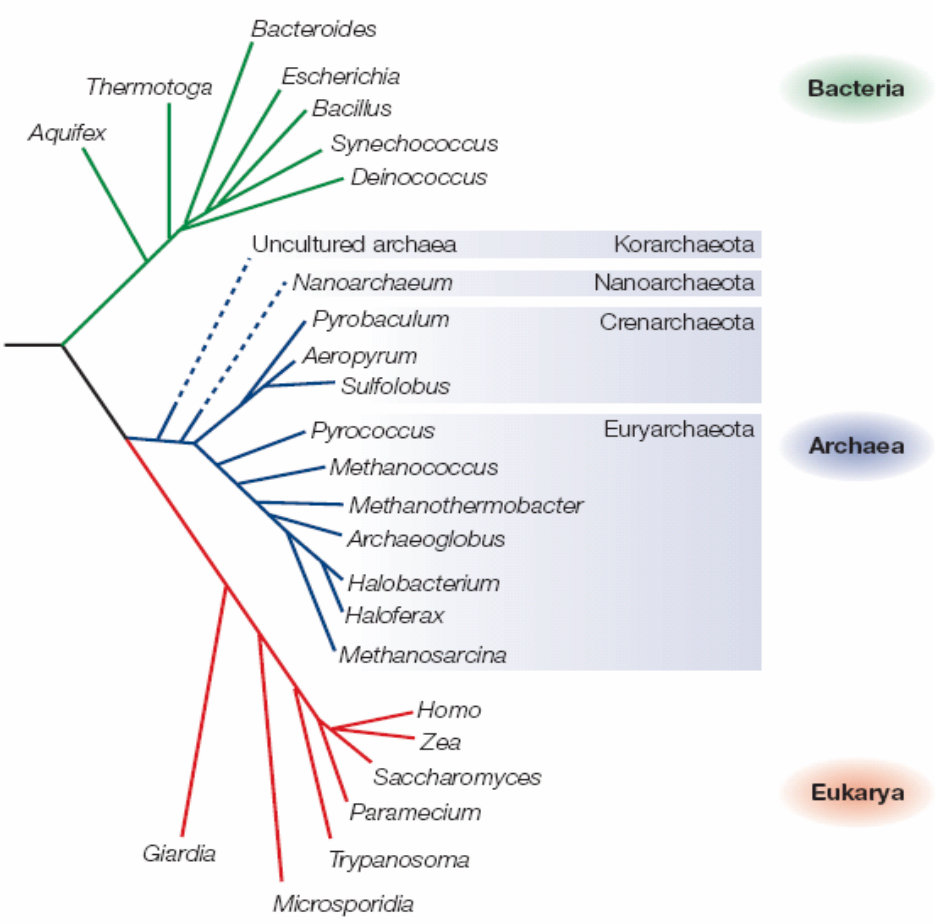


Figure 3.5: A phylogenetic tree of Archaea (Allers and Mevareck, 2005)

A phylogenetic tree based on small subunit ribosomal RNA (ssrRNA) sequences. The tree has been rooted by analysis of duplications in protein sequences (Olsen and Woese, 1997). In all Archaea, membranes are made from ether-linked lipids bonded to glycerol, and thus differ substantially from bacterial membranes. Like the eukaryotes, archaeal cell walls contain no peptidoglycan, again setting them apart from bacteria (Gaasterland, 1999).

Based on 16S rRNA analysis, the archaeal domain is split between four kingdom-level phyla: Euyarchaeto, Crenarchaeota, Korarchaeota and Nanoarchaeota (Rother and Metcalf, 2005). However, for the Korarchaeota, only their nucleic acids have been detected, and no organisms have been isolated or cultured. Based on their physiology, the Archaea can be organized into three types: methanogens (prokaryotes that produce methane); extreme halophiles (prokaryotes that live at very high concentrations of salt (NaCl); and extreme (hyper) thermophiles (prokaryotes that live at very high temperatures). In addition to the unifying archaeal features that distinguish them from Bacteria (i.e., no murein in cell wall, ether-linked membrane lipids, etc.), these prokaryotes exhibit other unique structural or biochemical attributes, which adapt them to their particular habitats. The Crenarchaeota consists mainly of hyperthermophilic sulfur-dependent prokaryotes and the Euryarchaeota contains the methanogens and extreme halophiles. The new group was placed on phyologenetic tree under Crenarchaeota/Euryarchaeota and named as Korarchaeota. ssrRNAs of the Korarchaeota have been obtained from hyperthermophilic environments similar to those inhabitated by Crenarchaeota. Nanoarchaeota are represented to date by only one species, *Nanoarchaeum equitans* (Rother and Metcalf, 2005) (Figure 3.5).

3.1.2.2 Methanogenic Archaea

The performance of the anaerobic reactor and the quality of the effluent depend on the activity of methanogens. Methanogenesis is defined as a rate-limiting step in the whole anaerobic treatment process due to the slow growth rate of the methanogens comparing with acidogens (Malina et. al., 1992; Noike et. al., 1985; Speece, 1983).

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 (Garcia et al., 2000).

Some of the microorganisms involved in the process of the methane production from acetate are the genera of *Methanosarcina* and *Methanothrix*, (Malina et. al., 1992; Noike et. al., 1985; Zehnder et. al., 1982) whereas Methano*thrix soehngenii, Methanosarcina*

barkeri, Methanobacterium sp., Methanococcus mazei are the examples of the most common species defined in the literature (Malina et. al., 1992; Fannin et. al., 1983).

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 *Mathanosaeta* contain gas vacuoles. The Gram reaction can be positive or negative even within members of the same genus (Garcia et al., 2000).

There is a great difference in the composition of the cell walls between methanogenic *Archaea* and *Bacteria*, and considerable diversity among the methanogens themselves. They do not contain murein, the peptidoglycan of *Bacteria*, which is compossed of muramic acid. Instead, they contain pseudomurein or protein subunits depending on the order. Methanogens are therefore insensitive to the antibiotics that inhibit the synthesis of cell walls in Bacteria, such as penicillin, cycloserine, and valinomycin. This particular feature has been employed to facilitate the isolation of pure strains of methanogens by elimination of contaminating non-methanogens (Garcia et al., 2000).

The 83 species of methanogens described so far (including six synonymous) are separated into three main nutritional categories: (a) 61 species (including five synonymous) of hydrogenotrophs oxidize H_2 and reduce CO_2 to form methane and among them 38 species (including three synonymous) of formatotrophs oxidize formate to form methane. (b) Twenty species (including one synonymous) of methylotrophs use methyl compounds as methanol, methylamines, or dimethylsulfide and of which 13 species are obligate methylotrophs. It has been proposed that metabolism of dimethylsulfide proceeds along a somewhat different route to that of methylamines and perhaps also to that of methanol. (c) Nine species (including 1 synonymous) of aceticlastic (or acetotrophic) methanogens utilize acetate to produce methane, with two species in this group being obligate acetotrophs (Garcia et al., 2000).

Table 3.2 illustrates taxonomy of methanogens based on both phenotypic as well as phylogenetic (comparative 16S rRNA sequencing) analyses (Madigan et al., 2002).

Table 3.2: Characteristics of Methanogenic Archea (Madigan et al., 2002)

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

The list of substrates for growth of methanogens may be divided into three groups (Table 3.3). It has been reported that at least ten substrates can be converted to methane by pure cultures of methanogens. Three classes of compounds including CO_2-type substrates, methyl substrates and acetate are listed in Table 3.3.

Table 3.3: Substrates converted to methane by various methanogenic archea (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

In the first group, the energy substrate (electron donor) is H_2, formate, or certain alcohols, and the electron acceptor is CO_2, which is reduced to methane. The ability to utilize H_2 as an electron donor for CO_2 reduction is almost universal among methanogens. Likewise, many methanogens also utilize formate, but the ability to utilize alcohols is less common (Bleicher et al., 1989; Zellner and Winter, 1987a). Some methanogens also utilize carbon monoxide as an electron donor, but growth is very slow (Daniels et al., 1977). CO_2 reduction is the major source of methane in certain habitats such as the rumen. In other environments, such as the sediments of freshwater lakes and certain bioreactors, only about one-third of the methane is formed from CO_2 reduction. However, this reaction is still very important for maintaining the very low concentrations of H_2 and formate typical of these anaerobic habitats and facilitating the process of interspecies electron transfer.

In the second group, the energy substrate is one of a variety of methyl-containing C-1 compounds, which can serve as substrates for a few taxa of methanogens. Usually these compounds are disproportionated. Some molecules of the substrate are oxidized to CO_2. The electron acceptors are the remaining methyl groups, which are reduced directly to methane (Table 3.4). Although dimethylselenide and methane thiol also serve as substrates for methanogenesis, these substrates do not support growth (Kiene et al., 1986). Methanogenesis from C-1 compounds is common where methyl-containing C-1 compounds are abundant. In marine sediments, trimethylamine may be formed from choline, glycine betaine, or trimethylamine oxide. In the large intestine of mammals, methanol may be formed from the anaerobic transformation of the methoxy groups of pectin. Dimethylsulfide is also common in anaerobic environments where it is formed from both methionine and the osmoregulant dimethylsulfoniopropionate.

In the third group, acetate is the major source of methane, but the ability to catabolize this substrate is limited to species of *Methanosarcina* and *Methanosaeta ("Methanothrix").* Acetate is present in many environments, and methane synthesis proceeds by an aceticlastic reaction, in which the methyl carbon of acetate is reduced to methane and the carboxyl carbon is oxidized to CO_2. Methanogenesis from acetate is common in anoxic freshwater sediments where the catabolism of acetate by other anaerobes is limited by the availability of alternate electron acceptors such as sulfate or nitrate.

The placement of most methanogens into three nutritional groups is not surprising and can be explained by the standard changes in free energies for methanogenesis (Table 3.4). The most favourable reaction is the reduction of CO_2 and H_2 and the least favorable is the acetoclastic reaction. So the natural pressure of selection has led to the evolution of many more hydrogenotrophic species than acetotrophs (Garcia et al., 2000).

Boone D.R. et al. (1993) have defined five orders which consist of ten families, 26 genera, and 74 validated species. We provide below the summary of the most recent taxonomic status of these species and a number of recently studied methanogens (Figure 3.6) (Garcia et al., 2000).

Table 3.4: Reaction and standard changes in free energies[a] for methanogenesis[b] (Garcia et al., 2000)

Reaction	**$\Delta G^{0'}$ (kJ/mol CH_4)**
$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$	-135.6
$4\,\text{Formate} \rightarrow CH_4 + 3CO_2 + 2H_2O$	-130.1
$2\,\text{Ethanol} + CO_2 \rightarrow CH_4 + 2\text{Acetate}$	-116.3
$\text{Methanol} + H_2 \rightarrow CH_4 + H_2O$	-112.5
$4\,\text{Methanol} \rightarrow 3CH_4 + CO_2 + 2H_2O$	-104.9
$4\,\text{Methylamine} + 2H_2O \rightarrow 3CH_4 + CO_2 + 4NH_4^+$	-75.0
$4\text{Trimethylamine} + 6H_2O \rightarrow 9CH_4 + 3\,CO_2 + 4NH_4^+$	-74.3
$2\,\text{Dimethylsulfide} + 2H_2O \rightarrow 3CH_4 + CO_2 + H_2S$	-73.8
$2\,\text{Dimethylamine} + 2H_2O \rightarrow 3CH_4 + CO_2 + 2NH_4^+$	-73.2
$4\,2\text{-Propanol} + CO_2 \rightarrow CH_4 + 4\text{Acetone} + 2H_2O$	-36.5
$\text{Acetate} \rightarrow CH_4 + CO_2$	-31.0

[a]: calculated from the free energy of formation of the most abundant ionic species at neutral pH. Thus, CO_2 is HCO_3^- +H^+ and formate is $HCOO^-$+H^+.

[b]: from Whitman W.B. et al., 1992.

Classification

1. Order Methanobacteriales: The order *Methanobacteriales* currently encompasses non-motile methanogens with pseudomurein cell walls and C20 and C40 isopranyl glycerol ethers in their membranes. The order contains two families namely Family I. *Methanobacteriacea*e and family II. *Methanothermaceae* (Figure 3.6).

Family I Methanobacteriaceae: Family *Methanobacteriaceae* contains four morphologically distinct genera. (a) The 13 species of the genus *Methanobacterium* are rod to filamentous cells. Some species are thermophilic and a few are alcaliphilic and found in various freshwater habitats. (b) The genus *Methanothermobacter* was proposed for the inclusion of thermophilic methanogens such as *M. thermoautotrophicum* and *M. wolfei.* (c) The seven members of genus *Methanobrevibacter* are neutrophilic mesophilic short rods, often forming pairs or chains and the G+C content varies from between 28 to 32 mol%. Each species inhabits a specialized habitat. (d) The two species of the genus *Methanosphaera* are Gram-positive spherical-shaped organisms which have been isolated from faeces of man and rabbit and are generally observed in the digestive tracts of animals. The G+C content is 23 to 26 mol%. Both species require both methanol and H_2 as substrates for methanogenesis and are unable to use H_2 plus CO_2 or formate. The type species is *M. stadtmaniae*.

Family II Methanothermaceae: Family *Methanothermaceae* consists of the single genus *Methanothermus* and its two species. Both species are extreme thermophiles and have been isolated from specific habitat (volcanic springs). The temperature optimum is 80^0C. The cells are rod-shaped, contain a double-layered wall and have a mol G+C content of 33-34%. As hydrogenotrophic methanogens, they use only hydrogen and carbon dioxide with prototrophic growth. The type species is *M. fervidus*.

2. Order Methanococcales: The order now contains two families and four genera (Figure 3.6) of hydrogenotrophic methanogens isolated essentially from marine and coastal environments. All species are irregular cocci, contain proteinaceous cell walls and are motile by a polar tuft of flagella. Cells lyse quickly in detergents. All species use both H_2 and formate as electron donors, and are prototrophs, except the three species of '*Methanocaldococcus*' and '*Methanoignis igneus*' which are unable to utilize formate.

Family I Methanococcaceaea: Family *Methanococcaceaea* contains two genera. (a) The genus *Methanococcus* includes five mesophilic species (including 1 synonymous) whose G+C content varies between 30 to 41 mol%. The type species is *M. vanniellii*. (b) The genus '*Methanothermococcus*' has been proposed to include the thermophilic species *M. thermolithotrophicus*.

Family II 'Methanocaldococcaceae': Family '*Methanocaldococcaceae'* has been recently proposed to include two thermophilic genera. The G+C ranges from 31 to 33 mol%.

(a) *'Methanocaldococcus jannaschii'*, an extreme thermophile isolated from a hydrothermal vent on the East Pacific rise, is the fastest growing methanogen known to date (generation time=30 min). (b) '*Methanoignis igneus'* is the only species in the new genus proposed by Boone et al (1993).

3. Order Methanomicrobiales: The order *Methanomicrobiales* comprises three families and nine genera of hydrogenotrophic methanogens (Figure 3.6).

Family I Methanomicrobiaceae: Family *Methanomicrobiaceae* contains seven genera with a variety of different morphologies which includes small rods, highly irregular cocci, and plane-shaped cells. The cell walls are proteinacceous and the lipids include both C20 and C40 isopranyl glycerol ethers.

(a) The genus *Methanomicrobium* includes the single mesophilic species, *M.mobile* whose G+C content is 49 mol%. It is a slightly curved rod, sluggishly motile with a polar flagellum. (b) The genus *Methanolacinia* has been created to include the reclassified species *Methanomicrobium paynteri*. (c) The genus *Methanogenium* contains five species isolated from various environments. Morphologically they are highly irregular cocci, stain Gram-negative and non-motile but do not posses flagella. Cell walls are composed of regular protein subunits. (d) The genus *Methanoculleus* consists of five mesophilic species (including one synonymous) of highly irregular non-motile cocci which stain Gram-negative and one thermophilic species. Formate is used by five species. (e) The genus *Methanoplanus* comprises three species of plane-shaped organisms with polar tuft of flagella. The cell walls contain at least one major glycoprotein. (f) Zellner et al. (1999) have proposed to reclassify *Methanogenium tationis* and *M. liminatans* in a new genus *Methanofollis*. These species use formate and have a G+C content of 54-60 mol%. (g) *Methanocalculus* is a newly described genus which encompasses the irregular coccoid *M.halotolerans*, an isolate from an offshore oil well.

Family II Methanocorpusculaceae: Family *Methanocorpusculaceae* contains one genus, *Methanocorpusculum*, and five species (including one synonymous) of mesophilic, small coccoid methanogens with monotrichous flagellation. They use H_2/CO_2 and formate and some species can use 2-propanal/CO_2.

Family III 'Methanospirillaceae': The creation of family *'Methanospirillaceae'* has been proposed by Boone et al. (1993) to include the single genus *Methanospirillum*. Members of the genus are mesophilic and have been reported from various habitats. However, only one species, *Methanosprillum hungatei*, has been described so far. Cells are curved rods and often form filaments several hundred µm in length. Cells present polar, tufted flagella and are sheathed.

4. Order 'Methanosarcinales': This new order proposed by Boone et al. (1993) regroups all the acetotrophic and/or methylotrophic methanogens into two families (Figure 3.6).

Family I Methanosarcinaceae: Family *Methanosarcinaceae* contains six genera and 21 species (including 1 synonymous). (a) The genus *Methanosarcina* represents the acetotrophic methanogens which predominate in many anaerobic ecosystems where organic matter is completely degraded to CH_4 and CO_2. (b) The genus *Methanolobus* contains five species. The type species, *M. tindarius* is an irregular mesophilic coccus isolated from coastal sediments, with a single flagellum, based on electron micrographs. (c) The genus *Methanococcoides* includes two species with *M. methylutens* as the type species. (d) The genus *Methanohalophilus* encloses four mesophilic, hyperhalophilic species. (e) The genus *'Methanosalsus'* has been recently proposed to reclassify *Methanohalophilus zhilinae* as '*Methanosalsus zhilinae'*, an alkaliphilic, halophilic species of methanogen isolated from an Egyptian lake and able to catobolize dimethylsulfide. The mol % G+C is 38. (f) The genus *Methanohalobium* is represented by only one extremely halophilic species, *M. evestigatum* growing at 25% NaCl and at 50^0C.

Family II 'Methanosaetaceae': Family *'Methanosaetaceae'* includes all the obligatory acetotrophic methanogens grouped into the genus *Methanosaeta* currently consists of two species.

5. Order 'Methanopyrales': Boone et al. (1993) have proposed to include the genus *Methanopyrus* into a new order, *'Methanopyrales'*. This order currently represents a novel group of methanogens growing at 110^0C and unrelated to all known methanogens. The single family *'Methanopyraceae,* includes only one species, *Methanopyrus kandleri* (Figure 3.6). The G+C content is 60 mol%.

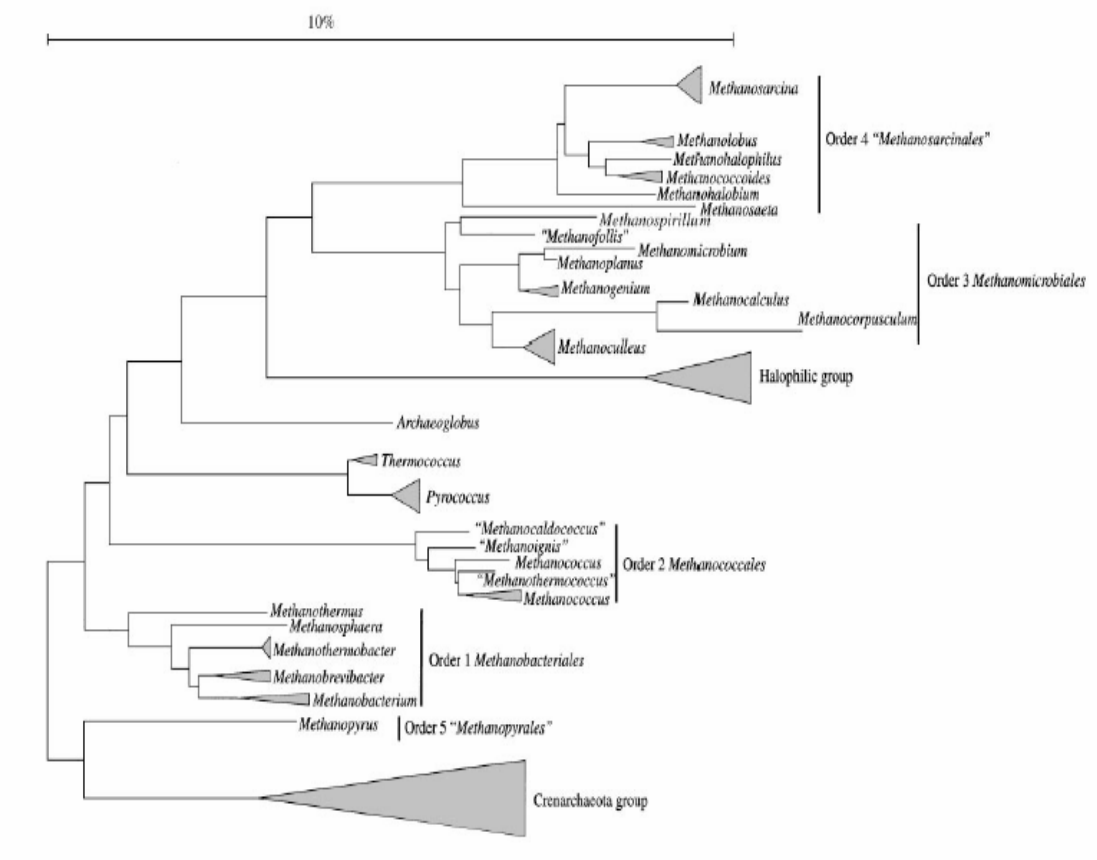


Figure 3.6: Updated phylogeny of methanogens, domain *Archaea*. Genus and family names shown in inverted commas identify changes proposed by Boone et al. (1993), which are yet to be taxonomically accepted and validated. Non-methanogens are indicated by their group names (large triangles) (Garcia et al., 2000)

3.1.3 Environmental Factors Affecting Anaerobic Treatment Process

The anaerobic treatment process is affected significantly by the operating conditions. As the process involves the formation of volatile acids, it is important that the rate of reaction be such that there is no accumulation of acids, which would result in the failure of the digester.

This, in turn, is governed by the loading rate and the influent strength. Temperature and pH are other important variables as the methane producing bacteria are sensitive to these as well (Rajeshwari et al., 2000).

Stability of anaerobic treatment requires a balanced activity of the mixed population of bacteria: this stability can be easily disturbed by different factors causing a rapid increase in the concentration of volatile fatty acids with a concurrent decrease in methane production. Anaerobic digestion failure can occur occasionally and it has been stated that methanogens may be the most sensitive members of the bacterial consortium (Codina et al., 1997).

It must be pointed out that methanogenic microorganisms seem to be extremely sensitive to certain environmental factors, in particular to oxygen, pH, ammonia, temperature and nutrients.

3.1.3.1 Effect of pH

Anaerobic reactions are highly pH dependent. The optimal pH range for methane producing bacteria is 6.8-7.2 while acid-forming bacteria, a more acid pH is desirable. The pH of an anaerobic system is typically maintained between methanogenic limits to prevent the predominance of the acid-forming bacteria, which may cause VFA accumulation. It is essential that the reactor contents provide enough buffer capacity to neutralize any eventual VFA accumulation, and thus prevent build-up of localized acid zones in the digester. In general, sodium bicarbonate is used for supplementing the alkalinity since it is the only chemical, which gently shifts the equilibrium to the desired value without disturbing the physical and chemical balance of the fragile microbial population (Rajeshwari et al., 2000).

3.1.3.2 Effect of Temperature

Anaerobic treatment is strongly influenced by temperature and can be grouped under one of the following categories: psychrophilic (0-20 ^{0}C), mesophilic (20-42 °C) and thermophilic (42-75 °C). The details of the bacterial processes in all the three temperature ranges are well established though a large section of the reported work deals

with mesophilic operation. Changes in temperature are well resisted by anaerobic bacteria, as long as they do not exceed the upper limit as defined by the temperature at which the decay rate begins to exceed the growth rate. In the mesophilic range, the bacterial activity and growth decrease by one half for each 10 ^{0}C drop below 35 ^{0}C. Thus, for a given degree of digestion to be attained, the lower the temperature, the longer is the digestion time.

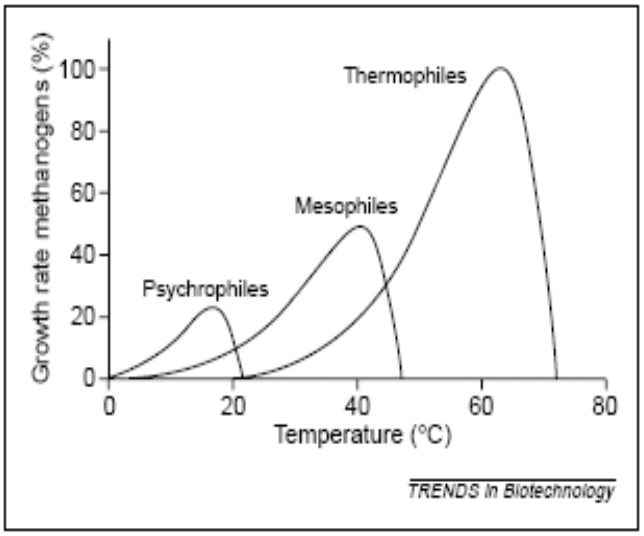


Figure 3.7: Relative growth rates of psychrophilic, mesophilic and thermophilic methanogens (Lettinga et al., 2001)

The effect of temperature on the first stage of the digestion process (hydrolysis and acidogenesis) is not very significant, as among the mixed population there are always some bacteria, which have their optimum within the range concerned. The second and third stages of decomposition can only be performed by certain specialized microorganisms (acetogenic and methanogenic bacteria) and thus, these are much more sensitive towards temperature change. However, an important characteristic of anaerobic bacteria is that their decay rate is very low at temperatures below at 15 ^{0}C. Thus, it is possible to preserve the anaerobic sludge for long periods without losing much of its activity. This is especially useful in the anaerobic treatment of wastewater from seasonal industries such as sugar mills (Rajeshwari et al., 2000).

Microorganisms are classified into “temperature classes” on the basis of the optimum temperature and the temperature span in which the species are able to grow and metabolize (Figure 3.7). The overlapping growth temperature ranges in Figure 3.7 indicate that there isn’t a clear boundary between these classic groups of psychrophilic, mesophilic and thermophilic microorganisms (Lettinga et al., 2001).

3.1.3.3 Effect of Nutrients

The presence of ions in the feed is a critical parameter since it affects the granulation and stability of reactors like UASB. The bacteria in the anaerobic digestion process requires micronutrients and trace elements such as nitrogen, phosphorus, sulphur, potassium, calcium, magnesium, iron, nickel, cobalt, zinc, manganese and copper for optimum growth. Although these elements are needed in extremely low concentrations, the lack of these nutrients has an adverse effect upon the microbial growth and performance. Methane forming bacteria have relatively high internal concentrations of iron, nickel, and cobalt. These elements may not be present in sufficient concentrations in wastewater streams from the processing of one single agroindustrial product like corn or potatoes or the wastewater derived from condensates. In such cases, the wastewater has to be supplemented with the trace elements prior to treatment. The required optimum C: N: P ratio for enhanced yield of methane has been reported to be 100:2.5:0.5. The minimum concentration of macro and micronutrients can be calculated based on the biodegradable COD concentration of the wastewater, cell yield and nutrient concentration in bacterial cells.

Table 3.5: The elemental composition of methane bacteria (Rajeshwari et al., 2000)

Macronutrients		Micronutrient	
Element	Concentration (mg/kg)	Element	Concentration (mg/kg)
N	65,000	Fe	1800
P	15,000	Ni	100
K	10,000	Co	75
S	10,000	Mo	60
Ca	4000	Zn	60
Mg	3000	Mn	20
		Cu	10

Table 3.5 presents the elemental composition of methane forming bacteria in the bacterial consortium. In general, the nutrient concentration in the influent should be adjusted to a value equal to twice the minimal nutrient concentration required in order to ensure that there is small excess in the nutrients needed (Rajeshwari et al., 2000).

3.1.3.4 Effect of Organic Loading Rate

In anaerobic wastewater treatment, loading rate plays an important role. In the case of nonattached biomass reactors, where the hydraulic retention time is long, overloading results in biomass washout. This, in turn, leads to process failure. Fixed film, expanded and fluidized bed reactors can withstand higher organic loading rate. Even if there is shock load resulting in failure, the system is rapidly restored to normal. In comparison to a CSTR system, fixed film and other reduction biomass reactors have better stability. Moreover, high degree of COD reduction is achieved even at high loading rates at short hydraulic retention configurations.

Table 3.6 gives the recommended COD loading rates with various reactor configurations. Anaerobic fluidized bed appears to withstand maximum loading rate compared to other high rate reactor.

Table 3.6: Characteristics of different reactor types (Rajeshwari et al., 2000)

Anaerobic Reactor Type	Start up period	Channeling effect	Effluent recycle	Gas solid separation device	Carrier package	Typical loading rates ($kgCOD/m^3d$)	HRT (day)
CSTR	-	Not present	Not required	Not required	Not essential	0.25-3	10-60
Contact	-	Non-existent	Not required	Not required	Not essential	0.25-4	12-15
UASB	4-16	Low	Not required	Essential	Not essential	10-30	0.5-7
Anaerobic filter	3-4	High	Not required	Beneficial	Essential	1-40	0.5-12
AAFEB	3-4	Less	Required	Not required	Essential	1-50	0.2-5
AFB	3-4	Non-existent	Required	Beneficial	Essential	1-100	0.2-5

3.1.3.5 Toxic Substances in Anaerobic Treatment and Inhibition

Anaerobic treatment is known as a sensitive process to inhibitory or toxic substances, which affect the activities of anaerobic bacteria. These substances may result from either influent waste stream or the metabolic activities of the digester bacteria themselves. Toxic compounds influence anaerobic digestion either by slowing down the rate of metabolism at low concentrations or killing the organism. Studies on toxicity revealed that some toxicants exhibit a reversible effect on the methanogens at the low concentrations. Methanogenesis is generally the most sensitive step to these materials although all groups involved in process can be affected. Common toxic substances in anaerobic digestion causing severe operational failures are volatile fatty acids especially propionate, sulfide, ammonia, heavy metals, cyanide, organic solvents and etc.

Sulphate is present in the wastewaters from many industrial processes, such as molasses fermentation (bakers yeast and ethanol), pulp and paper, pectin, wine distillery, palm oil, petroleum refineries, edible oil production and a variety of other chemical industries. The presence of sulphur-containing chemicals in wastewaters is of major concern in anaerobic wastewater treatment and hence warrants more detailed consideration in this thesis.

The toxicity level of sulphide is closely related to the free hydrogen sulphide concentration. This means that a low pH (<6.5) increases toxicity, whereas the presence of metals reduces toxicity due to precipitation. Capone et al. (1983) studied the interaction between sulphate reduction and methanogenesis, and found that different metals could markedly alter the flow of carbon in salt marsh sediments. Kroiss and Wabnegg (1983) have related methanogenesis inhibition to the level of free H_2S in solution and found that a free H_2S level of 50 mg/l inhibits acetoclastic methane producing bacteria by about 50%, while complete inhibition occurred at a free H_2S level of about 200 mg/l. Winifrey and Zeikus (1977) report a complete cessation of methane production at a sulphide concentration of 340 mg/l. Others report different values but in most cases there is no information concerning the pH in the reactor, which comparison difficult.

The general conclusion is that SRB inhibit methanogenic bacteria by outcompeting them for hydrogen and acetate. Lovlet et al. (1982) further concluded that methanogenic bacteria and SRB can coexist in the presence of sulphate, and that the outcome of competition at any time is a function of the rate of hydrogen production, the relative population sizes and sulphate availability. Thus, methanogenic bacteria and SRB should coexist in environments where the rate of uptake of hydrogen and acetate by the SRB is lower than the rate of hydrogen and acetate production.

Concentrations of soluble sulfide varying from 50 to 100 mg/l can be tolerated in anaerobic treatment with little or no acclimation required. With continuous operation and some acclimations, concentrations up to 200 mg/l of soluble sulfides can be tolerated with no significant inhibitory effect on anaerobic treatment.

3.2 Anaerobic Reactor Configurations

3.2.1 Suspended Growth Systems

3.2.1.1 Completely Mixed Digester

The simplest anaerobic reactor design for wastewater treatment applications is the conventional flow-through tanks without biomass recycle. Since the conventional completely mixed digester process does not incorporate a specific method for retaining and concentrating biomass, the average retention time of anaerobic microorganisms (SRT) is the same as the system hydraulic retention time (HRT). These systems are particularly suitable for wastewaters containing high concentrations of particulates or extremely high concentrations of soluble biodegradable organic materials (Figure 3.9). Due to the slow growth of methanogens, process stability can be limited by the short SRTs and large reactor volumes are required to maintain necessary SRTs. Because of the relatively low biomass concentrations and short operating SRTs, loading rates are typically low (1-10 kgCOD/m^3.day). The organic loading rates to conventional digester systems are usually expressed in terms of volatile solids (VS) since the predominant application of the process is to high particulate wastes. Loading rates of 0.5 to 6.0 kgVS/m^3.day are typical. If the internal mixing devices used are adequate, it provides

uniform conditions such as substrate, temperature and pH throughout the reactor and minimizes dead volume accumulation and flow channeling.

3.2.1.2 Anaerobic Contact Processes

This process is similar to the aerobic activated sludge process, in that cell recycling is used to maintain high biological solids retention at low HRT. Hence, good removal efficiencies can be obtained with small reactors. Since the anaerobic sludge is still actively producing gas when it exits from the reactor, problems have been experienced in getting it to settle quickly. Various methods have been used to get around this problem, including thermal shock and vacuum degasification. This system is suitable for treating effluents containing a high concentration of suspended solids.

In the anaerobic contact process (Figure 3.8), untreated wastes are mixed with recycled sludge solids and then processed in a reactor sealed off from the entry of air. After digestion, the mixture is separated in a clarifier and the supernatant is discharged as effluent and sent for further treatment. The settled anaerobic sludge is recycled to seed the incoming wastewater. Because of the low synthesis rate of anaerobic microorganisms, the excess sludge that must be disposed of is minimal. Its efficiency is limited by the difficulty in achieving sludge concentrations in the sedimentation tank, owing to the nature of the anaerobic sludge (Gavrilescu, 2002).

The first recorded instance of use of the anaerobic contact process occurred in 1955 (Schroepfer et al. 1955) where waste from a meat packing house (BOD 1.6 g/l) was treated successfully at retention times of only 12 hours at 35°C. BOD removals of 95% were obtained, at loading rates of 3.2 kg BOD per m^3 /day and, even at 25°C, removals of 95% were achieved.

Many food wastes can be treated efficiently using this process. With rum still age (COD 54.6 g/l) removals of 80% were obtained, at loading rates as high as 8.0 kg COD per m^3 day^{-1} (Roth and Lentz 1977). Raw sewage (COD 1.2 g/l) has been treated at 20°C, with low retention times (22 hours) in a contact process, and high removals (90%) were obtained.

While some full-scale plants are currently operating in Developed Countries, there are no known plants in Developing Countries. With high-strength industrial wastes, it would appear that other anaerobic processes (e.g., filter, ABR) would be just as efficient, easier to operate, and require less capital outlay.

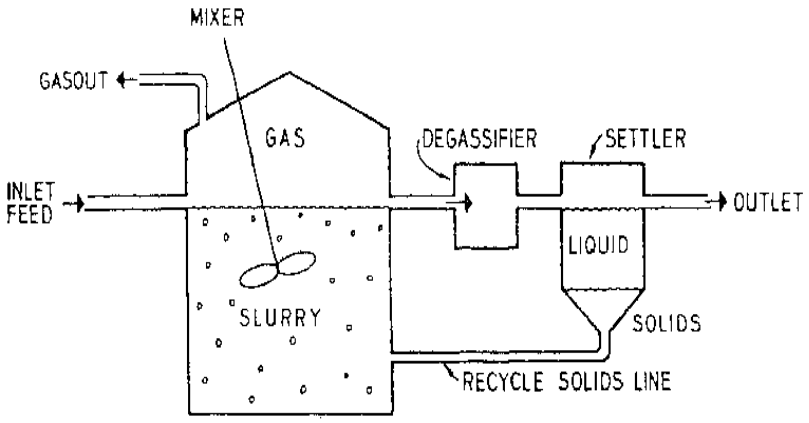


Figure 3.8: Anaerobic Contact Digester

The system SRT can be controlled independently from the HRT with the sludge recycle. Therefore, high treatment efficiency can be achieved by using short HRTs and smaller reactors due to the longer SRTs obtained with sludge recycle. Organic loading rates of 0.5 to 10 kgCOD m^{-3} day^{-1} can be applied to the reactor with HRTs of range between 0.5 and 5 days.

3.2.1.3 Upflow Anaerobic Sludge Blanket (UASB) Reactor

The UASB reactor was invented in the mid-1970s at the University of Wageningen and was applied at full-scale in the Ducth sugar industry (Gavrilescu, 2002). A UASB reactor essentially consists of gas-solids separator (to retain the anaerobic sludge within the reactor), an influent distribution system and effluent draw facilities (Rajeshwari et al., 2000). The sludge-blanket is composed of biological formed granules or particles and the wastewater, introduced in the bottom of the reactor, flows upwards through them (Figure 3.9). Treatment occurs at the contact with the granules (Gavrilescu, 2002). High treatment efficiency can be obtained with short hydraulic retention times and energy demand is low in the process. However, there are difficulties on the control of the granular sludge.

3.2.2 Supported (Attached) Growth Systems

3.2.2.1 Fixed Bed Processes

The anaerobic filter also known as fixed bed or fixed film, is suitable for the treatment of only effluent containing low concentrations of suspended solids. It is similar to the trickle filter in that microbial film grows of an inert solid support. This contactor is a column filled with various solid media. The waste flows upwards through the column, containing the media on which anaerobic bacteria grow. The advantage of this system is that it produces high concentrations of active biomass without the use of a settler (Gavrilescu, 2002). Fixed bed processes can be used for almost all types of industrial wastewaters with low (COD<1000 mg/l) to intermediate (COD>20000 mg/l) concentrations (Figure 3.9).

3.2.2.2 Anaerobic Expanded/Fluidized Bed Processes

In the anaerobic fluidized bed, the media for bacterial attachment and growth is kept in the fluidized state by drag forces exerted by the upflowing wastewater. The media used are small particle size sand, activated carbon, etc. Under fluidized state, each media provides a large surface area for biofilm formation and growth. It enables the attainment of high reactor biomass hold-up and promotes system efficiency and stability. This provides an opportunity for higher organic loading rates and greater resistance to inhibitors. In the expanded bed design, microorganisms are attached to an inert support medium such as sand, gravel or plastics as in fluidized bed reactor (Rajeshwari et al., 2000).

3.2.3 Hybrid Systems

The hybrid systems shown in Figure 3.9 have simple design and require no special gas or sludge separation device. This technology combines features of the upflow anaerobic sludge bed and upflow anaerobic filter processes and has been successfully employed at several complex chemical plants around the world (Rajeshwari et al., 2000). 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.

3.2.4 Two-phase Systems

Two-phase anaerobic digestion processes have been widely applied for waste treatment. The aim was to separate the acid and methane fermentation phases for providing more attention directed toward determining and satisfying the optimum environmental conditions for each microbial community in two separate reactor systems. Two-phase anaerobic digestion process offer significant advantages in comparison to single phase anaerobic waste treatment systems. These advantages primarily include increased process stability and control, a higher specific activity of methanogens and optimization of environmental conditions required for each separate reactor system (Demirel and Yenigün, 2005). The two-phase process has several potential benefits:

- Optimization of the conditions for the hydrolytic acidogenic group of bacteria and for the acetogenic-methanogenic group and subsequent production of the most appropriate acid metabolites for the methanogens and consequently an increase in the rate of substrate utilization and reduction in total reactor volume that cause savings in capital and operating costs (Ghosh et al., 1975),
- By proper control of acidification, increased stability due to the more heterogeneous nature of the bacterial population should result because the process would insure against organic and hydraulic overloading and fluctuations, with the first-stage acting as a metabolic buffer (Zoetemeyer, 1982). Materials toxic to methanogenic bacteria may also be removed in the first-stage,
- Fast growing, acidogenic biomass/sludge may be disposed of without the loss of methanogenic bacteria (Cohen, 1982),
- The product gas contains a higher methane content, thereby reducing gas clean-up costs where required (Ghosh et al., 1983).

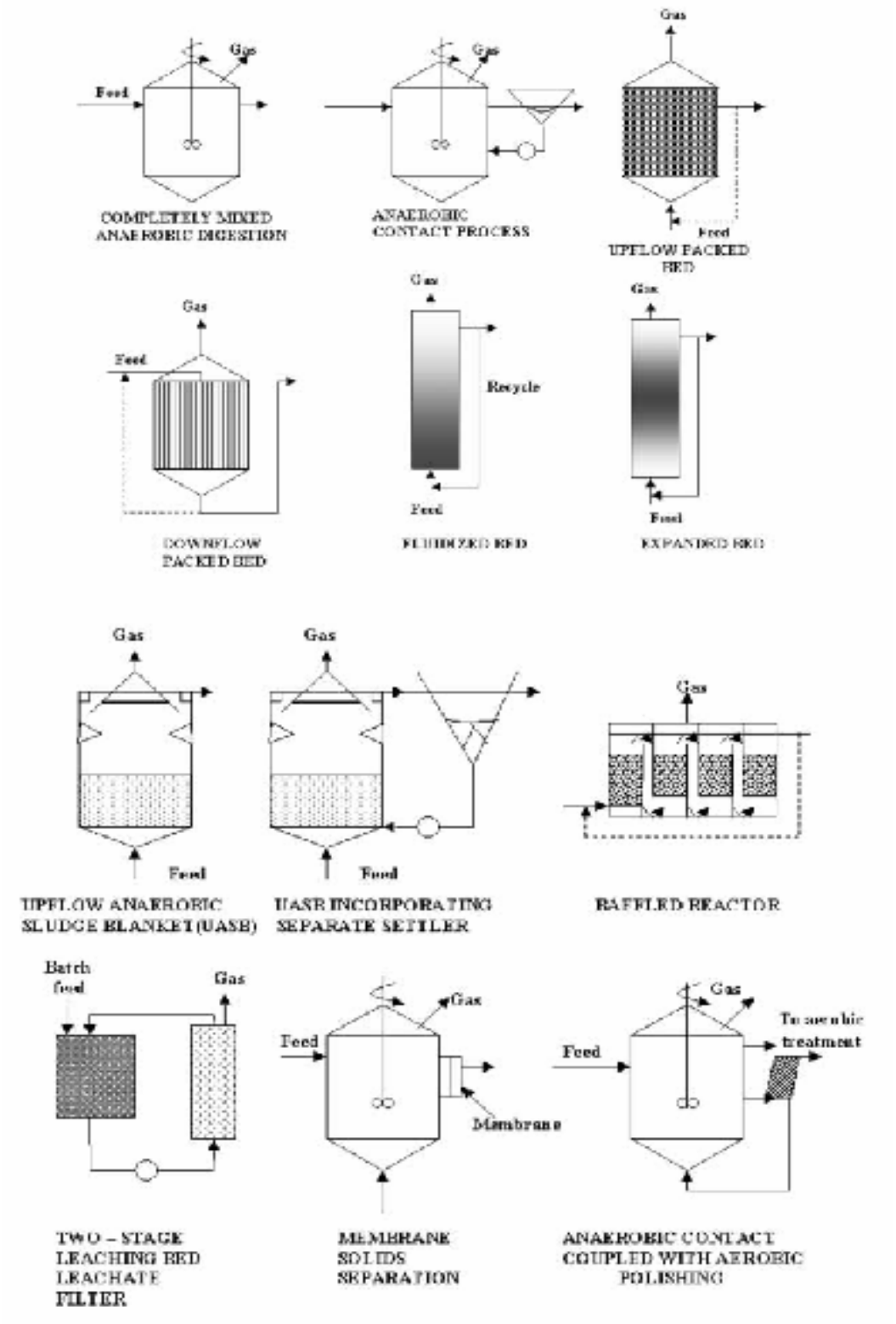


Figure 3.9: Typical processes in use for anaerobic waste treatment (Gavrilescu, 2002)

3.3 Identification and Quantification of Environmental Microorganisms

To start up and operate wastewater treatment systems properly the microbial diversity in the bioreactor should be investigated. These investigations should include the characteristics of microorganisms that lead to the formation of and stability of biomass, and the physiological and ecological properties of the microorganisms in the bioreactor. The application of anaerobic processes in wastewater treatment requires careful operation and monitoring the conventional parameters such as pH, alkalinity, temperature etc. Generally, little attention has been paid to the composition and activity of the microbial community compared to the conventional parameters during the operation of anaerobic reactors. However, an interdependent microbial community in anaerobic reactors reacts highly sensitively to sudden changes in environmental conditions and any imposed stress may lead to a change in species types, their relative population levels and their activity, which are ultimately reflected in the reactor performance. Therefore, maintenance of active methanogenic populations in an anaerobic reactor is critical for stable performance.

It is obvious that the performance of an anaerobic reactor is primarily determined by the amount of active microorganisms retained within the system. Besides, changes in operational and environmental conditions of the anaerobic reactor and within the microbial populations present in the reactor definitely affect each other mutually (Demirel and Yenigün, 2005). Knowledge about microbial diversity and activity of the seed biomass are needed for a successful start-up, however, as a general application, seed biomass is taken from another biological reactor unadapted to the new wastewater.

The microbial community of an anaerobic reactor is composed of large number of different organisms belonging to the *Eucarya, Bacteria* and *Archaea* domains. Anaerobic *Bacteria* and *Archaea* represent the major phylogentic groups within the anaerobic reactor community, and includes such physiological groups as hydrolytic bacteria, fermentative acidogenic bacteria, acetogenic syntrophic bacteria, hydrogenotrophic and acetotrophic methanogens, and sulfhate-reducing bacteria. For a better understanding and control of this diverse community, the groups must be analyzed

quantitatively. Notwithstanding the fact that methanogens and obligate anaerobic bacteria are difficult to cultivate in the laboratory, it is possible to enumerate and monitor these organisms by routine cultivation methods. It is, therefore, necessary to monitor any changes in the numbers or activities of methanogenic species in anaerobic reactors using available techniques such as fluorescence *in situ* hybridization (FISH), microscopic counts, most probable number (MPN), adenosine three phosphate (ATP), coenzyme F_{420}, dehydrogenic activity (DA) and specific methanogenic activity (SMA) tests under controlled conditions.

Most probable number (MPN) assays are methods used to quantify the number of microorganisms in aqueous samples without direct counting. Serial dilution of the sample estimates the density of microorganisms presents on the basis that one microorganism will produce a positive result after incubation. 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.

The speed with which specific genes can be isolated from the environment has rapidly made PCR methods favoured means of molecular analysis. The polymerase chain reaction (PCR) is a method for amplifying a sequence of DNA using a heat-stable polymerase and two 20-base primers, one complementary to the (+)-strand at one end of the sequence to be amplified and the other complementary to the (-)-strand at the other. PCR is applicable to the study of bacteria, fungi and viruses in a range of environmental samples, including soil, sediment, water, air and clinical samples.

Denaturing gradient gel electrophoresis (DGGE) is a technique that can be used to separate DNA fragments of the same length but differing in as little as a single base change. The basis of the technique is that DNA fragments that have different nucleotide sequences denature to differing degrees in the presence of a given concentration of denaturing chemicals (7M urea and 40 percent formamide, and temperature of 50-65^{0}C). The more denatured the DNA fragment, the lower its electrophoretic mobility in polyacrylamide gel. Because DGGE is based on analysis of nucleic acid sequences, it is

applicable to the study of bacteria, viruses and fungi in any environment from which DNA can be extracted.

Temperature gradient gel electrophoresis (TGGE) is a similar technique to DGGE. However, with TGGE the concentration of denaturing chemicals remains uniform whilst the temperature of the gel is increased gradually and uniformly, so that as the DNA passes down the gel, it encounters gradually increasing temperatures. The advantage of TGGE over DGGE is that as no chemical gradient is required, rapid high-throughput screening of samples is possible. However, the specificity of TGGE is slightly lower than DGGE.

A simpler approach to the quantification of microbial groups is presented by whole-cell fluorescence *in situ* hybridization (FISH) with rRNA-targeted, fluorescent oligonucleotide probes. Many specific probes and successful applications of *in situ* hybridization to detect different phylogenetic groups in the methanogenic community are known. Quantification of a methanogenic community by FISH can help in the optimization of the performance of an anaerobic reactor (Tay et al., 2001)

Fluorescence *in situ* hybridization (FISH) methods involve the labeling of specific nucleic acid sequences inside intact cells using so-called phylogenetic stains (DeLong et al., 1989). Because the oligonucleotide probe conferring the fluorescence is correlated to the ribosomal RNA (rRNA) of the cell, then the method provides an indication of growth rate, cell activity and viability (DeLong et al., 1989; Wallner et al., 1993).

FISH is based on oligodeoxynucleotide hybridization probes complementary to ribosomal RNA (rRNA) sequences that are diagnostic for selected phylogenetic groups. The microbial cells are made permeable to and hybridized with the radioactively labeled or fluorescent-dye labeled probes. The extent of hybridization is used as a measure of abundance and/or activity of the target population. When the probes are labeled with fluorescent dyes, they can be detected using a microscope (Rozzi et al., 2004).

rRNA is used as target molecule, because it can be found in all living organisms, is relatively stable and occurs in high copy numbers and because its content in microbial cells is proportional to the growth rate: therefore, the amount of fluorescent rRNA-

targeted probe that binds to cells should reflect growth rates and metabolic activities (Rozzi et al., 2004).

The main advantage of FISH against classical chemical stains is that it provides phylogenetic information on single microbial cells and requires no previous knowledge of the organism detected (Rozzi et al., 2004).

Bacteria and archaea contain 5S, 16S and 23S rRNAs with lengths of approximately 120, 1500 and 3000 nucleotides, respectively. In the vast majority of applications FISH probes target 16S rRNA. The public databases now include 16S rRNA sequences for most cultured microbial species, as well as numerous sequences directly retrieved from the environment (Amann et al., 2001).

A typical FISH protocol includes four steps: the fixation and permeabilisation of the sample; hybridization with the respective probes for detecting the respective target sequences as seen in Figure 3.10; washing steps to remove unbound probes; and the detection of labeled cells by microscopy or flow cytometry.

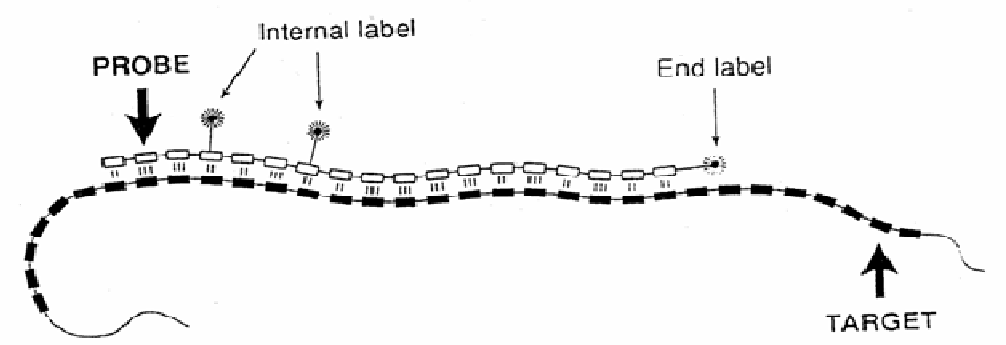


Figure 3.10: Hybridization of probe with target sequences

Prior to hybridization bacteria have to be fixed and permeabilized for penetration of the fluorescent probes into the cell and to protect the RNA from degradation by endogenous RNAses. Hybridization must be carried out under stringent conditions for proper annealing of the probe to the target sequence. For this crucial step of the FISH procedure, preheated hybridization buffer is applied to the sample containing fluorescently labelled probes complementary to the target RNA. Stringency can be adjusted by varying either the formamide concentration or the hybridization temperature. Formamide decreases the melting temperature by weakening the hydrogen bounds, thus

enabling lower temperatures to be used with high stringency. The hybridization takes place in a dark humid chamber, usually at temperatures between 37^0C and 50^0C. Hybridization time varies between 30 min and several hours. Afterwards slides are briefly rinsed with distilled water to remove unbound probe. Post-hybridization stringency washes are performed as required. To reduce the amount of toxic waste, varying the salt concentration instead of by using formamide can regulate stringency of the washing buffer. Finally, slides are rinsed with water again, dried and mounted (Moter A. and Göbel U. B., 2000).

CHAPTER 4 MATERIALS AND METHODS

4.1 Description of the Pulp and Paper Production Process

Modern Karton San. ve Tic. A.Ş. has started to produce packing paper based on waste paper with the capacity of 24 000 tons/year in 1981. There are Paper Machine1 (PM1), Paper Machine2 (PM2) and Paper Machine3 (PM3) paper production lines at Modern Karton. The third production line was started up in the year of 2000. The total capacity of PM1 and PM2 production lines is 100 000 ton/year, and PM3 production line is 200 000 ton/year. It is planned to build Paper Machine 4 (PM4) production line in addition to the existing paper production lines.

The detailed explanation of the production processes of PM4 paper production line is given below. The capacity of the line is 400 000 ton/year and after the below mentioned stages, short, medium and long fiber pulp and paper is made.

The raw material (waste paper) is fed into the pulpers by means of a conveyor. Pulping density (% fiber rate) is between 4.0-4.5 %. Pulper cleaning equipments removes the bigger pollutants such as plastics, which cannot pass through the sieves. The passed pulp is sent to the rough sand filters and the existing large items such as gravels, large sands and glass are taken out from the pulp. The large dirt and sands, which were removed former, are sent to the compactor (filterpress). The pulp coming from the pulper is sent to an intermediate tower. It is pumped to rough elimination equipments at 3% densities. The rough elimination equipments exist from 3 stages. The dirty outlet of the final stage is sent to the compactor. The density of the pulp passed through the rough elimination is decreased between 1.3-1.5 % and the small solid particles (like sand, glass) in the pulp are removed from the pulp. After thin sand cleaning, the pulp is divided into two parts after passing through a 0.2 mm slot sized 2 stage-pressured sieves. The density of the pulp, which can pass through the slots, is decreased to 0.7 % and is diluted. An important piece of the light materials such as wax and paraffin inside the pulp are removed from these reverse cleaners and sent to the disc filters to increase the density. The pulp taken from here is divided

into two parts after passing again through a pressured sieve. The passed part is called fiber; the part, which cannot pass, is called middle fiber. Fiber and middle fiber are sent into different towers and stored here for usage of paper machines. In the fractionation stage the pulp that cannot pass through 0.20 mm is called long fiber. As there is still dirt inside it, a thin cleaning is made with the density of 1.6 % by 3 stages pressured sieves. After this stage, in order to remove the light materials such as wax and paraffin inside the long fiber, it is sent to reverse cleaners. After removing most of the dirt here, it is sent to a long fiber disc filter in order to increase the density to 10 %. Here firstly the pulp's density is increased to 30 % from 10 % with pulp press. After that, the temperature is increased to 90-95 ^{0}C by giving steam in it. And it is passed through disperser. The dirt is broken into small pieces. The density is increased to 10 % again and sent to the storage tower for usage of the machine.

In all process of the production the recycling materials such as waste paper and pulp is used to protect the ecosystem and provides 5000 trees 8 meters-long uncut each day. The products are:

- Straw cardboard
- Straw fluting
- Fluting
- Testliner and Testliner 3
- White top testliner
- Modkraftliner

Straw cardboard is used in materials like core, conic etc. in different sectors. It is a kind of strong and environmentally safe cardboard. Straw fluting is obtained by the paste of mixed pulp and recycled paper. Using the foremost technological system based on 100 % recycled papers produces fluting and whereas not including the cellulose, it has the high technical and strength values. Testliner is a two-layer paper consists of fluting and modkraftpaper. Testliner 3 is a typical brown testliner. White top testliner is produced with the latest technology of the world based on select and 100 % recycled papers. Modkraftliner is a surface paper alternative for the kraft used packaging. 100 %-recycled papers make it.

4.2 Description of the Wastewater Treatment Plant

The system is treating wastewater for Paper Machine 1, Paper Machine 2, Modern Enerji Elektrik Üretimi Otoprodüktör A.Ş. and Modern Karton production lines. This system is built on 6500-m^2 areas.

Wastewater is treated with anaerobic and aerobic biological treatment. The wastewater called 'Black Liquor' from straw units and 'White Liquor' from paper machines is transmitted from different streams into the system.

White liquor; passing from coarse screen and hyper fine sieve is collected in the first sedimentation basin and transmitted into the white liquor equalization basin with volume of 120 m^3. From this basin water is fed with a stable flow (60-105 m^3/h) into the anaerobic tank. On the other side the black liquor is collected in an equalization tank and is fed into the anaerobic tank with stable flow (10-20 m^3/h). The temperature of the white and black liquor is increased to 37 ^{0}C, which is the most suitable temperature for bacteria's activity. The characteristics of white liquor and black liquor are given in Table 4.1.

Table 4.1: Characteristics of white liquor and black liquor

	White Liquor	Black Liquor
Flow rate, m^3 day^{-1}	2 000	500
COD, mg dm^{-3}	7 300-7 900	11 700-13 300
PH	5-7	8-9

The anaerobic contact reactor, with 10 000 m^3 volume and 16 m height, is the biggest anaerobic reactor in Turkey and forms biogas which equals to 5-6 ton fuel oil from the pollution load. The active volume of the anaerobic contact reactor is 9687 m^3 and active height is 15.5 m. The temperature and pH in the reactor are maintained within the ranges of 35-37 ^{0}C and 6.4-7.5 respectively. Wastewater from upper flow of the anaerobic tank is fed into the 350 m^3 lamella sedimentation tank. After the entrance that is designed in order to remove gas, the suspended solids are removed from the water at the lamella part. The sedimented waste is transmitted back into the anaerobic system and centrifugal separator removes the excess sludge.

The water that overflows from the lamella is fed into the aerobic tank with volume of 1000 m^3. In the aerobic tank dissolved oxygen is given with a diffuser until dissolved oxygen level of 2 mg/l is reached. The effluent water is fed into the final

sedimentation basin and the settled sludge is recycled back into the aeration basin. Centrifugal separator removes excess sludge.

The flow diagram of wastewater treatment process of Modern Karton-Corlu pulp and paper production is given in Figure 4.1.

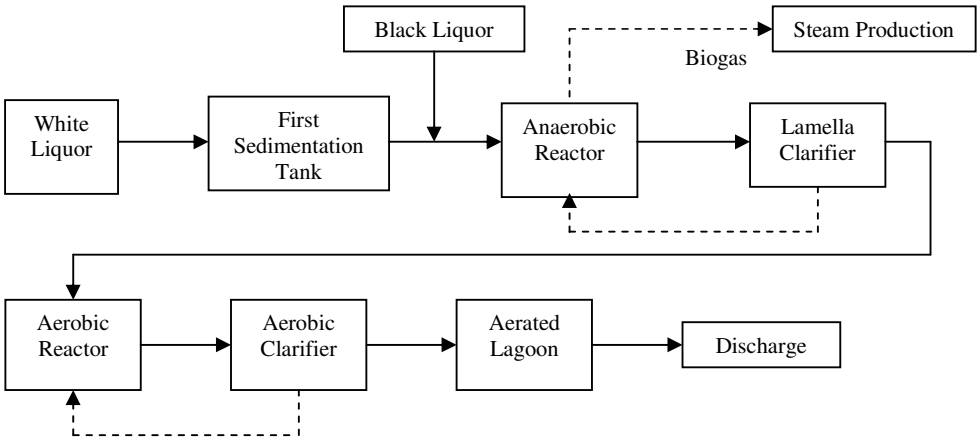


Figure 4.1: Flow diagram of wastewater treatment process of Modern Karton

An important amount of water consumption is seen during paper pulp preparation and paper production. The water consumption per tons of the product is about 10-100 m^3 at the similar plants. In contrast of the new built Paper Machine 4 (PM4) and the other production machines, PM4 has more equipment but needs less electrical power and water per good. It is foreseen that the water consumption is going to be 4.2 m^3/ton. 1.2 m^3 of this amount will be vaporized during drying the paper. Furthermore, heat recovery systems will be built in order to recover the evaporated amount. The rest 3 m^3/ton water will be discharged from the water treatment plant. The amount of the discharge is the minimum amount for protecting the quality of the good and supplying the running of the machine. In order to achieve this value, some equipment that gives various water qualities will take place to use the machine out of the treatment plant. These equipments are disc filters, microfiltration equipments and ultrafiltration systems in order to be used in more sensitive areas. The water amount that will be cleaned by ultrafiltration and reused is 62 m^3/h. This amount will decrease the clean water consumption minimum 1 m^3/ton. With this system the fall of the water turbidity will be more than 98 %, the inlet TSS value will decrease more

than 99 % from 100-500 mg/l and will be less than 5 mg/l. Furthermore there will be a microorganism removal more than 98 % in the water.

4.3 Wastewater Characteristics of the Anaerobic Contact Reactor

The anaerobic reactor is fed with two different streams. The wastewater called 'Black Liquor' with 7300-7900 mg/l COD and 2000 m^3/day volume from straw units and 'White Liquor' with 11700-13300 mg/l COD and 500 m^3/day volume from paper machines is transmitted from different streams into the system. Wastewater characteristics of the wastewaters are given in Table 4.2.

Table 4.2: Wastewater characteristics of the anaerobic contact reactor

	Anaerobic Contact Reactor
Influent COD, mg/l	8 200-9 000
Influent SO_4^{-2}, mg/l	850-950
SS, mg/l	16 600-18 000
VSS, mg/l	10 000-11 500
PH	5.6-6.6

4.4 Sampling

Triplicate samples were collected from three different heights (4m, 8m and 12 m) of the anaerobic contact reactor on July and August 2005. First samples were for SMA test and second and third samples were for FISH analysis. For SMA test, samples were transferred to the laboratory in cool-boxes maintained at 4°C or less. For FISH analysis, samples were transferred into sterile containers with the addition of 98% ethanol (1:1, v/v) on-site and transferred to the laboratory in cool-boxes maintained at 4°C or less. Upon arrival, samples were stored at -20°C and fixed within a week.

4.5 Analytical Methods

During the operation of the full-scale anaerobic contact reactor temperature, pH, COD and biogas production parameters were monitored. Suspended solids and

volatile suspended solids (SS/VSS) were measured. All analyses were carried out according to Standard Methods (APHA, 1997).

4.6 Description of Specific Methanogenic Activity (SMA) Test Equipment

The SMA test gives information about activity of acetoclastic methanogens and also provides information on potential loading capacity and optimum operating conditions of anaerobic reactor. The SMA is an indicator for evaluating the methanogenic activity of the biomass under a condition in which the supply of substrate is not a limiting factor (Han Sun-Kee et al., 2005).

The schematic diagram of SMA test unit is shown in Figure 4.2. The SMA test unit consisted of eight 1 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 60 rpm. Gas measurement system is shown in Figure 4.3 and contains a manometer and tubing for interconnection between the anaerobic reactor and the other units. This system has a solenoid valve, which has 3 ports. The valve is controlled with a pressure measurement device. There is a gas bulb for temporary storage of the gases and a line for interconnection anaerobic reactor and the units of the system. The solenoid valve was set so that the two normally open ports (1 and 2) communicate with the pressure measurement device and the gas bulb. When the third port was closed, the pressure in the reactor and in the bulb increased progressively. 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 valve was deactivated after an interval of time (3s for the complete release of the gases) and a new cycle was initiated.

A microcomputer Amstrad Model 1620 connected to the gas metering system by using an 8 channel analog input board model DAS 800 (supplied by Metrabyte Corporation) which was used to simultaneously monitor the gas production of the eight independent digesters.

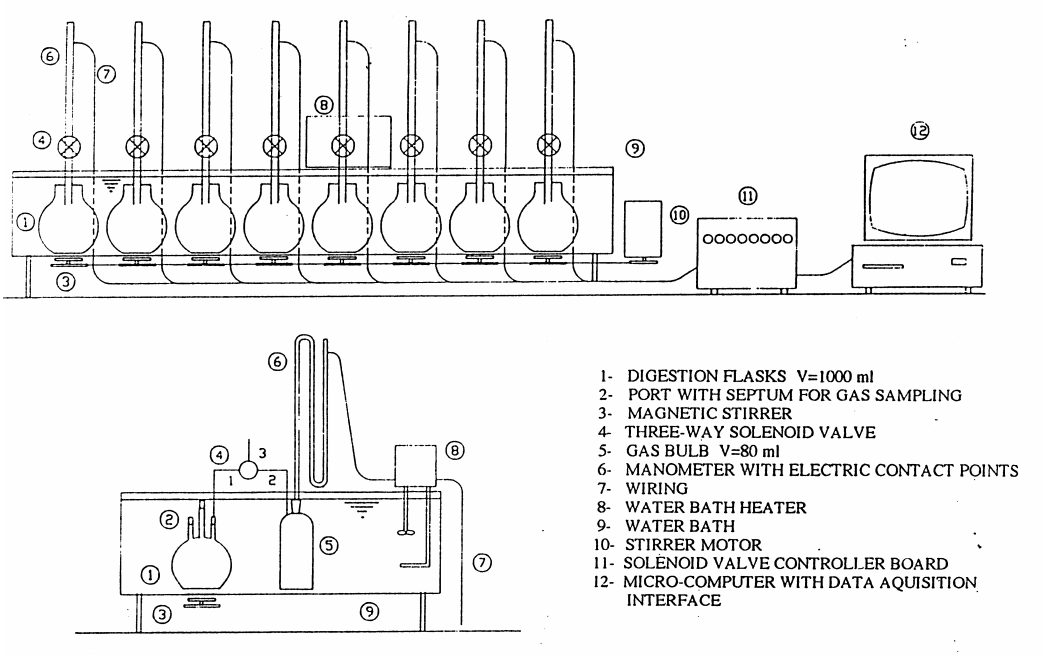


Figure 4.2: Experimental set-up for SMA test unit

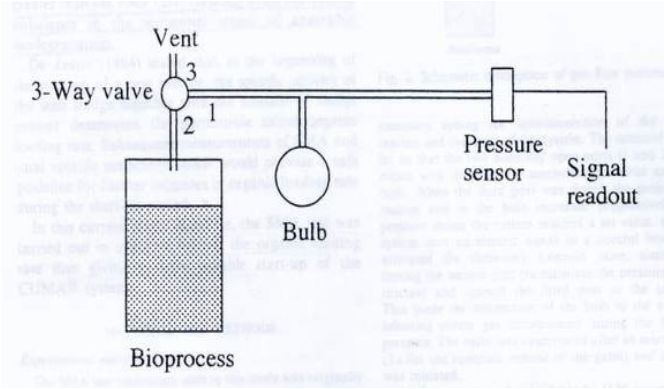


Figure 4.3: Schematic description of gas flow metering system

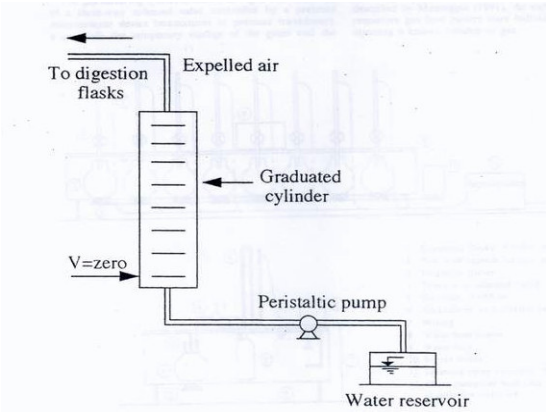


Figure 4.4: The device used for calibration

The device used for calibration of the eight digesters with their respective gas flow meters is shown in Figure 4.4 and was described by Monteggia (1991), the eight digesters and the respective gas flow meters were individually calibrated by injecting a known volume of gas.

4.6.1 Experimental procedure of specific methanogenic activity test

In this study, a fully computerized specific methanogenic activity (SMA) test unit originally developed by Monteggia (1991) and modified by Ince (1995a) was used to determine acetoclastic methanogenic activity. The laboratory routine for SMA test is given as follows:

1- 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).

2- The concentration of volatile suspended solid (VSS) in the reactors is about 2000 mg/l by diluting sludge sample with a mineral stock solution (Table 4.3).

Table 4.3: A mineral stock solution for SMA test* (Valcke and Verstraete, 1983)

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

*The pH of the solution should be adjusted to 6.8.

3- The each 1 L digestion flask is ensured to have 900 ml active volume.

4- Water level in monometer is adjusted by using respirometer.

5- The pH of the reactors should be adjusted to 6.8-7.0.

6- Reactors should be flushed with helium or nitrogen gas for a period of approximately 5-10 minutes at a pressure of 35-70 kN/m^2 to maintain anaerobic conditions in the reactor.

7- 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.

8- Temperature of the reactor content should be maintained 35±0.5 °C by heating water bath. Temperature should be dispersed equally all over the reactor.

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

10- Acetate as substrate is introduced to the SMA reactor (2000-4000 mg/l acetate).

11- For the last time, reactors are flushed with helium or nitrogen gas for a period of approximately 5-10 minutes.

12- 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.

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

14- Biogas production is saved automatically for every hour.

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

16- The volume of methane produced per unit of time and potential methane production rate is calculated using Equation 1-2.

4.6.2 Calculation of Specific Methanogenic Activity

The gas produced in the reactor is sent to a gas-washing flask. The methane content of the gas is measured by gas chromatograph. The volume of methane production per day and the potential methane production is calculated by the formulas expressed below:

$$D\ (ml\ CH_4/day) = A \times B \times C \times 24 \qquad (4.1)$$

A: Biogas production per hour

B: Methane content of biogas produced (%)

C: Valve factor

D: Volume of methane production per day (ml CH_4/day)

$$SMA\ (Specific\ methanogenic\ activity)\ (ml\ CH_4/gVSS.d) = D / (E \times F) \qquad (4.2)$$

E: Active volume of the SMA test reactor

F: Concentration of biomass in SMA test reactor

4.6.3 Feed and seed sludge for SMA tests

Acetate was used as feed during SMA tests, since approximately 72% of the methane formed during anaerobic digestion of complex substrate results from acetic acid (McCarty, 1964). Acetate concentrations in a range of 2000-4000 mg/l were initially tested in order to reach maximum potential methane production (PMP) rate during the SMA tests. Among those 3000 mg/l acetate concentration was found to be optimum.

The height of the anaerobic contact reactor is 16 m (active height is 15.5m). Samples were collected from three different heights of the anaerobic contact reactor (4, 8 and 12 meters up from the ground of the anaerobic contact reactor) on July and August 2005.

SS and VSS concentrations of the anaerobic contact reactor' sludge which belongs to three different heights in the reactor is given in Table 4.4. Reactor sludge was diluted to 2000 mg VSS l^{-1} for SMA tests as described in the laboratory routine.

Table 4.4: SS and VSS concentrations of the anaerobic contact reactor sludge

July, 2005	4m	8m	12m
SS, mg/l	11 200	8 130	8 153
VSS, mg/l	5 820	4 320	4 350

August, 2005	4m	8m	12m
SS, mg/l	14 780	7 320	8 720
VSS, mg/l	8100	3 960	4 740

4.7 Fluorescence *in situ* hybridization (FISH)

In order to assess the number of samples required at any one time and the number of random fields of view required counting any one sample, duplicate sludge samples were collected from each of the level of the anaerobic contact reactor for FISH analysis. The two samples were subdivided into two parts (four different hybridization for one sludge sample), and counts for 10 random fields of view were obtained. The average counting results and standard deviations were calculated according to four different hybridization results.

4.7.1 Standard paraformaldehyde (PFA) fixation

Sludge-ethanol mix (1:1, v/v) was washed once with phosphate-buffered saline (PBS) [130 mM NaCl p, 10 mM sodium phosphate, pH 7.2]) and resuspended in PBS in the ratio of 1:3 (v/v) and freshly prepared 4% paraformaldehyde (PFA) 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 PBS-absolute ethanol (1:1, v/v) and stored at -20°C (Harmsen et al., 1996).

4.7.2 Hybridization

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 pyrolidone, 5mM EDTA, pH 8.0, 25 mM NaH_2PO_4, pH 7.0, 0.1% SDS, 10-35% deionised formamide) and prehybridized at the intended hybridization temperature (Table 4.6) for 15 minutes (Amann et al., 1990a; Manz et al., 1992). After prehybridization, 2µl of probe (50 ng μl^{-1}) was added and incubated at the optimal hybridization temperature for the given probe for at least 4 hours or overnight.

The relative quantities of target methanogen groups in the whole microbial community in anaerobic sludge samples were determined using fluorescent rRNA targeted oligonucleotide probes. 16S rRNA-targeted oligonucleotide probes used in this study and their target microbial groups nucleotide sequences are listed in Table 4.5. Optimal hybridization conditions for each probe are also given in Table 4.6. All probes were made, labelled, and obtained commercially (Qiagen Corp.).

Whole microbial communities in sludge samples were also stained using DAPI (4', 6-diamidino-2-phenylindole) DNA stain to visualize intact cells in the samples. Phylogenetic tree for methanogens and the oligonucleotide probes designed for methanogens are also showed in Figure 4.5.

Table 4.5: 16S rRNA-targeted oligonucleotide probes used in this study

Probe	Target Group	Probe Sequence (5'-3')	Labelling (5')	Reference
MC1109	*Methanococcales*	GCAACATAGGGCACGGGTCT	CY3	Raskin et al., 1994a
MB310	*Methanobacteriales*	CTTGTCTCAGGTTCCATCTCCG	CY3	Raskin et al., 1994a
MG1200	*Methanogenium* relatives	CGGATAATTCGGGGCATGCTG	CY3	Raskin et al., 1994a
MS1414	*Methanosarcina* + relatives	CTCACCCATACCTCACTCGGG	CY3	Raskin et al., 1994a
MS821	*Methanosarcina*	CGCCATGCCTGACACCTAGGCCAGC	CY3	Raskin et al., 1994a
MX825	*Methanosaeta*	TCGCACCGTGGCCGACACCTAGC	TAMRA	Raskin et al., 1994a

Table 4.6: Optimum hybridization conditions for oligonucleotide probes

Probe	Formamide concentration	Hybridization temperature	Washing temperature	NaCl Concentration
MC1109	20%	46 °C	48 °C	225 mM
MB310	20%	46 °C	48 °C	225 mM
MG1200	30%	46 °C	48 °C	112 mM
MS1414	35%	46 °C	48 °C	84 mM
MS821	20%	46 °C	48 °C	225 mM
MX825	20%	46 °C	48 °C	225 mM

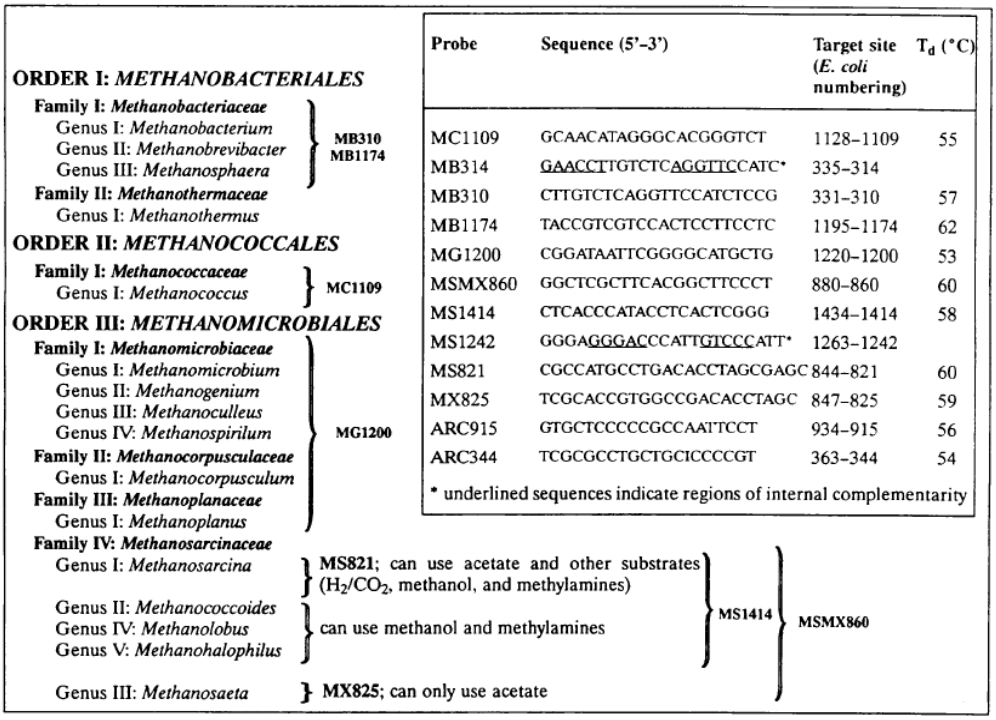


Figure 4.5: Oligonucleotide probes designed for methanogens and all Archaea (Raskin et al., 1994a)

4.7.3 Washing

Following the hybridization, 2µl 4', 6-diamidino-2-phenylindole (DAPI) DNA stain (last concentration should be adjusted to 3.3 µg/ml) is added and incubated at room temperature for 10 minutes. After incubation, 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 NaCl at the adjusted concentration according to the formula of Lathe (1985) at the optimal washing temperature (Table 4.6) before a final wash in MilliQ water (Manz et al., 1992). The cells were resuspended in 200µl of MilliQ water, and a 10-30 µl aliquot was placed on a gelatin-coated slide and air dried (Amann et al., 1990b). One drop of Citifluor antifadent (Citifluor Ltd., United Kingdom) was added to the sample, and a coverslip was applied to the preparation and sealed with nail polish before epifluorescence microscopy.

4.7.4 Observation

Observation was done under Olympus BX 50 Epifluorescence microscope equipped with a 100 W high-pressure mercury lamp and charged coupled device (CCD) camera. Images were processed and analyzed using Image-Pro Plus version 5 image analysis software (Media Cybernetics, USA).

CHAPTER 5 RESULTS

5.1 Performance of the Anaerobic Contact Reactor

The anaerobic contact reactor was operated with hydraulic retention time (HRT) of 4 days, sludge retention time (SRT) of 24 days and F/M (food/biomass) ratios of between 0.15-0.17 g COD/g VSS.day.

The applied temperature (34-37^{0}C) and pH (6.4-7.5) in the anaerobic contact reactor were within desired values (Appendix A and B).

The sludge volume index (SVI) of the anaerobic contact reactor varied between 39-67 ml/g (Appendix A and B).

MLSS, MLVSS and MLVSS/MLSS ratios of the anaerobic contact reactor varied between 16582-18063 mg/l, 10076-11534 mg/l and 0.59-0.65 respectively (Appendix A and B).

Influent BOD_5, effluent BOD_5 and BOD_5 removal efficiency varied between 1675-2435 mg/l, 551-661 mg/l and 62-76 % respectively (Appendix A and B).

Influent COD, Discharge COD and COD removal efficiency of wastewater treatment plant varied between 8294-8846 mg/l, 674-882 mg/l and 90-92%. Also discharge BOD_5 of wastewater treatment plant was varied between 255-365 mg/l respectively (Appendix A and B).

Changes in COD removal efficiency and organic loading rate (OLR) of the anaerobic contact reactor for the monitoring period for 5 months are given in Figure 5.1. During the same period, changes in methane yield of the anaerobic contact reactor are given in Figure 5.2.

Performance of the anaerobic contact reactor in terms of COD removal efficiency varied between 47% and 55% at OLRs in a range of 1.6 to 1.8 kg COD m^{-3} day^{-1} as seen in Figure 5.1.

The methane yield of the anaerobic contact reactor was varied between 0.18-0.20 m^3CH_4/kg $COD_{removed}$ as seen in Figure 5.2.

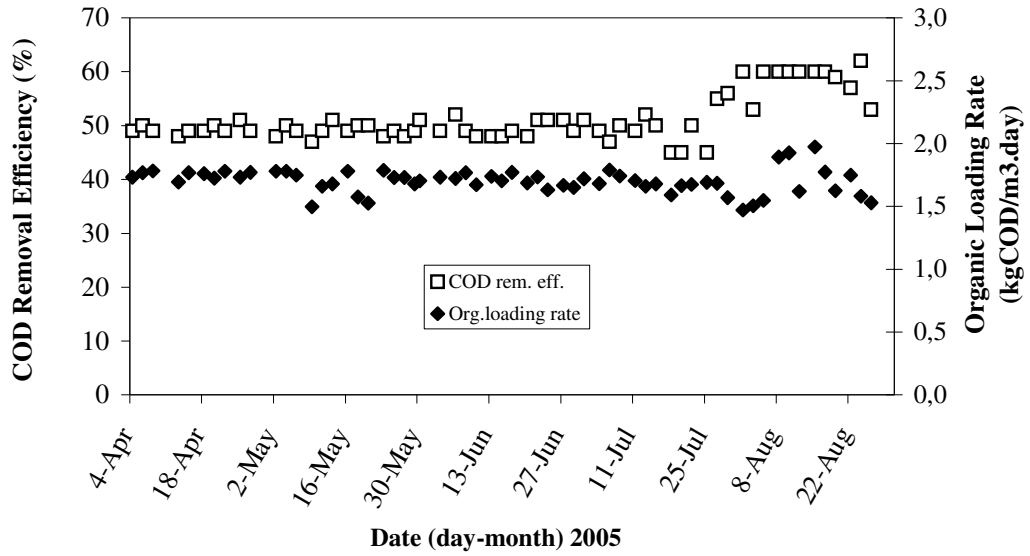


Figure 5.1: Changes in COD removal efficiency and organic loading rate (OLR) of the anaerobic contact reactor

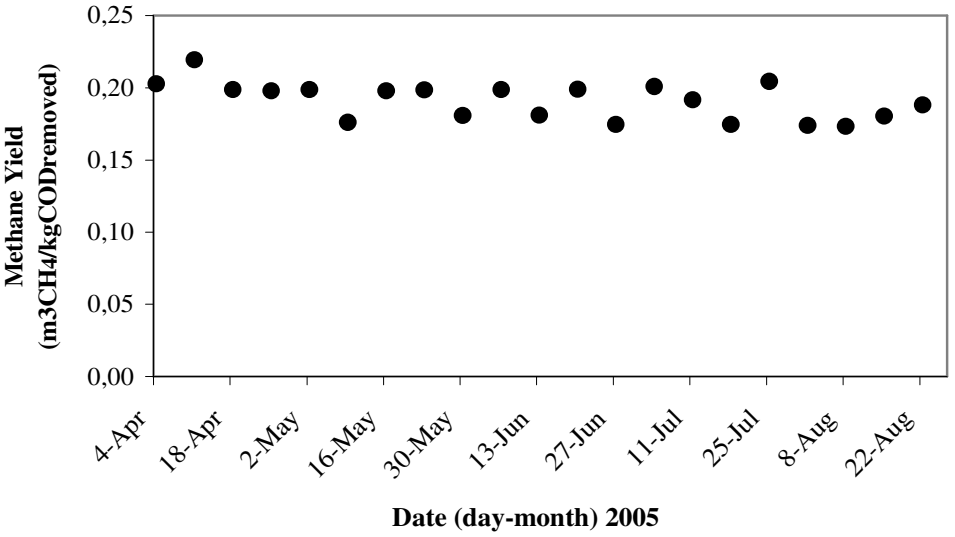


Figure 5.2: Methane yield of the anaerobic contact reactor

5.2 SMA test results

SMA tests had been carried out to determine the potential loading capacity and optimum operating conditions of the anaerobic contact reactor. The height of the anaerobic contact reactor is 16 m (active height is 15.5m). Samples were collected from 4, 8 and 12 meters up from the ground of the anaerobic contact reactor on July and August 2005. Acetate concentrations of 2000, 3000 and 4000 mg/l were used to determine the maximum potential methane production rate of the anaerobic sludge. Among those 3000 mg/l acetate concentration was found to be optimum.

PMP rates, AMP rates and AMP/PMP ratios are given in Table 5.1 and SMA test results are given in Figure 5.3.

Table 5.1: PMP rates, AMP rates and AMP/PMP ratios of the anaerobic contact reactor sludge

Date (2005)	Height	*PMP rate	Average *PMP rate	*AMP rate	AMP/PMP ratio
July	4m	350	283±76	58±3	0.17
	8m	300			0.20
	12m	200			0.29
August	4m	134	159±26	70±9	0.52
	8m	186			0.37
	12m	156			0.44

*PMP rate, AMP rate (ml CH_4 $gVSS^{-1}day^{-1}$)

According to the SMA test results, the potential methane production (PMP) rates of the sludge taken from 4, 8 and 12 meters height of the anaerobic contact reactor on July 2005 were 350 ml CH_4 $gVSS^{-1}day^{-1}$, 300 ml CH_4 $gVSS^{-1}day^{-1}$ and 200 ml CH_4 $gVSS^{-1}day^{-1}$ respectively (Table 5.1 and Figure 5.3). The highest activity was observed at 4m and the lowest activity was observed at 12m on July 2005. The average PMP rate was 283±76 ml CH_4 $gVSS^{-1}day^{-1}$ and the actual methane production (AMP) rate of the anaerobic contact reactor was 58±3 ml CH_4 $gVSS^{-1}day^{-1}$ on July 2005. The average PMP rate (283 ml CH_4 gVSS day^{-1}) of the sample was closer to PMP rate (300 ml CH_4 $gVSS^{-1}$ day^{-1}) of the anaerobic sludge with high activity reported in literature.

When the PMP rates were compared with AMP rates, the AMP/PMP ratios were evaluated to be 0.17, 0.20 and 0.29 on July 2005 respectively showing that anaerobic

sludge had been using only 20% of its potential acetoclastic methanogenic capacity on July 2005. When anaerobic reactors were run at an AMP/PMP ratio of 0.6-0.7, high operating stability and excellent COD removal could be achieved (Ince et al., 1995(a); Monteggia, 1991). The AMP/PMP ratio (0.2-0.3) on July 2005 showed that the anaerobic contact reactor was under loaded compared to its maximum loading capacity.

According to the SMA test results, the potential methane production (PMP) rates of the sludge taken from 4, 8 and 12 meters height of the anaerobic contact reactor on August 2005 were 134 ml CH_4 $gVSS^{-1}day^{-1}$, 186 ml CH_4 $gVSS^{-1}day^{-1}$ and 156 ml CH_4 $gVSS^{-1}day^{-1}$ respectively (Table 5.1 and Figure 5.4). The highest activity was observed at 8m and the lowest activity was observed at 4m on August 2005. The average PMP rate was 159 ml CH_4 $gVSS^{-1}day^{-1}$ and actual methane production (AMP) rate of the anaerobic contact reactor was 70±9 ml CH_4 $gVSS^{-1}day^{-1}$ in August 2005. The average PMP rate (159±26 ml CH_4 gVSS day^{-1}) of the anaerobic sludge was far from the PMP rate (300 ml CH_4 $gVSS^{-1}$ day^{-1}) of the sample with high activity reported in literature.

When the PMP rate was compared with AMP rate, the AMP/PMP ratio was evaluated to be 0.52, 0.37 and 0.44 on August 2005 respectively showing that anaerobic sludge had been using only 44% of its potential acetoclastic methanogenic capacity on August 2005. The AMP/PMP ratio (0.44<0.6-0.7) on August 2005 showed that the anaerobic contact reactor was under loaded compared to its maximum loading capacity.

Comparing SMA test results of July 2005 and August 2005, there were 62%, 38% and 22% losses in PMP rates of the sludge taken from 4, 8 and 12 meters height of the anaerobic contact reactor.

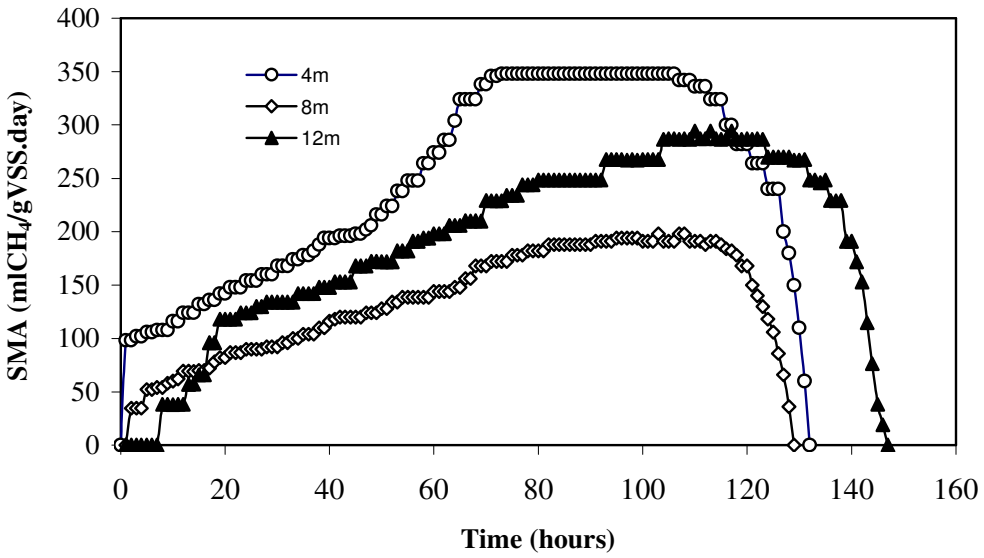


Figure 5.3: SMA test results of the sample taken on July 2005

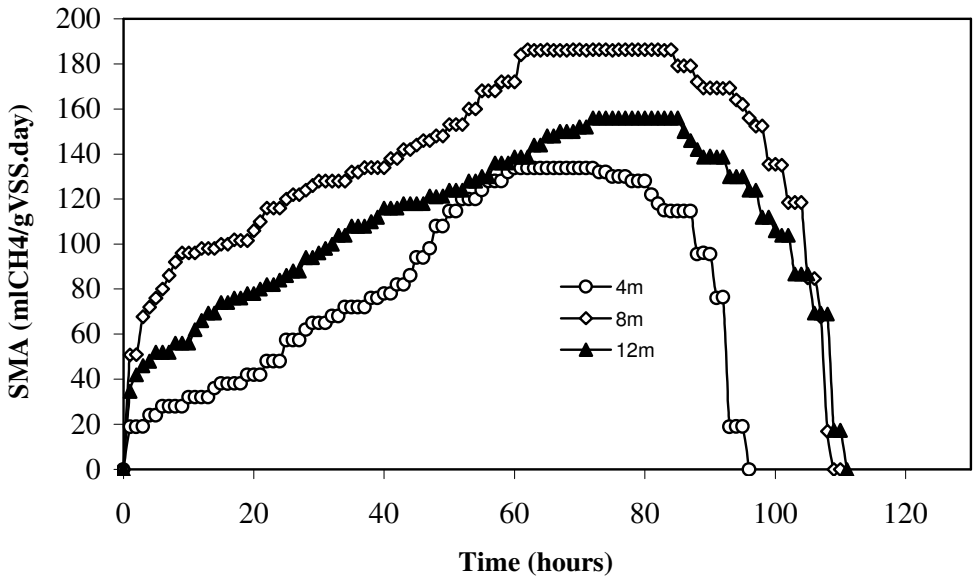


Figure 5.4: SMA test results of the sample taken on August 2005

5.3 Fluorescence *in situ* Hybridization (FISH) Results

The microbial community structure of anaerobic contact reactor sample was characterized using fluorescent rRNA targeted oligonucleotide probes specific for phylogenetically defined groups of methanogens. Whole microbial community in the anaerobic contact reactor samples was also stained using DAPI (DNA stain) to visualize intact cells in the samples. As seen in Table 5.2, when classifying and evaluating microorganisms according to utilized substrates, FISH results seem to be compatible with SMA test results. Image examples of *Methanococcales* in the anaerobic contact reactor sludge are given in Figure 5.5. Image examples of important microbial groups in the anaerobic contact reactor sludge are given in Appendix D. Figure 5.6 and 5.7 illustrates the results obtained with fluorescent rRNA targeted oligonucleotide probes in the analysis of the changes occurring in the microbial composition in the anaerobic contact reactor.

The changes of relative amount of *Methanobacteriales* spp. determined using MB310 probe in the whole microbial community according to time and reactor height is given in Figure 5.6. When FISH results of July 2005 were compared with FISH results of August 2005, *Methanobacteriales* spp. exceeded in numbers on July 2005. The relative amount of *Methanobacteriales* spp. in the whole microbial community on July 2005 was 6.8 % ± 0.4 % (mean ± standart deviation) at 4m, 8.8 % ± 0.2 % at 8m and 4.1 % ± 0.2 % at 12m. The relative amount of *Methanobacteriales* spp. in the whole microbial community on August 2005 was 1 % ± 0.2 % at 4m, 4 % ± 0.1 % at 8m and 1.9 % ± 0.1 % at 12m.

The changes of relative amount of *Methanococcales* spp. determined using MC1109 probe in the whole microbial community according to time and reactor height is given in Figure 5.6. When FISH results of July 2005 was compared with FISH results of August 2005, on July 2005 *Methanococcales* spp. were observed in every height of the anaerobic contact reactor and on August 2005 *Methanococcales* spp. were observed only at 4m and 12 m. The relative amount of *Methanococcales* spp. in the whole microbial community on July 2005 was 16 % ± 0.8 % at 4m, %13 ± 0.7 % at 8m and 2.4 % ± 0.3 % at 12m. The relative amount of *Methanococcales* spp. in the whole microbial community on August 2005 was 2.5 % ± 0.4 % at 4m, 4.5 % ± 0.3 % at 12m.

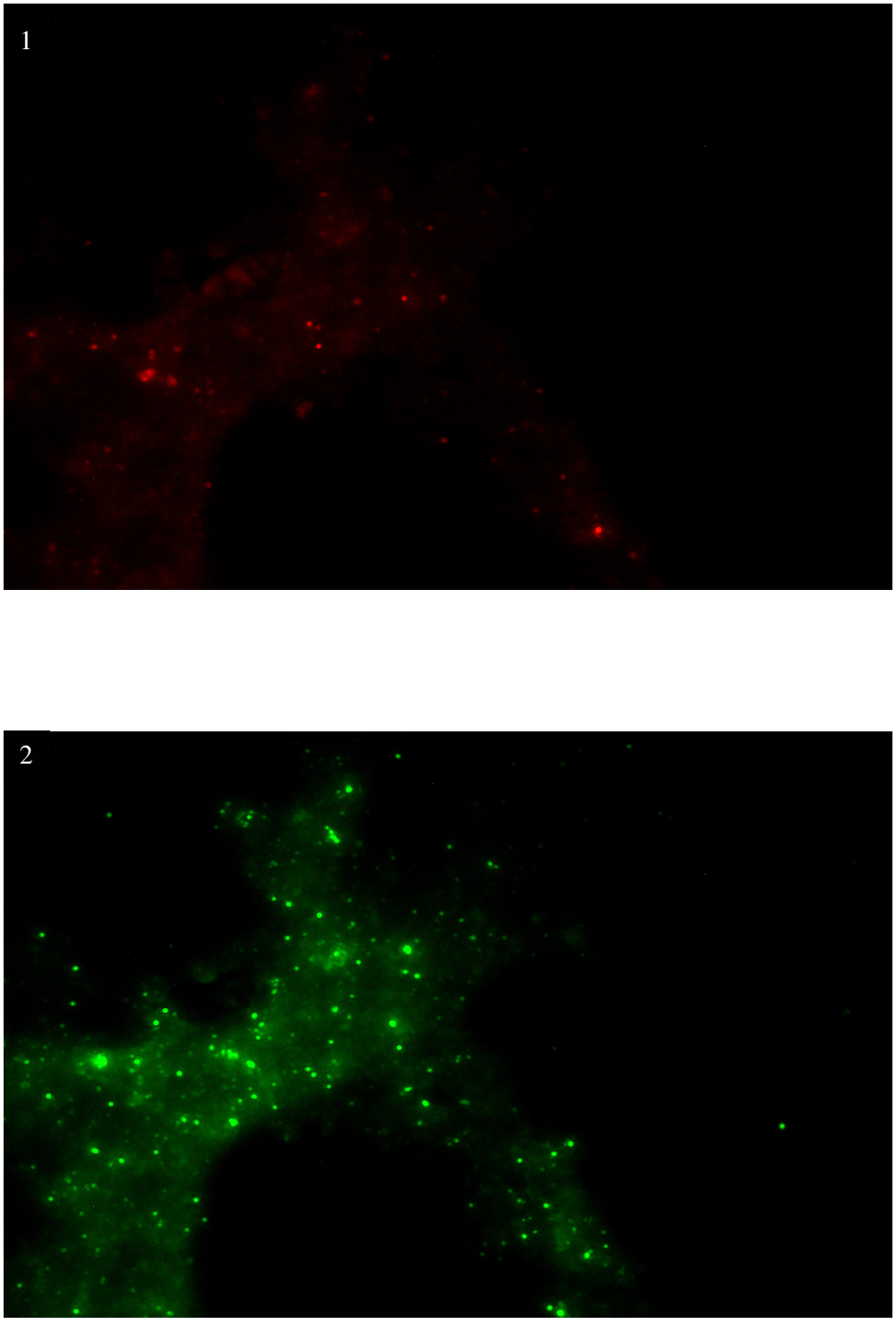


Figure 5.5: Image examples of *Methanococcales* in the anaerobic contact reactor sludge, 1: Determined cell with hybridization probe (MC1109-*Methanococcales*), 2: Determined cell with DAPI (DNA stain)

Table 5.2: FISH quantification results using Image Pro Plus 5 a) July 2005 b) August 2005

a)

Target Group	**Probe**	**The relative amount of methanogens in the whole microbial community**		
		4m	**8m**	**12m**
Methanobacteriales	MB310	6.8% ± 0.4%	8.8%±0.2%	4.1%±0.2%
Methanococcales	MC1109	16% ± 0.8%	13%±0.7%	2.4%±0.003
Methanogenium relatives	MG1200	6%±0.3%	-	-
Methanosarcina	MS821	14%±0.9%	9%±0.4%	3%±0.4%
Methanosarcina +relatives	MS1414	14%±0.5%	9%±0.1%	3%±0.9%
Methanosaeta	MX825	7%±0.2%	4.2%±0.2%	3%±0.2%

b)

Target Group	**Probe**	**The relative amount of methanogens in the whole microbial community**		
		4m	**8m**	**12m**
Methanobacteriales	MB310	1%±0.2%	4%±0.1%	1.9%±0.1%
Methanococcales	MC1109	2.5%±0.4%	-	4.5%±0.3%
Methanogenium relatives	MG1200	-	-	-
Methanosarcina	MS821	5%±0.7%	8.5%±0.6%	8%±0.4%
Methanosarcina +relatives	MS1414	5%±0.4%	8.5%±0.4%	8%±0.1%
Methanosaeta	MX825	-	-	-

The changes of relative amount of *Methanobactericeae* spp. determined using MG1200 probe in the whole microbial community according to time and reactor height is given in Figure 5.6. When FISH results of July 2005 was compared with FISH results of August 2005, on July 2005 *Methanobactericeae* spp. were observed only at 4m and on August *Methanobactericeae* spp. were not observed at any height. The relative amount of *Methanobactericeae* spp. in the whole microbial community on July 2005 was 6 % ± 0.3 % at 4m.

The changes of relative amount of *Methanomicrobiales* spp. determined using MS821 probe in the whole microbial community according to time and reactor height is given in Figure 5.6. When FISH results of July 2005 was compared with

FISH results of August 2005, while on July 2005 decreased the relative amount of *Methanomicrobiales* spp. along the anaerobic contact reactor was observed on August 2005 increased the relative concentration of *Methanomicrobiales* spp. along the anaerobic contact reactor was observed. The relative amount of *Methanomicrobiales* spp. in the whole microbial community in the same sample taken on July 2005 was 14 % ± 0.9 % at 4m, 9 % ± 0.4 % at 8m and 3 % ± 0.4 % at 12m. The relative amount of *Methanomicrobiales* spp. in the whole microbial community in the same sample taken on August 2005 was 5 % ± 0.7 % at 4m, 8.5 % ± 0.6 % at 8m and 8 % ± 0.4 % at 12m.

The changes of relative amount of *Methanosarcina* spp., *Methanococcoides* spp., *Methanolobus* spp. and *Methanolophilus* spp determined using MS1414 probe in the whole microbial community according to time and reactor height is given in Figure 5.6. When FISH results of July 2005 were compared with FISH results of August 2005, on July 2005 and on August 2005 *Methanosarcina* spp., *Methanococcoides* spp., *Methanolobus* spp. and *Methanophilus* spp. were observed in every height of the anaerobic contact reactor. The relative amount of *Methanosarcina* spp., *Methanococcoides* spp., *Methanolobus* spp. and *Methanophilus* spp. in the whole microbial community on July 2005 was 14 % ± 0.5 % at 4m, 9 % ± 0.1 % at 8m and 3% ± 0.9 % at 12m. The relative amount of *Methanosarcina* spp., *Methanococcoides* spp., *Methanolobus* spp. and *Methanophilus* spp. in the whole microbial community on August 2005 was 5 % ± 0.4 % at 4m, 8.5 % ± 0.4 % at 8m and 8 % ± 0.1 % at 12m.

The changes of relative amount of *Methanosaeta* spp. determined using MX825 probe in the whole microbial community according to time and reactor height is given in Figure 5.6. While *Methanosaeta* spp. was observed in the sample taken on July 2005, *Methanosaeta* spp. was not observed in the sample taken on August 2005. The relative amount of *Methanosaeta* spp. in the whole microbial community on July 2005 was 7 % ± 0.2 % at 4m, 4.2 % ± 0.2 % at 8m and 3 % ± 0.2 % at 12m.

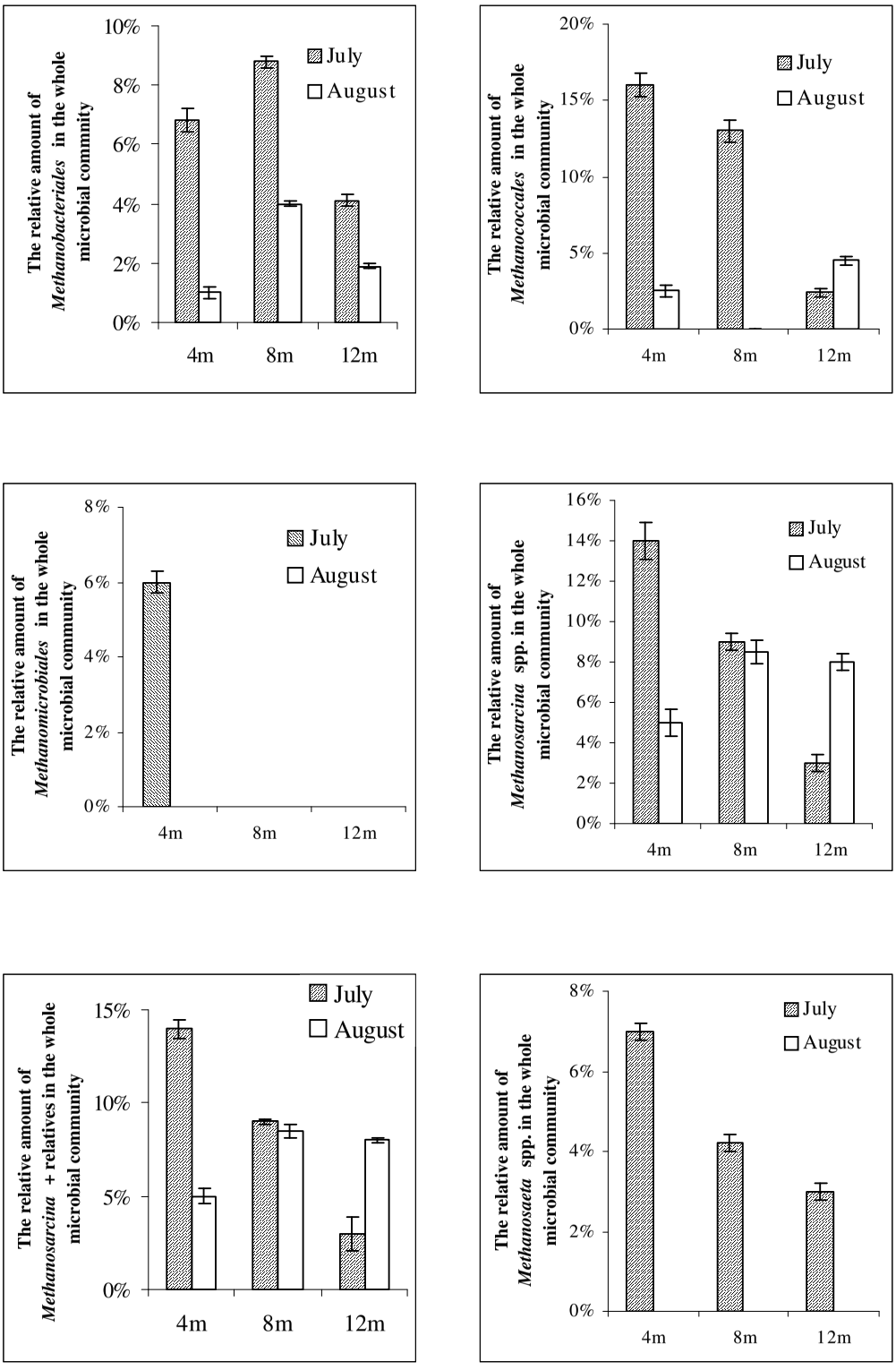
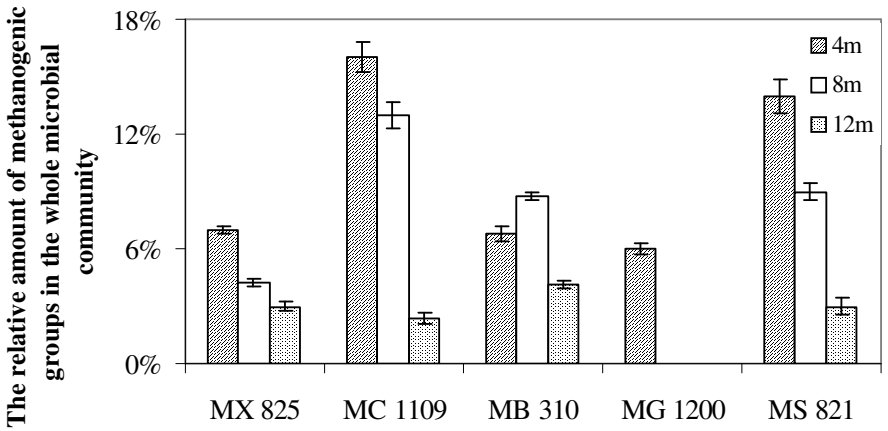
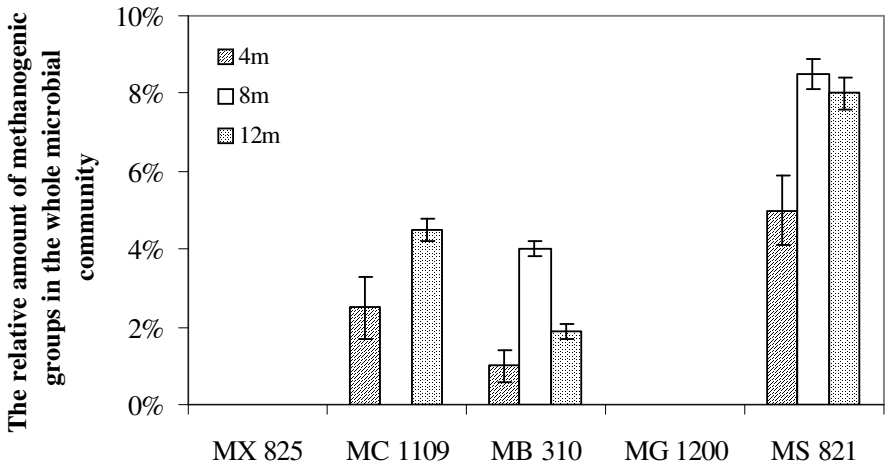


Figure 5.6: The changes in relative amount of methanogens in the whole microbial community depend on reactor height and time



(a)



(b)

Figure 5.7: The changes in relative amount of methanogens in the whole microbial community depend on reactor height and time a) July 2005 b) August 2005 (MX825-*Methanosaeta* spp., MC1109-*Methanococcales,* MB310- *Methanobacteriales,* MG1200-*Methanogenium* relatives, MS821-*Methanosarcina* spp.)

CHAPTER 6 DISCUSSION

In the pulp and paper industry, there are various points of wastewater generation. There are variations in the COD, inhibitors and the degradability upon the source of the wastewaters (Rajeshwari et al., 2000).

The applied HRT (4 days) was in a range of stated in literature for the successful applications treating pulp and paper mill effluents (0.5-5 days) (Savant et al., 2005; Rintala et al., 1999). The applied temperature (35-37 ^{0}C) and pH (6.5-7.5) in the anaerobic contact reactor were within desired values.

COD removal efficiency values of similar anaerobic reactors, treating pulp and paper mill effluents, are reported 40-80% in literature (Savant et al., 2005; Rintala et al., 1999). Observed COD removal efficiency (47-55%) values of anaerobic contact reactor stayed in the lower limit according to literature. But methane yield (0.18-0.20 m^3CH_4/kg $COD_{removed}$) values of the anaerobic contact reactor were higher than the methane yield (0.08-0.16 m^3CH_4/kg $COD_{removed}$) values of similar anaerobic reactors treating pulp and paper mill effluents in literature (Savant et al., 2005; Rintala et al., 1999). In such a case, it can be said that the performance of the anaerobic contact reactor has showed a good performance during the monitoring period of 5 months.

Nutrients are not added into the anaerobic contact reactor and COD/N/P ratio in the anaerobic contact reactor is not known. It is previously reported in literature that the anaerobic reactors treating pulp and paper mill effluents are operated successfully with 176:5:1 ratio (COD/N/P) and generally extra addition of nutrient isn't necessary for maintaining this ratio (Ammary et al., 2004).

Organic loading rate values of similar anaerobic reactors, treating pulp and paper mill effluents, are reported 0.5-5 kg COD m^{-3} day^{-1} in literature (Savant et al., 2005; Rintala et al., 1999). Observed OLRs (1.6-1.8 kg COD m^{-3} day^{-1}) of anaerobic contact reactor stayed in the lower limit. It is previously reported in literature that increasing OLRs (from 0.75 to 3 kg COD m^{-3} day^{-1}) of completely stirred anaerobic

reactors have useful effects on methane activity and system performance (Rincon et al., 2006).

Applied F/M ratio (0.15-0.17 g COD/g VSS.day) was quite lower than the typical F/M ratios of similar anaerobic reactors (0.5-1.0 g COD/g VSS.day) (Speece, 1996). It is previously reported in literature that increasing F/M ratios of operated anaerobic reactors with low F/M ratio have positive effects on methane activity and system performance (Baier and Delavy, 2005).

According to the SMA test results of the sludge taken from 4, 8 and 12 meters height of the anaerobic contact reactor on July 2005 and August 2005, AMP/PMP ratio was evaluated to be 0.20 and 0.40 respectively. These ratios showed that the anaerobic contact reactor was under loaded compared to its maximum loading capacity. When anaerobic reactors were run at an AMP/PMP ratio of 0.6-0.7, high operating stability and excellent COD removal could be achieved (Ince et al., 1995(a); Monteggia, 1991).

Comparing SMA test results of July 2005 and August 2005, there were 62%, 38% and 22% losses in PMP rates of the sludge taken from 4, 8 and 12 meters height of the anaerobic contact reactor. The average PMP rate was 283±76 ml CH_4 $gVSS^{-1}$ day^{-1} and the actual methane production (AMP) rate of the anaerobic contact reactor was 58±3 ml CH_4 $gVSS^{-1}day^{-1}$ on July 2005. The average PMP rate (283 ml CH_4 gVSS day^{-1}) of the sample was closer to PMP rate (300 ml CH_4 $gVSS^{-1}$ day^{-1}) of the anaerobic sludge with high activity reported in literature. The average PMP rate was 159 ml CH_4 $gVSS^{-1}day^{-1}$ and actual methane production (AMP) rate of the anaerobic contact reactor was 70±9 ml CH_4 $gVSS^{-1}day^{-1}$ on August 2005. It can be interpreted that the anaerobic contact reactor sludge has lost its good quality from steady-state conditions on July 2005 to start-up and operation conditions after 15 days maintenance. It can be seen that AMP rates from July to August didn't change very much. Because of the system were run at low loading rates, the decreases in PMP rates didn't affect the discharge quality of wastewater. Also during the maintenance of the system for short times the discharge quality of the wastewater didn't change because of running the system at low loading rates.

The all-operational parameters (such as pH, temperature, organic loading, hydraulic retention time etc.) except F/M ratio were maintained within desired ranges. The

reason behind of this could be due to retaining high amount of sludge within anaerobic contact reactor resulting in an F/M ratio, which is relatively much lower than the typical values reported for similar reactors. Pulling out more sludge from anaerobic contact reactor or increasing applied OLRs can increase applied F/M ratio. But when applying these suggestions, the system potential should be taken into consideration and OLRs should be increased while AMP/PMP ratio is remained between 0.6-0.7.

FISH results seem to be compatible with SMA test results. Acetoclastic methanogenic activity and relative abundance of acetoclastic methanogens and hydrogenetrophic methanogens were decreased as the reactor height increased. The relative amount of acetoclastic methanogens (*Methanosaeta* spp., and *Methanosarcina* spp.) has decreased in the ratio of 47 % from July 2005 to August 2005. At the same time acetoclastic methanogenic activity has decreased in the ratio of 44 %.

In parallel with acetoclastic methanogenic activity loss, the relative concentration of hydrogenotrophic methanogens has decreased in the ratio of 67 %. It is generally known that sulfate reducing bacteria (SRB) converts acetate to H_2/CO_2 (Delbe's et al., 2001). Consequently, it can be said that during the production of methane from acetate in the anaerobic contact reactor, in addition to acetoclastic methane production, converting acetate to H_2/CO_2 by sulfate reducing bacteria and after that converting this generated H_2/CO_2 to CH_4 by hydrogenetrophic methanogens could occur during this monitoring period. This methane production type can be seen just only in the anaerobic systems, which are applied in the stress (Schnurer et al., 1994, Peterson and Ahring, 1992 and 1999). For maintaining all the operational parameters except F/M ratio within desired ranges, the cause of the stress in the system could be operating the anaerobic contact reactor in the low F/M ratios. It can be considered that the methanogens and sulfate reducing bacteria (SRB) syntrophically interacting with each other instead of competing for electron and carbon source within the reactor.

Even though the reactor is completely stirred tank, SMA and FISH results, the concentration of methanogenic community at 4, 8 and 12 meters height of the anaerobic contact reactor, the relative quantities of specific species and acetoclastic methane activities have been showed important differences. Because of that there

was not a homogenous dispersion in the anaerobic contact reactor. The cause of this problem could be the completely stirring problem of anaerobic contact reactor. For testing this problem, a trace matter can be poured into the anaerobic contact reactor and the changes of the concentration of a trace matter can be observed at the reactor effluent.

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APPENDIX A: Operational Data

Table A.1: Operational data of anaerobic reactor in months between April and August 2005

Date	Influent COD (mg/l)	Effluent COD (mg/l)	COD Rem. Eff. (%)	OLR ($kgCOD/m^3.day$)	Methane Yield ($m^3CH_4/kgCOD_{removed}$)
4-Apr-05	6929	3534	49	1,732	0,20
6-Apr-05	7070	3535	50	1,768	
8-Apr-05	7133	3638	49	1,783	
11-Apr-05	6780	3458	49	1,695	0,22
13-Apr-05	6775	3523	48	1,694	
15-Apr-05	7075	3608	49	1,769	
18-Apr-05	7046	3593	49	1,762	0,20
20-Apr-05	6902	3451	50	1,726	
22-Apr-05	7116	3629	49	1,779	
25-Apr-05	6933	3397	51	1,733	0,20
27-Apr-05	7083	3612	49	1,771	
29-Apr-05	6714	3424	49	1,679	
2-May-05	7118	3701	48	1,780	0,20
4-May-05	7121	3561	50	1,780	
6-May-05	6996	3568	49	1,749	
9-May-05	5988	3174	47	1,497	0,18
11-May-05	6641	3387	49	1,660	
13-May-05	6714	3290	51	1,679	
16-May-05	7124	3633	49	1,781	0,20
18-May-05	6295	3148	50	1,574	
20-May-05	6106	3053	50	1,527	
23-May-05	7151	3719	48	1,788	0,20
25-May-05	6918	3528	49	1,730	
27-May-05	6920	3598	48	1,730	
30-May-05	6818	3341	51	1,705	0,18
1-Jun-05	6831	3484	49	1,708	
3-Jun-05	6927	3533	49	1,732	
6-Jun-05	6886	3305	52	1,722	0,20
8-Jun-05	7075	3608	49	1,769	
10-Jun-05	6690	3479	48	1,673	
13-Jun-05	6956	3617	48	1,739	0,18
15-Jun-05	6813	3543	48	1,703	
17-Jun-05	7083	3612	49	1,771	
20-Jun-05	6747	3508	48	1,687	0,20
22-Jun-05	6938	3400	51	1,735	
24-Jun-05	6531	3200	51	1,633	
27-Jun-05	6667	3267	51	1,667	0,17
29-Jun-05	6599	3365	49	1,650	

Date	Influent COD (mg/l)	Effluent COD (mg/l)	COD Rem. Eff. (%)	OLR ($kgCOD/m^3.day$)	Methane Yield ($m^3CH_4/kgCOD_{removed}$)
1-Jul-05	6878	3370	51	1,720	
4-Jul-05	6726	3430	49	1,682	0,20
6-Jul-05	7159	3794	47	1,790	
8-Jul-05	6969	3485	50	1,742	
11-Jul-05	6826	3481	49	1,707	0,19
13-Jul-05	6641	3188	52	1,660	
15-Jul-05	6722	3361	50	1,681	
18-Jul-05	6361	3499	45	1,590	0,17
20-Jul-05	6660	3663	45	1,665	
22-Jul-05	6695	3348	50	1,674	
25-Jul-05	6762	3719	45	1,691	0,20
27-Jul-05	6740	3033	55	1,685	
29-Jul-05	6280	2763	56	1,570	
1-Aug-05	5886	2354	60	1,471	0,17
3-Aug-05	6016	2828	53	1,504	
5-Aug-05	6196	2478	60	1,549	
8-Aug-05	7565	3026	60	1,891	0,17
10-Aug-05	7702	3081	60	1,925	
12-Aug-05	6478	2591	60	1,620	
15-Aug-05	7896	3158	60	1,974	0,18
17-Aug-05	7095	2838	60	1,774	
19-Aug-05	6496	2663	59	1,624	
22-Aug-05	7000	3010	57	1,750	0,19
24-Aug-05	6326	2404	62	1,582	
26-Aug-05	6113	2873	53	1,528	

Table A.2: pH and temperature of the anaerobic contact reactor in months between April and August 2005

Date	pH	Temperature (^{0}C)	Date	pH	Temperature (^{0}C)
4-Apr-05	7,5	37,1	17-Jun-05	7,5	36,9
6-Apr-05	6,8	36,9	20-Jun-05	7,1	37,0
8-Apr-05	7,1	37,0	22-Jun-05	6,5	36,6
11-Apr-05	7,2	36,9	24-Jun-05	6,9	37,0
13-Apr-05	7,2	37,4	27-Jun-05	7,0	36,6
15-Apr-05	7,3	36,6	29-Jun-05	7,2	37,1
18-Apr-05	6,6	37,0	1-Jul-05	6,6	36,9
20-Apr-05	7,3	36,6	4-Jul-05	7,3	37,1
22-Apr-05	6,6	37,0	6-Jul-05	6,9	37,1
25-Apr-05	6,9	37,2	8-Jul-05	7,1	37,5
27-Apr-05	6,7	36,9	11-Jul-05	7,1	36,9
29-Apr-05	7,2	36,7	13-Jul-05	7,0	37,1
2-May-05	7,0	37,0	15-Jul-05	6,8	37,5
4-May-05	6,7	36,8	18-Jul-05	6,9	36,6
6-May-05	7,1	36,9	20-Jul-05	7,3	36,7
9-May-05	6,8	36,7	22-Jul-05	6,5	36,8
11-May-05	7,2	37,0	25-Jul-05	7,1	37,0
13-May-05	6,6	37,1	27-Jul-05	7,2	36,6
16-May-05	7,3	37,3	29-Jul-05	6,6	36,6
18-May-05	7,4	37,4	1-Aug-05	6,9	37,4
20-May-05	6,8	36,7	3-Aug-05	7,3	37,2
23-May-05	6,5	37,3	5-Aug-05	6,8	37,0
25-May-05	6,9	36,9	8-Aug-05	6,6	37,2
27-May-05	7,2	37,0	10-Aug-05	7,3	37,0
30-May-05	6,9	36,8	12-Aug-05	6,7	36,6
1-Jun-05	7,1	36,9	15-Aug-05	6,7	36,8
3-Jun-05	6,7	37,4	17-Aug-05	6,9	37,1
6-Jun-05	6,8	37,5	19-Aug-05	7,4	37,0
8-Jun-05	7,1	36,6	22-Aug-05	6,8	37,4
10-Jun-05	7,4	36,7	24-Aug-05	6,6	36,6
13-Jun-05	6,7	36,8	26-Aug-05	6,8	37,0
15-Jun-05	7,1	37,0			

Table A.3: MLSS, MLVSS and sludge volume index (SVI) values of the anaerobic contact reactor in months between April and August 2005

Date	MLSS (mg/l)	MLVSS (mg/l)	MLVSS/MLSS	SVI (ml/g)
4-Apr-05	18230	11470	0,63	46
11-Apr-05	18520	12410	0,67	55
18-Apr-05	16850	11240	0,67	36
25-Apr-05	17250	11200	0,65	52
2-May-05	16850	11240	0,67	57
9-May-05	16850	9960	0,59	43
16-May-05	17580	11200	0,64	68
23-May-05	17420	11230	0,64	41
30-May-05	17460	10230	0,59	78
6-Jun-05	17980	11240	0,63	41
13-Jun-05	18260	10240	0,56	62
20-Jun-05	16540	11260	0,68	63
27-Jun-05	16540	9870	0,60	54
4-Jul-05	17580	11365	0,65	36
11-Jul-05	17460	10840	0,62	70
18-Jul-05	16840	9870	0,59	54
25-Jul-05	18452	11560	0,63	36
1-Aug-05	15460	9840	0,64	85
8-Aug-05	16800	9800	0,58	41
15-Aug-05	17600	10200	0,58	43
22-Aug-05	17250	10640	0,62	49

Table A.4: Influent BOD_5, effluent BOD_5 and BOD_5 removal efficiency values of the anaerobic contact reactor in months between April and August 2005

Date	Influent BOD_5 (mg/l)	Effluent BOD_5 (mg/l)	BOD_5 Rem.Eff. %
4-Apr-05	2370	580	76
11-Apr-05	2660	620	77
18-Apr-05	2150	550	74
25-Apr-05	1720	520	70
2-May-05	2430	620	74
9-May-05	2370	520	78
16-May-05	2150	530	75
23-May-05	2460	580	76
30-May-05	2410	650	73
6-Jun-05	1760	560	68
13-Jun-05	2410	620	74
20-Jun-05	1740	560	68
27-Jun-05	1620	660	59
4-Jul-05	1670	657	61
11-Jul-05	1450	680	53
18-Jul-05	1560	654	58
25-Jul-05	2150	720	67
1-Aug-05	1860	620	67
8-Aug-05	2560	650	75
15-Aug-05	2050	580	72
22-Aug-05	1600	600	63

Table A.5: Influent COD, discharge COD, COD removal efficiency and effluent BOD_5 values of the wastewater treatment plant in months between April and August 2005

Date	Influent COD (mg/l)	Effluent COD (mg/l)	COD Rem. Eff. (%)	Effluent BOD_5 (mg/l)
4-Apr-05	8528	841	90	313
6-Apr-05	8750	757	91	
8-Apr-05	8884	764	91	
11-Apr-05	8352	726	91	335
13-Apr-05	8448	726	91	
15-Apr-05	8768	821	91	
18-Apr-05	8728	755	91	297
20-Apr-05	8456	739	91	
22-Apr-05	8904	762	91	
25-Apr-05	8616	743	91	281
27-Apr-05	8794	885	90	
29-Apr-05	8188	719	91	
2-May-05	8852	762	91	335
4-May-05	8732	801	91	
6-May-05	8616	849	90	
9-May-05	7570	802	89	281
11-May-05	8380	842	90	
13-May-05	8398	899	89	
16-May-05	8816	957	89	286
18-May-05	8584	809	91	
20-May-05	8410	654	92	
23-May-05	8832	766	91	313
25-May-05	8622	741	91	
27-May-05	8516	865	90	
30-May-05	8498	730	91	351
1-Jun-05	8502	732	91	
3-Jun-05	8540	742	91	
6-Jun-05	8458	737	91	302
8-Jun-05	8768	758	91	
10-Jun-05	8374	716	91	
13-Jun-05	8634	745	91	335
15-Jun-05	8576	730	91	
17-Jun-05	8814	759	91	
20-Jun-05	8432	867	90	302
22-Jun-05	8650	780	91	
24-Jun-05	8148	839	90	
27-Jun-05	8354	857	90	356
29-Jun-05	8260	848	90	
1-Jul-05	8604	884	90	
4-Jul-05	8286	864	90	355
6-Jul-05	8838	920	90	
8-Jul-05	8680	896	90	
11-Jul-05	8506	877	90	367
13-Jul-05	8342	830	90	
15-Jul-05	8546	852	90	
18-Jul-05	8204	891	89	392

Date	Influent COD (mg/l)	Effluent COD (mg/l)	COD Rem. Eff. (%)	Effluent BOD_5 (mg/l)
20-Jul-05	8388	769	91	
22-Jul-05	8411	838	90	
25-Jul-05	9352	947	90	288
27-Jul-05	8914	741	92	
29-Jul-05	8862	678	92	
1-Aug-05	8278	740	91	240
3-Aug-05	8020	810	90	
5-Aug-05	8544	920	89	
8-Aug-05	8900	900	90	190
10-Aug-05	8920	650	93	
12-Aug-05	9040	790	91	
15-Aug-05	8910	800	91	400
17-Aug-05	8292	450	95	
19-Aug-05	8580	560	93	
22-Aug-05	8640	600	93	200
24-Aug-05	8600	450	95	
26-Aug-05	8472	560	93	

APPENDIX B: Operational Data of Anaerobic Contact Reactor and Wastewater Treatment Plant

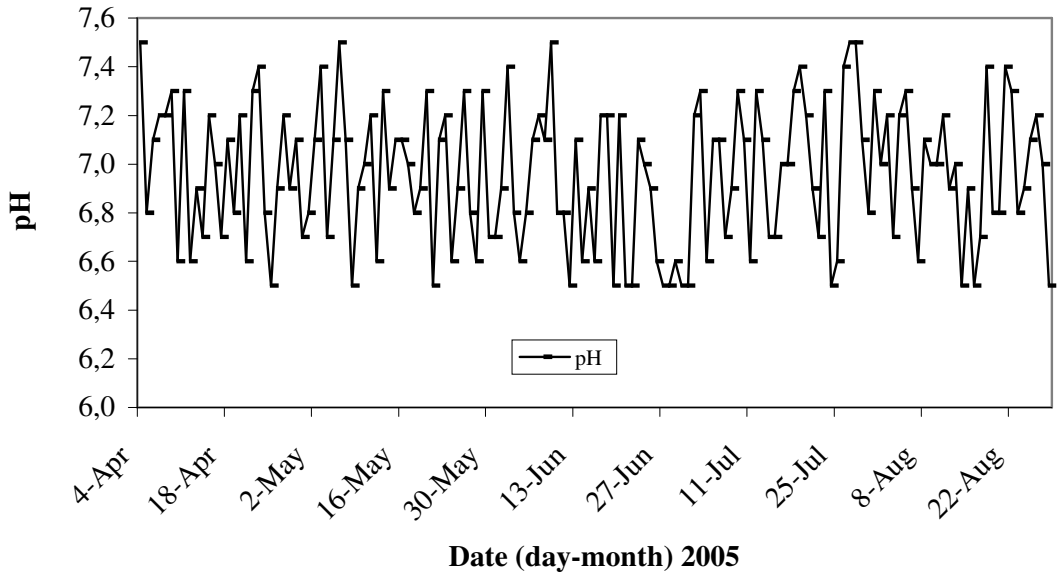

Figure B.1: Changes in pH of the anaerobic contact reactor

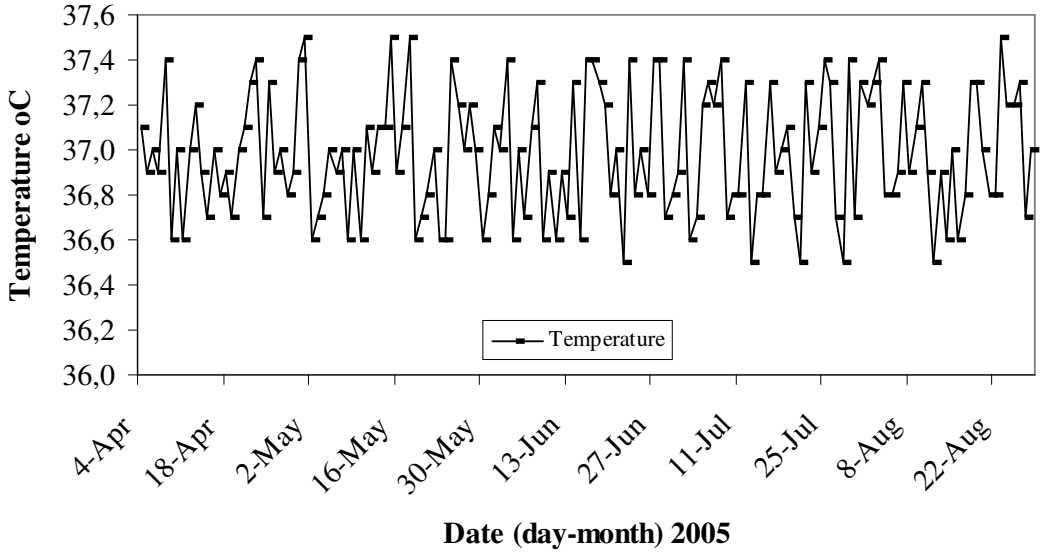

Figure B.2: Changes in temperature of the anaerobic contact reactor

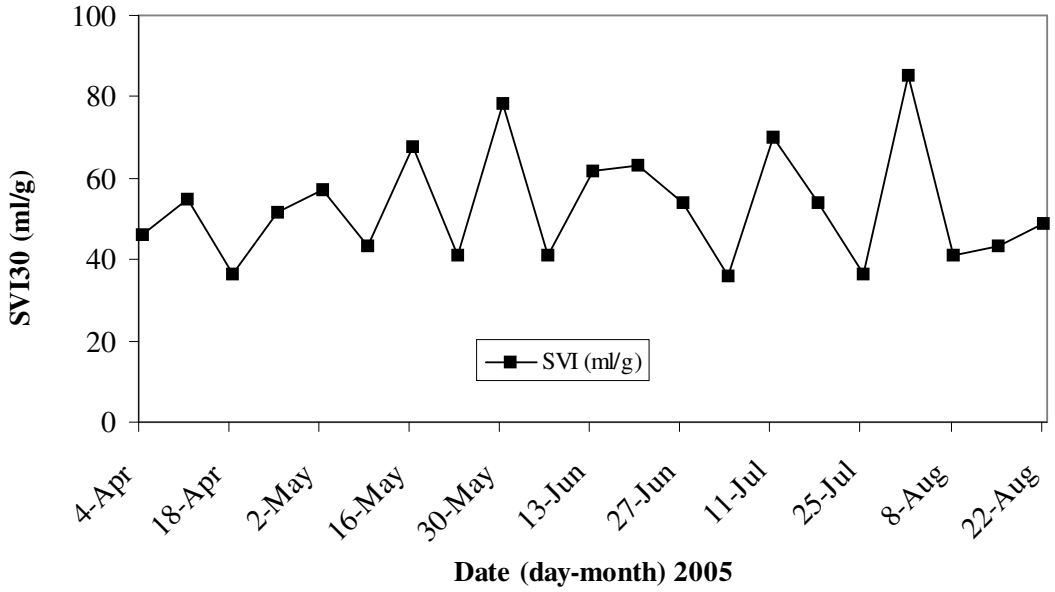

Figure B.3: Changes in sludge volume index (SVI) of the anaerobic contact reactor

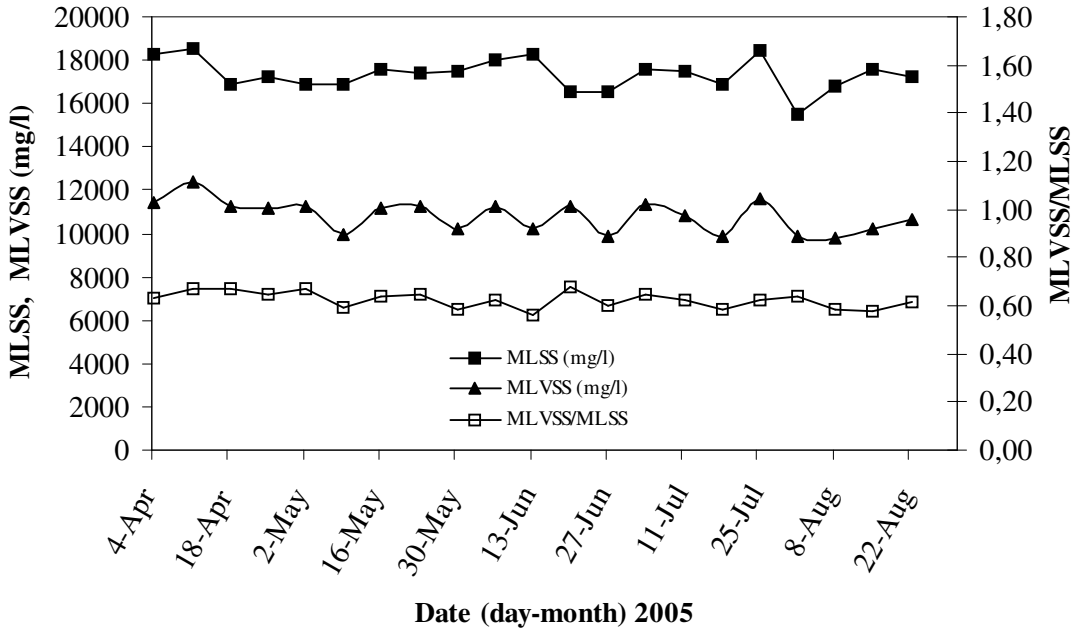

Figure B.4: Changes in suspended solids (MLSS) and volatile suspended solids (MLVSS) of the anaerobic contact reactor

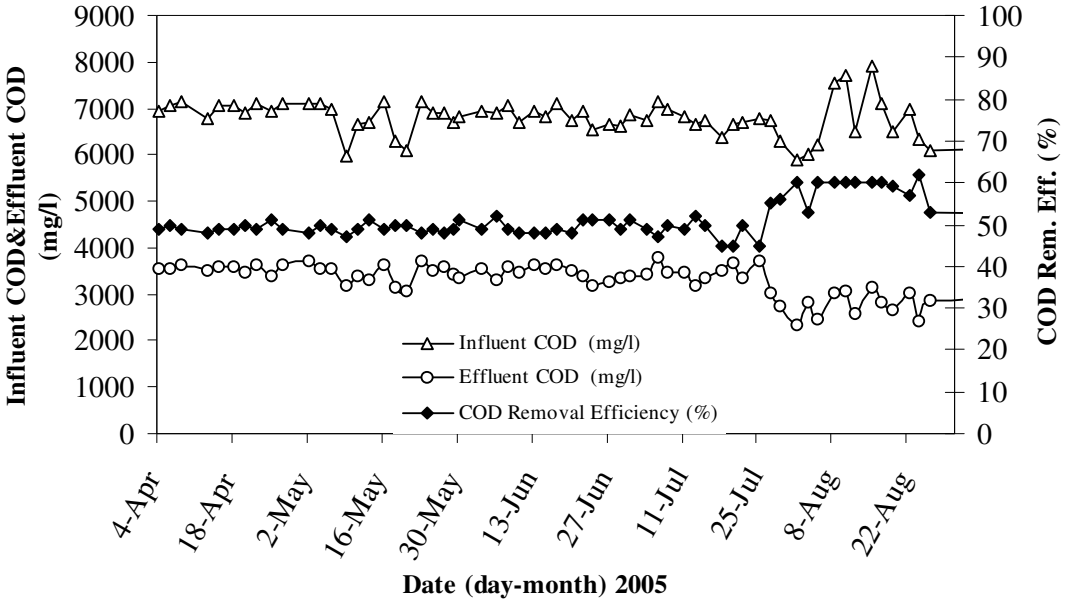

Figure B.5: Changes in influent COD, effluent COD and COD removal efficiency of the anaerobic contact reactor

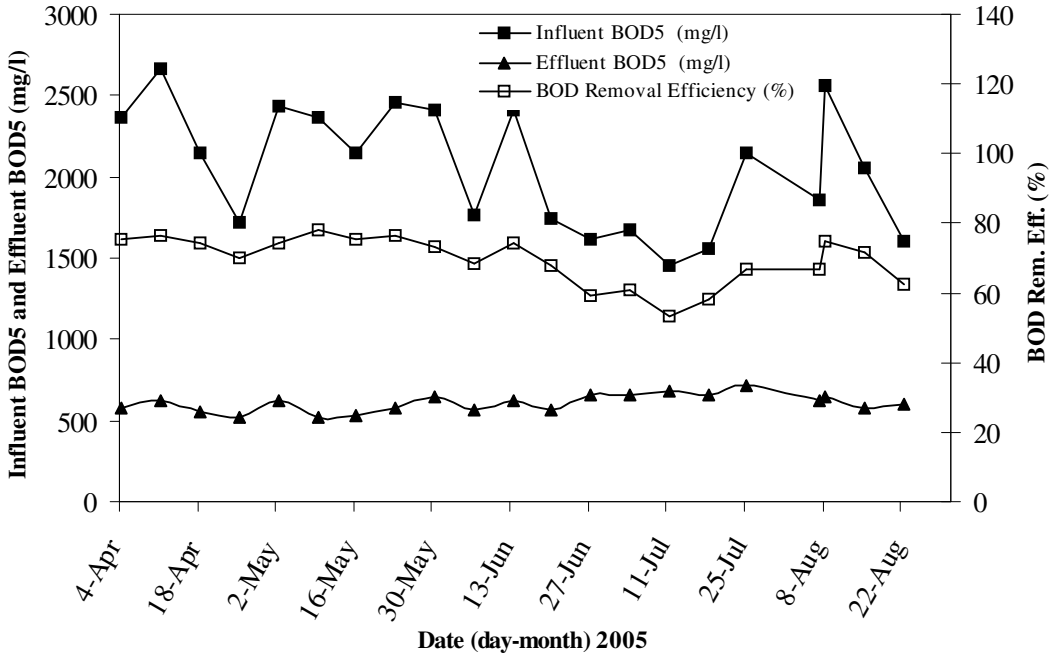

Figure B.6: Changes in influent BOD_5, effluent BOD_5 and BOD_5 removal efficiency of the anaerobic contact reactor

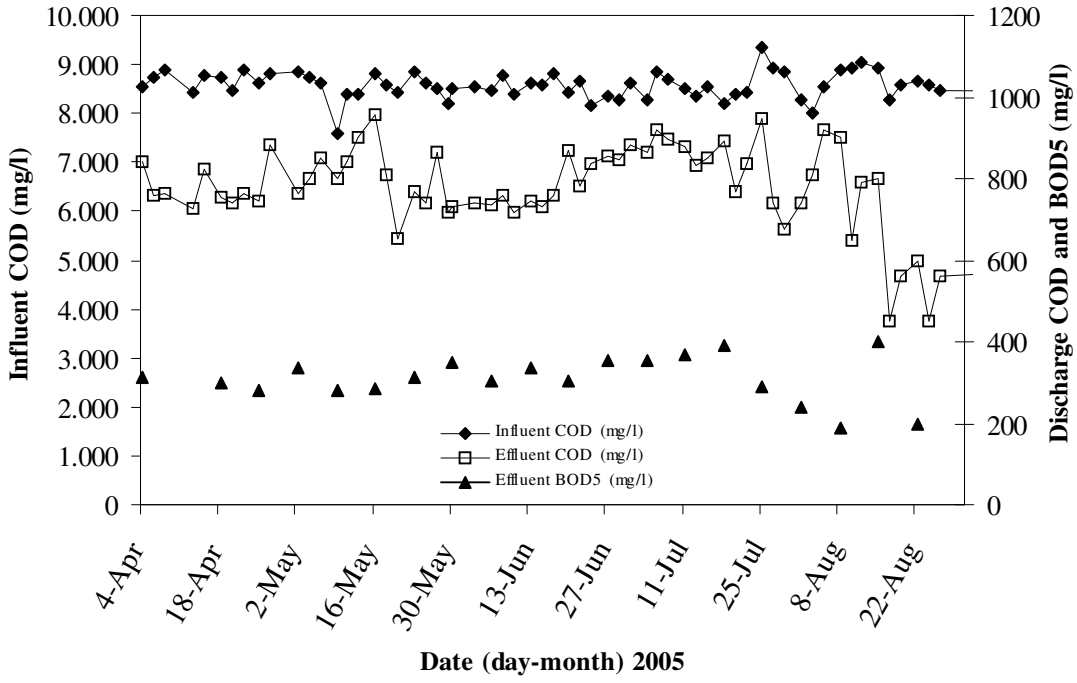

Figure B.7: Performance of the wastewater treatment plant

APPENDIX C: SMA Test Results

Table C.1. SMA test results for 3000 mg/l acetate concentration on July 2005

4m			8m			12m		
Time (hour)	**Pulse (1/hour)**	**SMA ($mlCH_4$/gVSS.day)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4$/gVSS.day)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4$/gVSS.day)**
0	0	0	0	0	0	0	0	0
1	5	98	1	2	35	1	0	0
2	5	98	2	2	35	2	0	0
3	5	102	3	2	35	3	0	0
4	5	102	4	3	52	4	0	0
5	6	106	5	3	52	5	0	0
6	6	106	6	3	54	6	0	0
7	6	108	7	3	54	7	2	38
8	6	108	8	3	58	8	2	38
9	6	108	9	3	60	9	2	38
10	6	116	10	4	62	10	2	38
11	6	116	11	4	69	11	2	38
12	6	124	12	4	69	12	3	57
13	6	124	13	4	69	13	3	57
14	6	124	14	4	70	14	3	66
15	7	132	15	4	70	15	3	66
16	7	132	16	4	72	16	5	96
17	7	136	17	5	78	17	5	96
18	7	136	18	5	82	18	6	118
19	7	142	19	5	82	19	6	118
20	7	142	20	5	87	20	6	118
21	8	148	21	5	87	21	6	118
22	8	148	22	5	87	22	6	124
23	8	148	23	5	90	23	6	124
24	8	154	24	5	90	24	6	124
25	8	154	25	5	90	25	7	130
26	8	154	26	5	90	26	7	130
27	8	160	27	5	92	27	7	134
28	8	160	28	5	92	28	7	134
29	8	160	29	5	92	29	7	134
30	9	168	30	6	96	30	7	134
31	9	168	31	6	96	31	7	134
32	9	168	32	6	100	32	7	134
33	9	174	33	6	100	33	7	142
34	9	174	34	6	104	34	7	142
35	9	178	35	6	104	35	7	142
36	9	178	36	6	104	36	7	142
37	10	182	37	6	110	37	8	148
38	10	188	38	6	110	38	8	148
39	10	194	39	7	116	39	8	148
40	10	194	40	7	118	40	8	153
41	10	194	41	7	120	41	8	153
42	10	196	42	7	120	42	8	153
43	10	196	43	7	120	43	8	153
44	10	196	44	7	120	44	9	168
45	10	198	45	7	120	45	9	168
46	10	198	46	7	124	46	9	168
47	11	202	47	7	124	47	9	172

4m			8m			12m		
Time (hour)	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**
48	11	206	48	7	124	48	9	172
49	11	216	49	7	128	49	9	172
50	11	216	50	7	128	50	9	172
51	12	224	51	8	134	51	9	172
52	12	224	52	8	134	52	10	182
53	12	238	53	8	139	53	10	182
54	12	238	54	8	139	54	10	182
55	13	248	55	8	139	55	10	191
56	13	248	56	8	139	56	10	191
57	13	248	57	8	139	57	10	194
58	14	264	58	8	139	58	10	194
59	14	264	59	8	144	59	10	198
60	14	274	60	8	144	60	10	198
61	14	274	61	8	144	61	10	198
62	15	286	62	8	144	62	11	206
63	15	286	63	9	148	63	11	206
64	16	304	64	9	148	64	11	206
65	17	324	65	9	156	65	11	210
66	17	324	66	9	156	66	11	210
67	17	324	67	10	168	67	11	210
68	17	324	68	10	168	68	11	210
69	18	338	69	10	168	69	12	229
70	18	338	70	10	172	70	12	229
71	18	346	71	10	172	71	12	229
72	18	346	72	10	172	72	12	229
73	18	348	73	10	172	73	12	234
74	18	348	74	10	178	74	12	234
75	18	348	75	10	178	75	12	234
76	18	348	76	10	178	76	13	244
77	18	348	77	11	182	77	13	244
78	18	348	78	11	182	78	13	244
79	18	348	79	11	182	79	13	248
80	18	348	80	11	182	80	13	248
81	18	348	81	11	188	81	13	248
82	18	348	82	11	188	82	13	248
83	18	348	83	11	188	83	13	248
84	18	348	84	11	188	84	13	248
85	18	348	85	11	188	85	13	248
86	18	348	86	11	188	86	13	248
87	18	348	87	11	188	87	13	248
88	18	348	88	11	188	88	13	248
89	18	348	89	11	188	89	13	248
90	18	348	90	11	191	90	13	248
91	18	348	91	11	191	91	13	248
92	18	348	92	11	191	92	14	267
93	18	348	93	11	191	93	14	267
94	18	348	94	11	194	94	14	267
95	18	348	95	11	194	95	14	267
96	18	348	96	11	194	96	14	267
97	18	348	97	11	194	97	14	267
98	18	348	98	11	194	98	14	267
99	18	348	99	11	191	99	14	267
100	18	348	100	11	191	100	14	267
101	18	348	101	11	191	101	14	267
102	18	348	102	11	198	102	14	267
103	18	348	103	11	191	103	15	287
104	18	348	104	11	191	104	15	287
105	18	348	105	11	191	105	15	287
106	18	348	106	11	198	106	15	287
107	18	342	107	11	198	107	15	287
108	18	342	108	11	191	108	15	287

4m			8m			12m		
Time (hour)	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**
109	18	342	109	11	191	109	15	294
110	18	336	110	11	191	110	15	287
111	18	336	111	11	188	111	15	287
112	18	336	112	11	191	112	15	294
113	17	324	113	11	191	113	15	287
114	17	324	114	11	188	114	15	287
115	17	324	115	11	184	115	15	287
116	16	300	116	11	182	116	15	294
117	16	300	117	10	178	117	15	287
118	15	282	118	10	168	118	15	287
119	15	282	119	10	168	119	15	287
120	15	282	120	9	150	120	15	287
121	14	264	121	8	140	121	15	287
122	14	264	122	8	130	122	15	287
123	14	264	123	7	118	123	14	270
124	13	240	124	6	106	124	14	270
125	13	240	125	5	86	125	14	270
126	13	240	126	4	66	126	14	270
127	10	200	127	2	36	127	14	270
128	9	180	128	0	0	128	14	267
129	8	150				129	14	267
130	6	110				130	14	267
131	3	60				131	13	248
132	0	0				132	13	248
						133	13	246
						134	13	248
						135	12	229
						136	12	229
						137	12	229
						138	10	191
						139	10	191
						140	9	172
						141	8	153
						142	6	115
						143	4	76
						144	2	38
						145	1	19
						146	0	0

Table C.2 SMA test results for 3000 mg/l acetate concentration on August 2005

4m			8m			12m		
Time (hour)	**Pulse (1/hour)**	**SMA ($mlCH_4$/gVSS.day)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4$/gVSS.day)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4$/gVSS.day)**
0	0	0	0	0	0	0	0	0
1	1	19	1	3	51	1	2	35
2	1	19	2	3	51	2	2	42
3	1	19	3	4	68	3	3	46
4	1	24	4	4	72	4	3	48
5	1	24	5	4	76	5	3	52
6	1	28	6	5	80	6	3	52
7	1	28	7	5	86	7	3	52
8	1	28	8	5	92	8	3	56
9	1	28	9	6	96	9	3	56
10	2	32	10	6	96	10	3	56
11	2	32	11	6	96	11	4	62
12	2	32	12	6	98	12	4	66
13	2	32	13	6	98	13	4	69
14	2	36	14	6	98	14	4	69
15	2	38	15	6	100	15	4	74
16	2	38	16	6	100	16	4	74
17	2	38	17	6	102	17	4	76
18	2	38	18	6	102	18	4	76
19	2	42	19	6	102	19	5	78
20	2	42	20	6	106	20	5	78
21	2	42	21	6	110	21	5	80
22	3	48	22	7	116	22	5	82
23	3	48	23	7	116	23	5	82
24	3	48	24	7	116	24	5	84
25	3	57	25	7	120	25	5	86
26	3	57	26	7	122	26	5	88
27	3	57	27	7	122	27	5	88
28	3	62	28	7	124	28	5	94
29	3	65	29	7	126	29	5	94
30	3	65	30	8	128	30	6	96
31	3	65	31	8	128	31	6	98
32	4	68	32	8	128	32	6	100
33	4	68	33	8	128	33	6	104
34	4	72	34	8	128	34	6	104
35	4	72	35	8	132	35	6	108
36	4	72	36	8	132	36	6	108
37	4	72	37	8	134	37	6	108
38	4	76	38	8	134	38	6	110
39	4	76	39	8	134	39	6	112
40	4	78	40	8	134	40	7	116
41	4	78	41	8	138	41	7	116
42	4	82	42	8	138	42	7	116
43	4	82	43	8	142	43	7	118
44	5	86	44	8	142	44	7	118
45	5	94	45	9	144	45	7	118
46	5	94	46	9	146	46	7	118
47	5	98	47	9	146	47	7	121
48	6	108	48	9	148	48	7	121
49	6	108	49	9	148	49	7	121
50	6	115	50	9	153	50	7	124
51	6	115	51	9	153	51	7	124
52	6	120	52	9	153	52	7	124
53	6	120	53	9	160	53	7	128
54	6	120	54	9	160	54	7	128
55	6	124	55	10	168	55	8	130
56	7	128	56	10	168	56	8	130
57	7	128	57	10	168	57	8	136

4m			8m			12m		
Time (hour)	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**	**Time (hour)**	**Pulse (1/hour)**	**SMA ($mlCH_4/gVSS.day$)**
58	7	128	58	10	172	58	8	136
59	7	132	59	10	172	59	8	136
60	7	134	60	10	172	60	8	139
61	7	134	61	11	184	61	8	139
62	7	134	62	11	186	62	8	139
63	7	134	63	11	186	63	8	144
64	7	134	64	11	186	64	8	144
65	7	134	65	11	186	65	9	148
66	7	134	66	11	186	66	9	148
67	7	134	67	11	186	67	9	150
68	7	134	68	11	186	68	9	150
69	7	134	69	11	186	69	9	150
70	7	134	70	11	186	70	9	152
71	7	134	71	11	186	71	9	152
72	7	134	72	11	186	72	9	156
73	7	132	73	11	186	73	9	156
74	7	132	74	11	186	74	9	156
75	7	130	75	11	186	75	9	156
76	7	130	76	11	186	76	9	156
77	7	130	77	11	186	77	9	156
78	7	128	78	11	186	78	9	156
79	7	128	79	11	186	79	9	156
80	7	128	80	11	186	80	9	156
81	6	122	81	11	186	81	9	156
82	6	118	82	11	186	82	9	156
83	6	115	83	11	186	83	9	156
84	6	115	84	11	186	84	9	156
85	6	115	85	11	179	85	9	156
86	6	115	86	11	179	86	9	150
87	6	115	87	11	179	87	8	146
88	5	96	88	10	172	88	8	142
89	5	96	89	10	169	89	8	139
90	5	96	90	10	169	90	8	139
91	4	76	91	10	169	91	8	139
92	4	76	92	10	169	92	8	139
93	1	19	93	10	169	93	8	130
94	1	19	94	10	164	94	8	130
95	1	19	95	10	162	95	8	130
96	0	0	96	9	156	96	7	124
			97	9	152	97	7	124
			98	9	152	98	6	112
			99	8	135	99	6	112
			100	8	135	100	6	106
			101	8	135	101	6	104
			102	7	119	102	6	104
			103	7	119	103	5	87
			104	7	119	104	5	87
			105	5	85	105	5	87
			106	5	85	106	4	69
			107	4	68	107	4	69
			108	1	17	108	4	69
			109	0	0	109	1	17
			110	0	0	110	1	17
						111	0	0

APPENDIX D: Fluorescence *in situ* hybridization (FISH) results

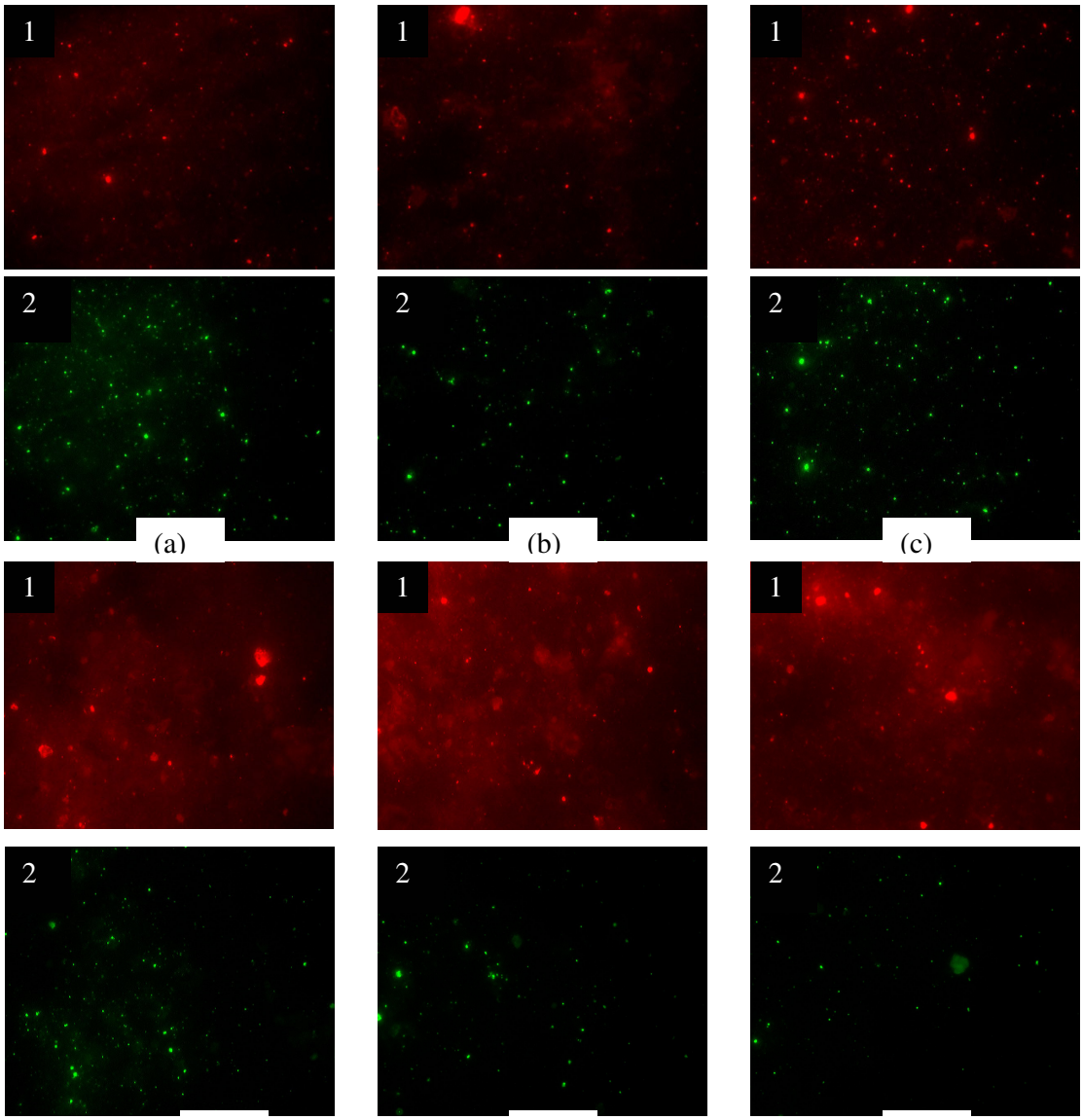

Figure D.1: Image examples of important methanogenic groups in the anaerobic contact reactor sludge at 4m on July 2005, 1: Determined cells with hybridization probes, 2: Determined cells with DAPI (DNA stain) a) MB310- *Methanobacteriales* b) MC1109-*Methanococcales* c) MG1200-*Methanogenium* relatives d) MS821-*Methanosarcina* spp. e) MS1414-*Methanosarcina* +relatives f) MX825-*Methanosaeta* spp.

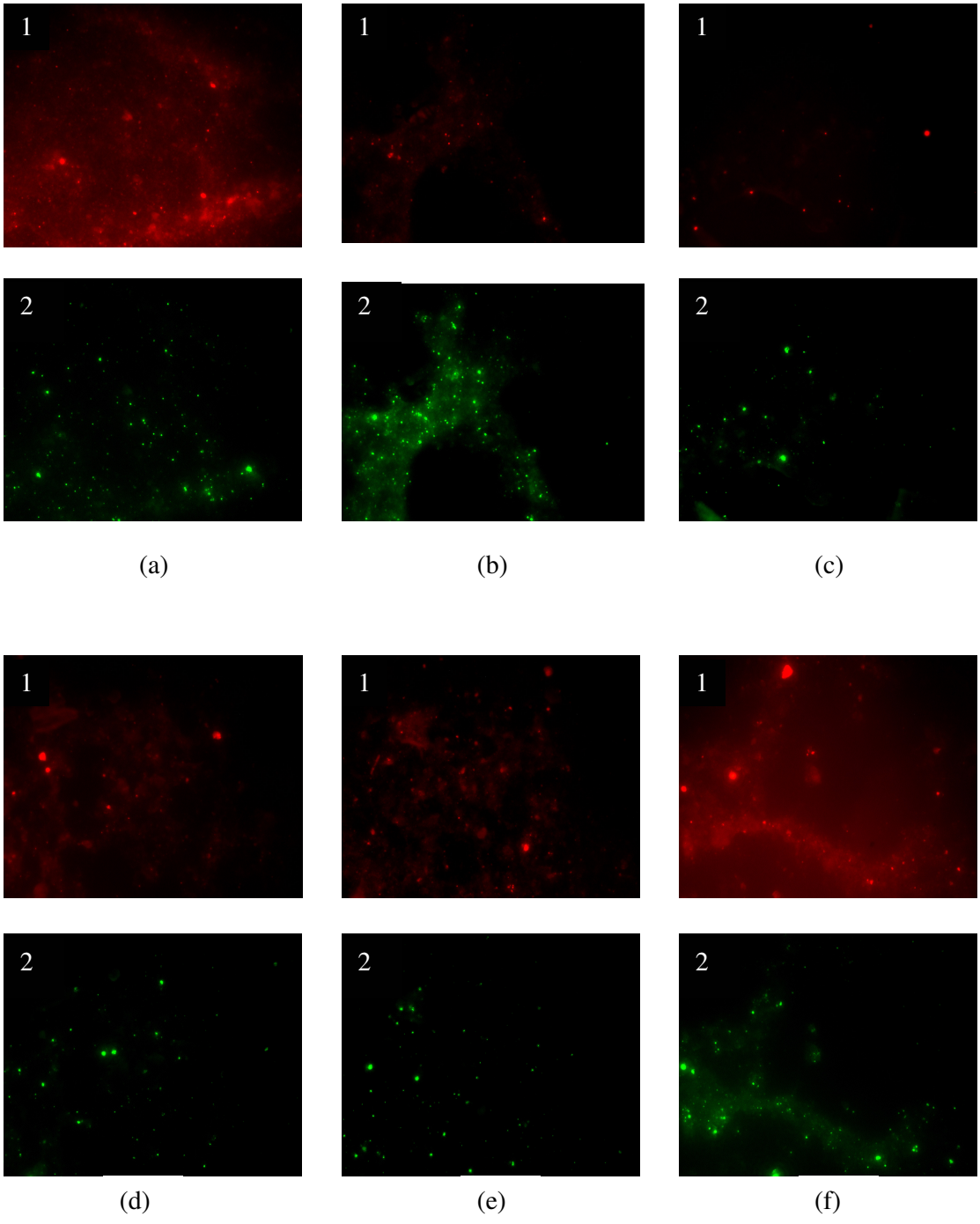

Figure D.2: Image examples of important methanogenic groups in the anaerobic contact reactor sludge at 8m on July 2005, 1: Determined cells with hybridization probes, 2: Determined cells with DAPI (DNA stain) a) MB310- *Methanobacteriales* b) MC1109-*Methanococcales* c) MG1200-*Methanogenium* relatives d) MS821-*Methanosarcina* spp. e) MS1414-*Methanosarcina* +relatives f) MX825-*Methanosaeta* spp.

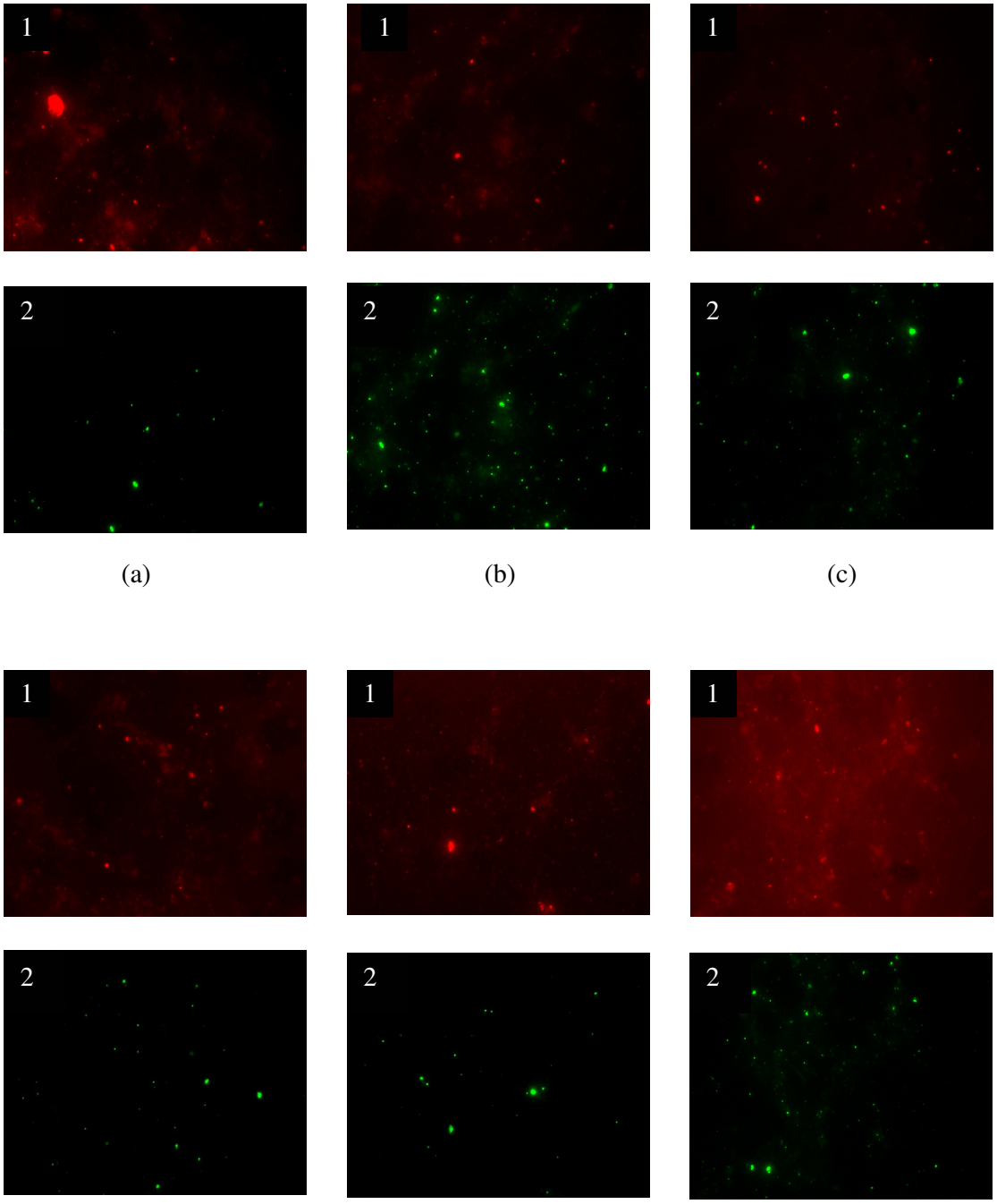

Figure D.3: Image examples of important methanogenic groups in the anaerobic contact reactor sludge at 12m on July 2005, 1: Determined cells with hybridization probes, 2: Determined cells with DAPI (DNA stain) a) MB310- *Methanobacteriales* b) MC1109-*Methanococcales* c) MG1200-*Methanogenium* relatives d) MS821-*Methanosarcina* spp. e) MS1414-*Methanosarcina* +relatives f) MX825-*Methanosaeta* spp.

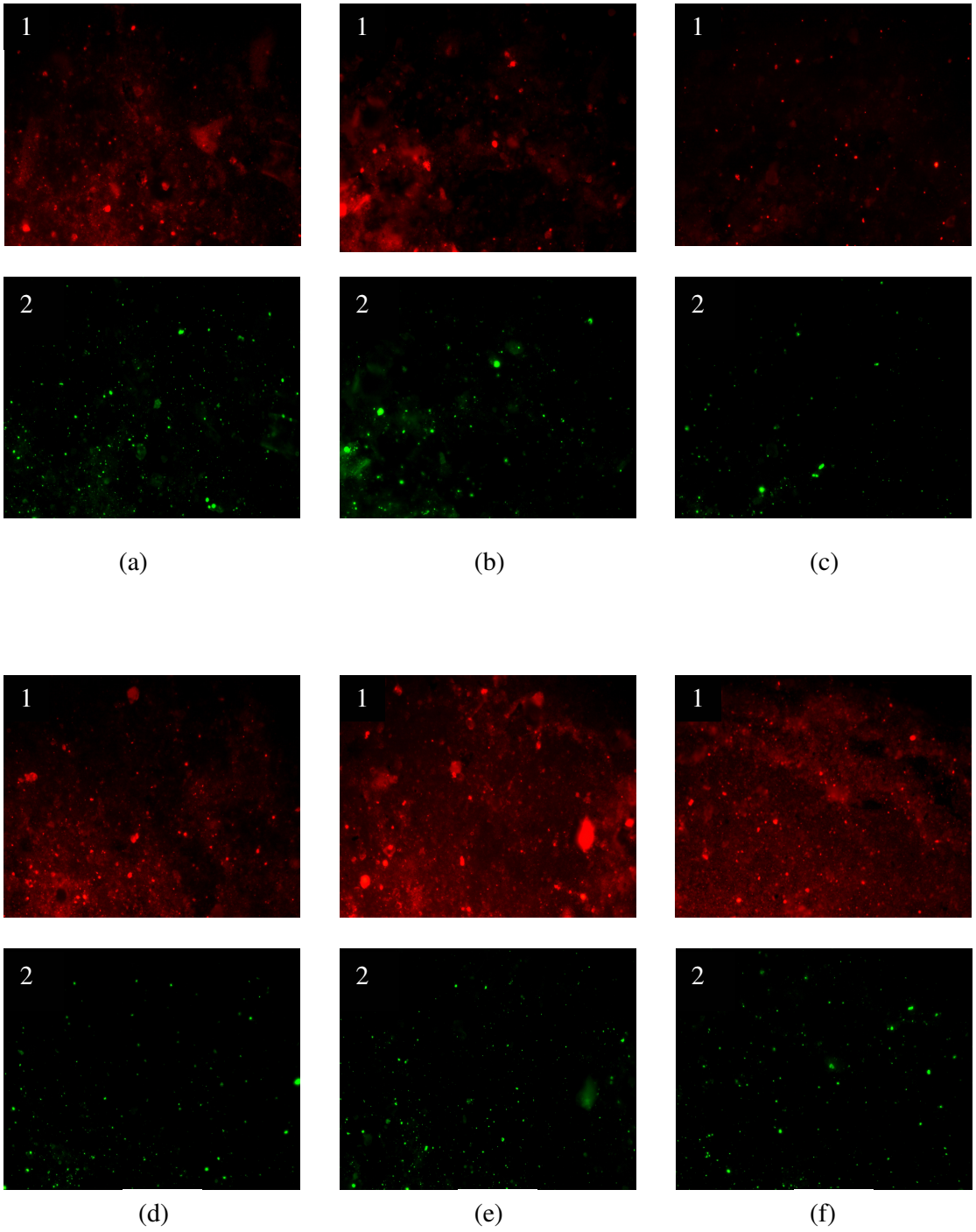

Figure D.4: Image examples of important methanogenic groups in the anaerobic contact reactor sludge at 4m on August 2005, 1: Determined cells with hybridization probes, 2: Determined cells with DAPI (DNA stain) a) MB310- *Methanobacteriales* b) MC1109-*Methanococcales* c) MG1200-*Methanogenium* relatives d) MS821-*Methanosarcina* spp. e) MS1414-*Methanosarcina* +relatives f) MX825-*Methanosaeta* spp.

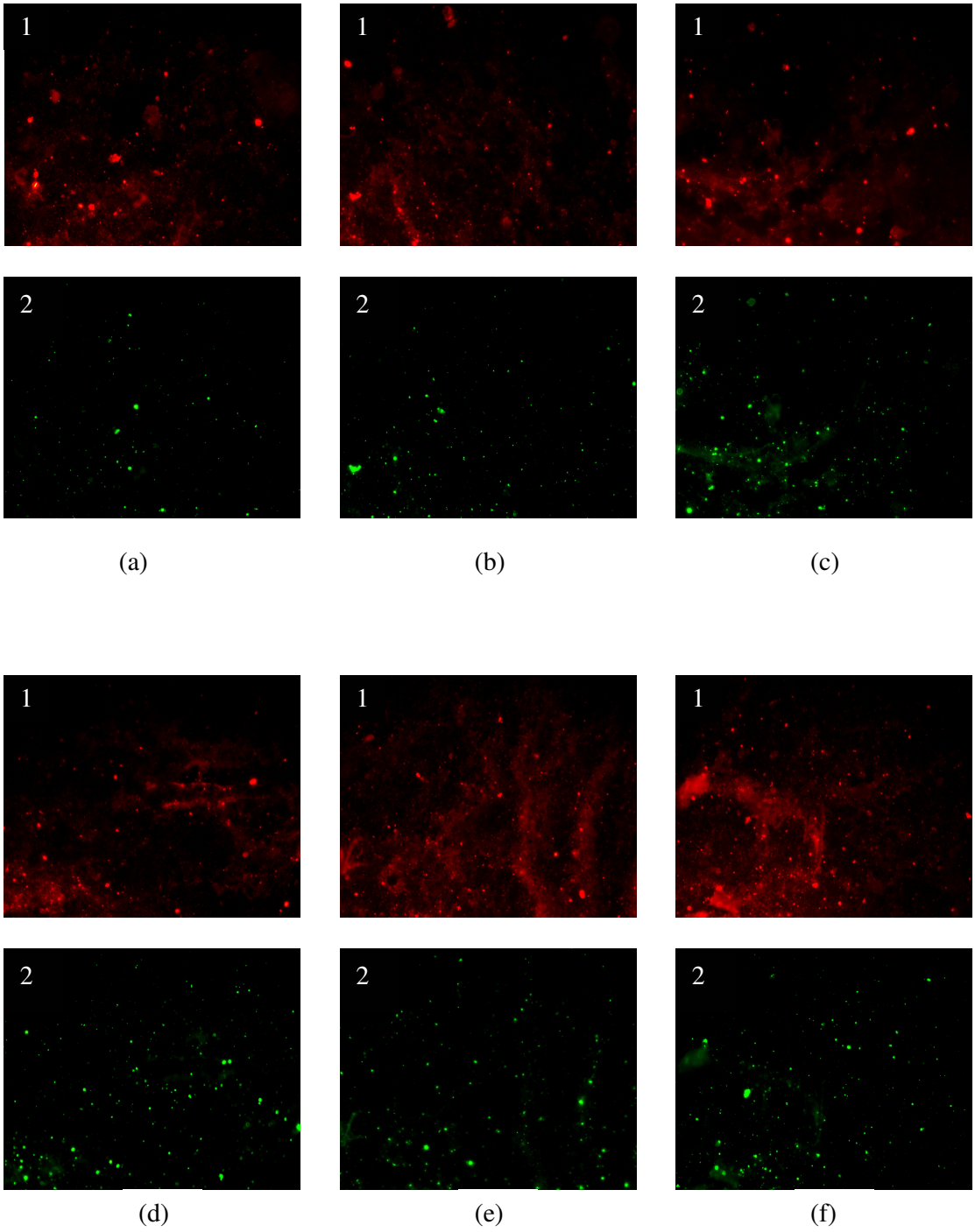

Figure D.5: Image examples of important methanogenic groups in the anaerobic contact reactor sludge at 8m on August 2005, 1: Determined cells with hybridization probes, 2: Determined cells with DNA stain DAPI a) MB310- *Methanobacteriales* b) MC1109-*Methanococcales* c) MG1200-*Methanogenium* relatives d) MS821-*Methanosarcina* spp. e) MS1414-*Methanosarcina* +relatives f) MX825-*Methanosaeta* spp.

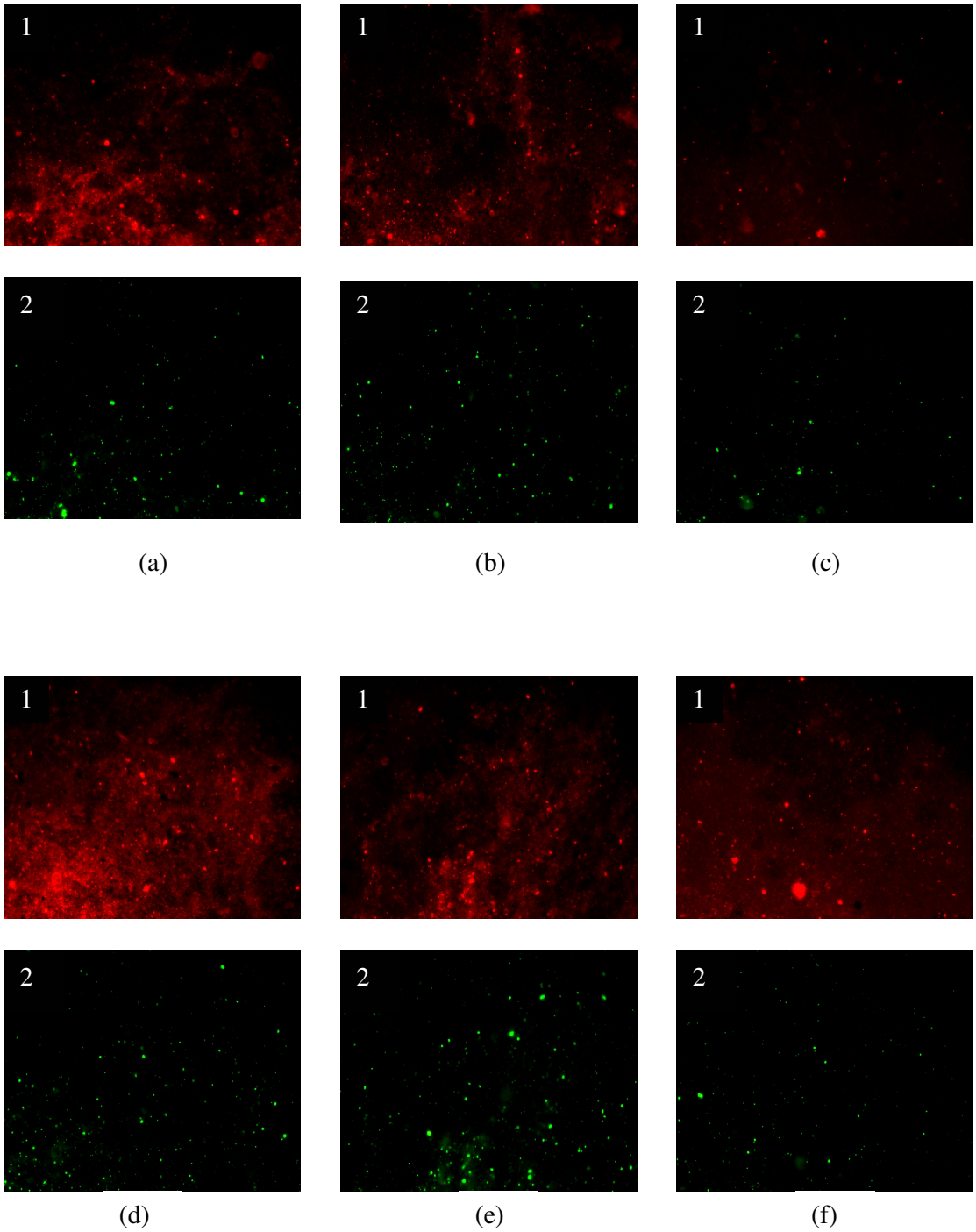

Figure D.6: Image examples of important methanogenic groups in the anaerobic contact reactor sludge at 12m on August 2005, 1: Determined cells with hybridization probes, 2: Determined cells with DAPI (DNA stain) a) MB310-*Methanobacteriales* b) MC1109-*Methanococcales* c) MG1200-*Methanogenium* relatives d) MS821-*Methanosarcina* spp. e) MS1414-*Methanosarcina* +relatives f) MX825-*Methanosaeta* spp.

CURRICULUM VITAE

Sevgi Ulugöl was born in Istanbul, 1978. She graduated from Pertevniyal High School. She enrolled to the Istanbul University, Environmental Engineering Department in 1996. She graduated from the department in 2000. After that, she worked at Aksa Enerji Uretim A.S. from 2000 to 2003. She started to MSc degree education in the Istanbul Technical University-Environmental Biotechnology Department in 2002. She is still working at Akenerji Elektrik Uretim A.S. as an environmental specialist since 2003.