

**A COMPARATIVE EVALUATION OF ANAEROBIC TREATMENT OF
LOW STRENGTH WASTEWATERS AT MESOPHILIC AND
PSYCHROPHILIC TEMPERATURES**

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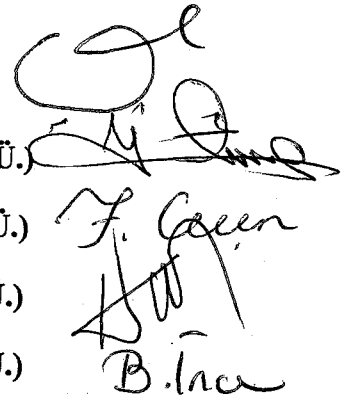
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**DÜŞÜK KİRLİLİK YÜKÜNE SAHİP ATIKSULARIN HAVASIZ
ARITIMININ MEZOFİLİK VE PSİKROFİLİK SICAKLIKLARDA
KARŞILAŞTIRMALI OLARAK DEĞERLENDİRİLMESİ**

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ABBREVIATIONS

COD_{tot}	: Total Chemical Oxygen Demand
COD_{rem}	: Chemical Oxygen Demand Removed
COD_{sol}	: Soluble Chemical Oxygen Demand
CSLM	: Confocal Scanning Laser Microscopy
FISH	: Fluorescent <i>In-Situ</i> Hybridization
HRT	: Hydraulic Retention Time
HRT_{avr}	: Average Hydraulic Retention Time
NH₄-N	: Ammonium Nitrogen
OLR	: Organic Loading Rate
pH_{avr}	: Average pH
rRNA/DNA	: Ribosomal Ribonucleic/Deoxyribonucleic Acid
SRT	: Sludge Retention Time
TKN	: Total Kjeldahl Nitrogen
TSS	: Total Suspended Solids
UASB	: Upflow Anaerobic Sludge Bed
VFAs	: Volatile Fatty Acids
VSS	: Volatile Suspended Solids

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LIST OF SYMBOLS

ARC915	: General probe for <i>Archaea</i>
Cy3, Cy5	: Cyanine dyes
DAPI	: 4',6'-diamidino-2-phenylindole hydrochloride
EUB338	: General probe for most bacteria
EURY498	: Oligonucleotide probe for most euryarchaeota
F/M	: Food to microorganism ratio
K_s	: Half-velocity coefficient
MB310	: Oligonucleotide probe for <i>Methanobacteriales</i>
MC1109	: Oligonucleotide probe for <i>Methanococcus</i> spp.
MG1200	: Oligonucleotide probe for <i>Methanogenium</i> relatives
MS821	: Oligonucleotide probe for <i>Methanosarcina</i> spp.
MX825	: Oligonucleotide probe for <i>Methanosaeta</i> spp.
Q_{gas}	: Daily biogas production
SARCI645	: Oligonucleotide probe for genus <i>Methanosarcina</i>
S₀, S₁	: Influent, effluent COD _{sol} concentration
T_{avr}	: Average Temperature
U	: Specific substrate utilization rate
X	: Average volatile suspended solid concentration in the reactor

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ABSTRACT

Anaerobic digestion has been generally applied to medium and high strength wastewaters. Recently, growing efforts have been spent in order to establish the feasibility of high rate anaerobic digestion to the treatment of diluted wastewaters. Thus, applications of anaerobic processes are now being extended to the treatment of dilute industrial wastewaters, and even anaerobic sewage treatment is considered achievable under certain conditions. The most appropriate anaerobic system to treat domestic wastewater has still been considered as the UASB reactor because of its simplicity, low investment and operation costs and the long favorable experience in the treatment of a wide range of wastewaters. Many recent researches conducted at various temperatures revealed that temperature is not a limiting factor in anaerobic treatment applications if the appropriate process design is chosen. It is reported that if the organisms are acclimatized to desired temperatures, the reactors can be operated even under psychrophilic conditions and psychrophilic anaerobic treatment becomes an attractive option for wastewaters, which are discharged at moderate to low temperatures. Since domestic sewage has a temperature lower than 35°C, heating is required during mesophilic anaerobic treatment. Thus, psychrophilic anaerobic treatment systems allow substantially lower treatment costs due to their ability to operate at low temperatures (10-20°C). Despite some drawbacks, successful anaerobic treatment of many dilute streams, which are also low in temperature, without the addition of heat would be a significant achievement in reducing the investment and operation costs and energy needs.

Two important methanogenic genera in anaerobic digestion, namely *Methanosaeta* and *Methanosarcina*, are mainly responsible for methane production from acetate. 16S rRNA/DNA based microbial identification techniques have become, over the past decade, an appropriate tool for detecting and studying microorganisms in many biological treatment systems. Among these microbial identification techniques, Fluorescent *in situ* hybridization (FISH) has been used widely to characterize the microbial population located in anaerobic systems. Operating temperature is reported as one of the most important parameters for anaerobic treatment of wastewater that selects the dominant bacterial flora and determines the growth rate. Since the activity of methanogens is strongly affected by temperature, an optimum operating temperature should be maintained in the system.

The main objectives of this study are evaluating the possibilities of optimizing performance of a high-rate anaerobic system treating low strength wastewaters at low temperatures and of achieving more feasible system due to no heating requirement.

Besides, dominant microbial communities in two lab-scale UASB reactors operated at mesophilic ($35\pm 2^{\circ}\text{C}$) and psychrophilic ($10\pm 1^{\circ}\text{C}$) temperatures and fed with synthetic domestic wastewater and raw domestic sewage were identified by FISH technique in combination with Confocal Scanning Laser Microscopy (CSLM). The variation in microbial diversity and alteration in granular structure were also investigated. Thus, in addition to classical operational parameters, process performances of both high rate anaerobic reactors treating low strength wastewater at different temperatures were evaluated by investigating the variations in microbial diversity.

Results indicated around 82% average total chemical oxygen demand (COD_{tot}) removal at around 11°C (psychrophilic) at an organic loading rate (OLR) of $2 \text{ kgCOD/m}^3\cdot\text{day}$ while at the same OLR around 90% average COD_{tot} removal was observed at 35°C (mesophilic) in synthetic sewage treatment. On the other hand, average COD_{tot} removals were obtained as 49% and 35% for the mesophilic and psychrophilic UASB reactors treating raw domestic sewage, respectively at an OLR around $0.7 \text{ kgCOD/m}^3\cdot\text{day}$. Low removal efficiencies during raw domestic sewage treatment could be due to high amounts of sea water additions into the sewerage system that caused dilute influent concentrations during sampling periods. Results also indicated that the use of 16S rRNA-targeted probes, was well suited to the study of the microbial ecology in anaerobic treatment systems and *Methanosaeta* were found as the dominant species in both types of granules.

DÜŞÜK KİRLİLİK YÜKÜNE SAHİP ATIKSULARIN HAVASIZ ARITIMININ MEZOFİLİK VE PSİKROFİLİK SICAKLIKLARDA KARŞILAŞTIRMALI OLARAK DEĞERLENDİRİLMESİ

ÖZET

Havasız arıtma orta ve yüksek kirlilik yüklerine sahip atıksularda yıllardan beri uygulanmaktadır. Son yıllarda, seyreltik atıksuların yüksek hızlı anaerobik sistemler ile arıtılabilirliklerinin fizibilitesi değerlendirilmektedir. Bu sebeple, anaerobik arıtma seyreltik endüstriyel atıksularda uygulanmaya başlanmış olup hatta evsel atıksuların bile belirli koşullar altında havasız arıtılabilirliklerinin gerçekleştirilebileceği düşünülmektedir. Uygulamadaki kolaylıkları, düşük ilk yatırım ve işletme maliyetleri ve çeşitli atıksuların arıtımlarındaki uzun süreli deneyimlerden dolayı, evsel atıksuların anaerobik arıtımında en uygun sistemlerin yukarı akışlı çamur yataklı (YAÇY) reaktörler olduğu düşünülmektedir. Son yıllarda farklı sıcaklık değerlerinde yapılan birçok araştırma, uygun proses tasarımı seçildiği takdirde, sıcaklık parametresinin havasız arıtma uygulamalarında kısıtlayıcı bir faktör olmadığını göstermiştir. Organizmalar istenilen sıcaklık değerlerine adapte edilirse, reaktörlerin psikrofilik (düşük sıcaklıklarda yaşayabilen) şartlar altında dahi işletilebilecekleri ve psikrofilik havasız arıtmanın, orta ve düşük sıcaklıklarda deşarj edilen atıksular için çok uygun bir alternatif olabileceği belirtilmektedir. Genelde evsel atıksuların deşarj edildikleri sıcaklık değerleri 35°C'nin altında olduğu için, mezofilik havasız arıtma sırasında ısıtma ihtiyacı duyulmaktadır. Bu sebeple, düşük sıcaklıklarda (10-20°C) işletilebilen havasız arıtma sistemleri çok daha düşük arıtma maliyetlerine olanak sağlayabilirler. Bazı dezavantajları olmasına rağmen, düşük sıcaklıklara sahip seyreltik atıksuların ilave ısıtma ihtiyacı göstermeyerek arıtılmaları, ilk yatırım ve işletme maliyetlerini ve enerji ihtiyaçlarını önemli ölçüde azaltmaktadır.

Havasız çürütme proseslerinde iki önemli metanojen tür olan *Methanosaeta* ve *Methanosarcina*, esas olarak asetati kullanarak metan üretimini sağlarlar. 16S rRNA/DNA esaslı mikrobiyolojik tanımlama teknikleri, son yıllarda, birçok arıtma sistemlerinde bulunan mikroorganizmaların tesbit ve incelenmesinde uygun bir yöntem olmaktadır. Mikrobiyolojik tanımlama teknikleri arasında en fazla kullanılan Floresan *yerinde* hybridizasyon (FISH) tekniği ile anaerobik sistemlerde bulunan mikrobiyolojik popülasyonlar tanımlanabilmektedir. Atıksuların havasız arıtılmaları sırasında baskın bakteri floralarının ve büyüme hızlarının seçimi sırasında göz önüne alınması gereken en önemli parametrenin işletme sıcaklığı olduğu belirtilmektedir. Sıcaklığın metanojen aktiviteleri üzerinde önemli etkileri olduğu için, sistem içersinde en uygun işletme sıcaklığının sağlanması gerekmektedir.

Çalışmanın amacı düşük kirlilik yüklerine sahip seyreltik atıksuların, düşük sıcaklık şartlarında yüksek hızlı YAÇY reaktör ile havasız arıtılabilirliklerinin değerlendirilmesi ve ilave ısıtma ihtiyacı göstermemesinden dolayı işletme maliyeti açısından daha ekonomik bir alternatif olarak incelenmesidir. Bu çalışmada, biri mezofilik ($35\pm 2^{\circ}\text{C}$), diğeri ise psikrofilik ($10\pm 1^{\circ}\text{C}$) sıcaklık şartlarında işletilmiş iki adet laboratuvar ölçekli YAÇY reaktör önce evsel atıksuyu karakterize eden sentetik atıksu ile, daha sonra ham evsel atıksu ile beslenmiştir. Her iki reaktördeki baskın mikrobiyolojik türler FISH tekniği kullanılarak konfokal tarayıcı lazer mikroskopu (CSLM) ile tanımlanmıştır. Deneysel çalışma süresince, mikrobiyolojik çeşitlilikteki değişim ve granül çamur yapılarında meydana gelen farklılıklar araştırılmıştır. Böylece seyreltik atıksuların farklı sıcaklıklarda arıtıldığı yüksek hızlı havasız reaktörlerin performansları, konvansiyonel izleme parametrelerinin yanında mikrobiyolojik çeşitlilik incelenerek değerlendirilmiştir.

Sonuçlar, sentetik atıksu arıtımında 11°C 'de (psikrofilik) ve $2\text{ kgKOI/m}^3\cdot\text{gün}$ 'lük organik yükleme hızı (OYH)'nda %82'lere varan ortalama KOI_{top} giderimi göstermiştir. Aynı organik yükleme hızında 35°C 'deki (mezofilik) ortalama KOI_{top} giderimi ise %90 olarak bulunmuştur. Diğer taraftan, ham evsel atıksuyun arıtıldığı mezofilik ve psikrofilik YAÇY reaktörlerde elde edilen ortalama KOI_{top} giderimleri $\text{OYH}=0.7\text{ kgKOI/m}^3\cdot\text{gün}$ 'de sırasıyla %49 ve %35 olarak gözlenmiştir. Ham evsel atıksuyun arıtılması sırasındaki düşük giderim verimlerine, numune alma döneminde kanalizasyon sistemine verilen deniz suyundan kaynaklanan seyrelmenin sebep olduğu düşünülmektedir. 16S rRNA-odaklı oligonükleotid probalar, havasız sistemlerin mikrobiyolojisi hakkında etkili sonuçlar vermiş ve her iki granül çamurunda, *Methanosaeta* (MX825) türünün baskın tür olduğu gözlenmiştir.

1. INTRODUCTION

1.1 Significance of the Study

Anaerobic systems have been used for the treatment of medium to high strength wastewaters such as industrial wastewaters for years. However in the last 20 years, anaerobic treatment was also successfully applied for the treatment of low strength wastewaters. In anaerobic processes, organic materials are mainly converted to methane and carbon dioxide in the absence of oxygen. Mostly, domestic sewage represents a low strength complex wastewater consisting of particulate organic materials such as carbohydrates, proteins and lipids. Not only are domestic wastewaters characterized as low strength wastewaters but they are also characterized as partially soluble wastewaters. Low COD concentrations in the influent result in very low substrate levels inside the reactor and in low biogas productions as well. Not only sewerage systems, but also a number of industrial processes such as alcoholic and soft drink bottling industries, paper recycle and papermaking mills, fruit and vegetable canneries, and malting and brewery processes discharge dilute wastewaters. Although most anaerobic treatment processes are relatively efficient in the removal of organic matter and suspended solids, they are not very efficient in removing nutrients such as nitrogen and phosphorus. Thus, the anaerobic treatment of sewage could be considered as a pre-treatment alternative for the removal of the organic content whereas the nutrients should be removed in a proceeding process appropriate for nutrient removal. Recently, high-rate anaerobic systems emerged as a viable alternative for the treatment of many industrial and municipal wastewaters and are gaining popularity and importance. Among the most economic high-rate anaerobic systems, the UASB reactor system, developed by Lettinga and his coworkers in 1970's, has received widespread acceptance and has been successfully used in the treatment of several types of wastewaters.

Modern 'high rate' anaerobic treatment systems are typically applied to the treatment of high-strength wastewaters at temperatures in the mesophilic range (25 to 40°C). Thus, full-scale applications of anaerobic treatment are restricted to wastewaters with a temperature exceeding 18°C. Temperatures of many wastewaters are lower than the mesophilic range (<25°C) and normally they have to be heated for efficient treatment. In several studies it was reported that anaerobic processes are sensitive to sudden changes in environmental conditions. The temperature induced, exposure time and bacterial composition of sludge are the main factors which determine the effect of a temperature shock. Unstable performance and in some extreme cases complete failure of the system could be seen when anaerobic reactors under mesophilic conditions were exposed to a temperature shock. Thus, temperature should be controlled carefully in anaerobic treatment of wastewaters.

1.2 Aim and the Scope of the Study

The main objectives of this study are evaluating the optimum performance of a high-rate anaerobic system treating low strength wastewaters and raw domestic sewage at low temperatures and achieving more feasible system without heating requirement. Besides, the dominant microbial communities in the two lab-scale UASB reactors operated at mesophilic ($35\pm 2^\circ\text{C}$) and psychrophilic ($10\pm 1^\circ\text{C}$) temperatures were identified by using the FISH technique in combination with Confocal Scanning Laser Microscopy (CSLM). One of the UASB reactor used in the study was operated at mesophilic conditions whereas the other one was operated at sub-mesophilic conditions at the beginning and psychrophilic conditions as the study continued. In the first part of the study, the anaerobic treatability of the synthetic wastewater characterizing domestic sewage was investigated. The study was carried out by feeding raw domestic sewage into UASB reactors at both temperatures. The comparison was based on classical operating parameters such as COD removal rates and biogas yields at increased OLR's in the effluents by monitoring temperature, pH, alkalinity, and nutrient concentrations. The variation in microbial diversity and alteration in granular structure were also investigated.

2. LITERATURE REVIEW

2.1 Anaerobic Treatment

The process resulting in methanogenesis needs the conversion of simple organic compounds to volatile acids and other intermediates by acidogens. In anaerobic processes, organic materials are converted to methane and carbon dioxide in the absence of oxygen (**Pavlostathis and Giraldo-Gomez, 1991**). During the reduction of the organic content of a waste stream, the use of microbial consortium is involved (**Ndon and Dague, 1997**). Complex particulate organic materials are degraded by a multistep process of sequential and parallel reactions in which complex polymeric materials such as polysaccharides, proteins and lipids (fat and grease) are firstly hydrolyzed by extracellular enzymes to soluble products of a size small enough for the allowance of their transport across cell membrane. Secondly, short-chain fatty acids, alcohols, carbon dioxide, hydrogen, and ammonia are produced by the fermentation or anaerobic oxidation of these relatively simple, soluble compounds. Principal Volatile Fatty Acids (VFAs) produced by the acidogens involve; acetic acid, propionic acid, butyric acid, isobutyric acid, and valeric acid (**Speece, 1996**). The short chain fatty acids except acetate are converted to acetate, hydrogen gas and carbon dioxide. Methanogenesis occurs from carbon dioxide reduction by hydrogen and from acetate in the last step (**Pavlostathis and Giraldo-Gomez, 1991**). These steps are performed by several types of bacteria namely:

- hydrolytic-fermentative bacteria that hydrolyze and ferment complex organic matter to simple organic compounds (formate, acetate, propionate, butyrate and other fatty acids, ethanol, etc.), hydrogen and carbon dioxide;
- hydrogen-producing acetogenic bacteria in combination with hydrogen-consuming acetogenic bacteria by which metabolic products from the first group are converted mainly into acetate and hydrogen (or formate);

- carbon dioxide-reducing methanogens, and aceticlastic methanogens that convert acetate, or hydrogen/carbon dioxide and formate to methane (and carbon dioxide in the case of acetate degradation)

The complex metabolism is illustrated in Figure 2.1. From Figure 2.1, maximizing acetate production and its subsequent conversion to methane is shown. H_2/CO_2 , methanol and methylamines are more favorable methanogenic substrates than acetate generally. Before acetate is used, the preferred substrate is depleted first.

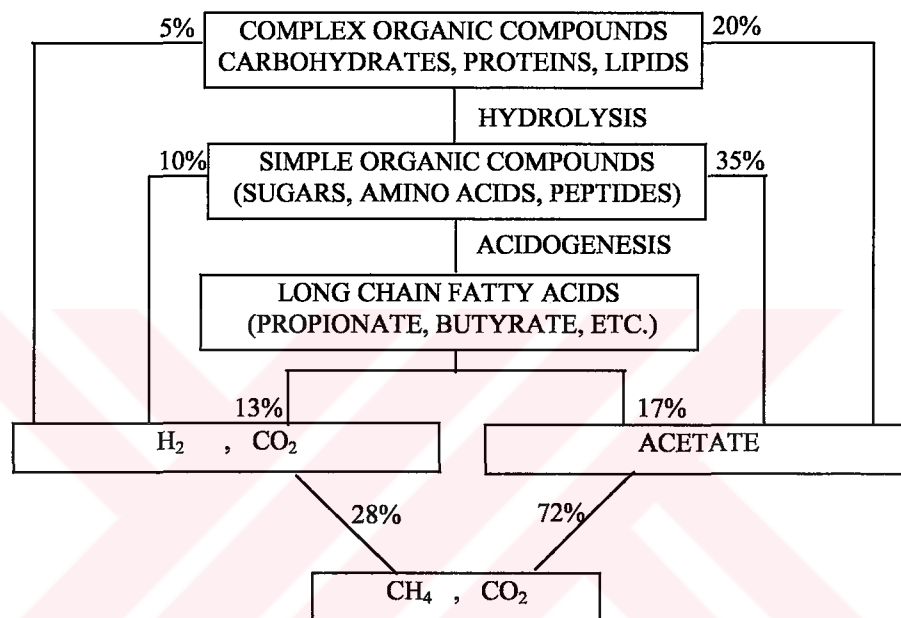


Figure 2.1: Anaerobic metabolism (Elmitwalli, 2000)

Sulfate-reducing bacteria can partially or completely mineralize the fermentation products to hydrogen sulfide or carbon dioxide via sulfate reduction (if sulfate is present). However, three microbial trophic groups or four metabolic steps (hydrolysis, fermentation, syntrophic acetogenesis and methanogenesis) are generally considered due to the fact that, sulfate reduction is not significant in most anaerobic reactors except for wastewaters consisting of high sulfate concentrations (Iza, 1991).

Although biodegradable organic compounds are effectively removed in anaerobic treatment, mineralized compounds like NH_4^+ , PO_4^{3-} , and S^{2-} are left in the solution necessitating an additional post-treatment (Lettinga, 1996).

Changing environmental conditions have adverse effects on anaerobic bacteria because they appear to be 'conservatives' and not to have the capacity to adapt

rapidly to changing environmental conditions (Lens et al., 2001). Besides, anaerobic systems can recover slowly after a toxic shock which can take days to weeks as well as higher residual BOD and COD are left (Mergaert et al., 1992). Other main well-known disadvantages of anaerobic bacteria are their slow growth and the corresponding problem of long detention times that were reported to be overcome by advanced reactor concepts, e.g. fixed-bed and fluidised-bed reactors or the upflow anaerobic sludge blanket reactor technology. Compared to aerobic systems, anaerobic techniques are superior due to low energy input and methane recovery as a valuable by-product (Schink, 2001).

Microbial interactions in anaerobic systems still require to be examined much more, even though great advances have been made since the discovery of *Archaea* (formerly *Archaeobacteria*) and the placement of the methanogens in this domain. The ecology of methanogens in anaerobic treatment processes should be well understood for an effective control at start-up and during operation (Raskin et al., 1994).

2.1.1 Microbiology of anaerobic processes

Bacteria populations arise from individual cells and metabolically similar populations such as sulfate- and sulfur reducing bacteria involve groupings referred to as guilds. These sets of guilds (e.g., sulfate- and sulfur reducing bacteria and methanogenic *Archaea*) conducting interdependent physiological processes form microbial communities. Microorganisms also form natural assemblages at air-water interfaces and in suspensions, such as anaerobic digesters. They preferentially aggregate to form flocs or granules (Davey and O'Toole, 2000).

The multispecies microconsortia in anaerobic reactors can result from an association between metabolically cooperative organisms, and their proximity facilitates interspecies substrate exchange and the removal or distribution of metabolic products. The degradation of complex organic matter into methane and carbon dioxide during anaerobic digestion needs the interaction of at least three guilds. The catabolism is initiated by fermentative bacteria producing acids and alcohols that are then readily utilized as substrates by acetogenic bacteria. The methanogens obtain energy from converting acetate, carbon dioxide, and hydrogen to methane at the final stage. Thus, very efficient cooperation and mutual dependence can occur within a

biofilm providing an ideal environment for the establishment of syntrophic relationships (Schink, 1997).

Microorganisms are classified into 'temperature classes' on the basis of optimum temperature and the temperature span in which the species are able to grow and metabolize. The overlapping growth temperature ranges indicate that there is not a clear boundary between these classic group of psychrophilic, mesophilic and thermophilic microorganisms. However, the bacterial growth rates of anaerobic groups are well determined. The growth rates of psychrophilic methanogens are comparably slower than mesophilic and thermophilic methanogens. Moreover, most reactions in the biodegradation of organic matter require more energy to proceed at low temperature than at a temperature optimum of 37°C (URL 2.1).

Four different groups of microorganisms, primary fermenting bacteria, secondary fermenting bacteria, and two types of methanogens degrade organic matter to methane and carbon dioxide in discrete steps in anaerobic treatment. Among them, methanogens produce methane as an internal part of their energy metabolism. Methanogens are found at large numbers and belong to *Archaea*. Methanogens may represent all basic morphological types such as; cocci and packets of cocci, rods of different shape and size, spirillum, and filamentous forms. They are dependent on other organisms for the supply of substrates in most anaerobic environments. Methanogens have the ability to use H_2 that plays a key regulatory role and controls the types of products made by fermentative bacteria. Even though large amounts of H_2 are produced, H_2 concentrations are normally very low in a well-operated anaerobic treatment system because of the fact that methanogens use large capacities of H_2 . The most important substrate for methane production is acetate which is a major product of fermentative metabolism. Acetate using methanogens consist of members of the genera *Methanosarcina* and *Methanosaeta*. *Methanosarcina* spp. have faster growth rates, higher apparent half saturation constant values for acetate use and higher threshold acetate values than *Methanosaeta* spp. (Çallı, 2003).

Methanosaeta and *Methanosarcina* are known to grow by an acetoclastic reaction, producing methane from acetate among the many methanogenic genera. *Methanosaeta concilii* is solely an acetoclastic bacterium and is the only mesophilic

species of its genus, other species being thermophiles. *Methanosarcina barkeri* is metabolically the most versatile of all the mesophilic methanogenic bacteria isolated in pure culture, because it can form methane from H₂ and CO₂ (hydrogenotroph), from methanol and methylamines (methylotroph), and from acetate (acetoclast) (Rocheleau et al., 1999).

Cells belong to the genus of *Methanosarcina*, known to utilize acetate for methane production, have been shown to be common in anaerobic reactors (Sorensen et al., 1997). Generally, the granules are composed of *Methanosaeta* spp. rather than *Methanosarcina* spp. in high rate anaerobic sludge bed reactors (Çallı, 2003). Investigation of the microbial diversity in the bioreactors is very important during the operation period. Thus, identification of the microorganisms should be done for formation, stability, and physiological properties of the biomass in anaerobic reactors that is a key factor during the evaluation of the process performance.

2.1.1.1 Microbiology of anaerobic granules

Different types of anaerobic bacteria compose the granular sludge which is an aggregate and much attention has been focused on the microbial structure and composition of granular sludge. Usually, the granules have a complex layered structure. On the outer surface, mainly fermentative bacteria and hydrogenotrophic methanogens exist and the inner layer is occupied by acetoclastic methanogens and H₂-producing bacteria. Besides, the juxtaposition of different types of anaerobic bacteria is detected in granules. The change of bacterial composition and function of bacteria requires investigation during the granulation. Studies should also focus on the syntrophic association between H₂-producing acetogenic bacteria and methanogens in granular sludge. Microcolonies responsible for propionate degradation are mutualistic associations of *Syntrophobacter* and *Methanobrevibacter*, or *Syntrophomonas* associated or unassociated with *Methanobrevibacter* or *Methanospirillum hungatei* in granular sludge (Jianrong et al., 1997).

Methanosaeta spp. are known to grow only on acetate and are filamentous organisms. *Methanosarcina* spp. grow either as single coccoidal cells or in large

clumps up to 1 to 3 mm in diameter that consist of large numbers of individual cells surrounded by a thick polymeric wall. *Methanosarcina* spp. are also able to grow on substrates such as methanol, methylamines, and sometimes hydrogen and carbon dioxide. *Methanosaeta* spp. have a lower growth rate at high acetate concentrations than *Methanosarcina* spp., and their affinity for acetate is 5 to 10 times higher. These kinetic data show that a low acetate concentration in the effluent from a UASB reactor results in a selection for granules dominated by *Methanosaeta* spp. However, since *Methanosarcina* spp., unlike *Methanosaeta* spp., can grow on several substrates, their role in UASB reactors can not be based only on their ability to use acetate. It was reported that there was no correlation between the effluent concentrations of acetate from UASB reactors and the ratio of *Methanosaeta* to *Methanosarcina*. It was also reported that probably other factors were involved in selection, such as macro and micro nutrients and the hydraulic loading of the reactor (Schmidt and Ahring, 1999).

Jianrong et al. (1997) reported that *Methanosaeta* was well associated with H₂-producing acetogenic bacteria and even with hydrogenotrophic methanogens-*Methanobrevibacter* spp. Hydrogenotrophic methanogens are considered to normally associate with acetogens. *Methanosaeta* can effectively convert acetate (the end product of propionate and butyrate) to methane. Thus, the syntrophic association between *Methanosaeta* and H₂-producing acetogenic bacteria might play an important role in the degradation of substrate, especially the short chain fatty acids such as propionate and butyrate. *Methanosarcina* clumps and *Methanospirillum* were also observed and *Methanosarcina mazei* existed in granules.

The adhesion of cells is dependent mainly on the interactions of cell wall, e.g., surface charges. *Methanosaeta* might play an important role in the formation of network of granular sludge because of the fact that the arrangement of cells were observed very close (less than 60 nm in distance). This is induced and controlled by the operational conditions in the reactor (Jianrong et al., 1997).

Different groups of bacteria play an important function in the occurrence of the syntrophic association in granules. The layered structure and juxtapositioning arrangement favor the metabolism of substrate in granules, especially the interspecies

hydrogen transfer. Hydrogenotrophic methanogens such as *Methanobrevibacter* and *Methanospirillum* are considered to associate with the acetogens due to their faster growth rates and good metabolic activity (Jianrong et al., 1997).

The nature of the substrate also plays a very important role in the microstructure of the granules. It was reported that granules degrading soluble carbohydrates exhibited a layered structure, while those degrading glutamate exhibited a rather uniform structure. This can be explained by the different rates of acidogenesis and substrate diffusion (Fang et al., 1994). They also reported that *Methanosaeta* (formerly *Methanothrix*) was the key structural element in all anaerobic granules which suggests that these filaments likely play an important role in sludge granulation. Various different species of acidifiers, acetogens and methanogens can be represented by filamentous microorganisms in anaerobic digesters. However, one of the most well known filamentous methanogenic bacteria is the acetoclastic *Methanosaeta*. Different morphologies from filaments longer than 1000 units to short filaments of 5-10 units could be observed for this bacterium which is the dominant acetoclastic species under low substrate concentrations (Alves et al., 2000).

Consequently, microorganisms are immobilized in densely packed granules and high conversion rates of alcohols and fatty acids can be reached in anaerobic systems. Besides, the architecture of syntrophic consortia has been studied in methanogenic granular sludge. Usually, a layered structure of the granule, in which a central core of acetoclastic methanogens is surrounded by a layer of hydrogen- or formate-consuming methanogens, was proposed. The outer layer of the granule contains most likely bacteria which hydrolyze and acidify complex organic matter (Harmsen et al., 1996).

2.1.2 Identification of microbial communities in anaerobic processes

The identification and quantification of members of particular microbial communities and a clear understanding of the functional relationship between members are needed before full appreciation and possible management of the complex processes. To attain this goal, recent technological advances have been made (Davey and O'Toole, 2000).

Microbial communities of anaerobic treatment processes have primarily been examined by classical parameters (such as VSS) or used microscopic or culture-based counts which are informative but may not be adequate (Akarsubasi et al., 2005).

There are two identification techniques such as classical identification techniques and molecular identification techniques. Among classical identification techniques, cultivation dependent methods and microscopic analyses can be listed. In molecular identification techniques, immuno detection, membrane lipid fatty acid analysis, ribosomal RNA/DNA based methods and Fluorescent *In-Situ* Hybridization (FISH) methods are gaining importance (Çalli, 2003).

The spatial distribution of methanogens in methanogenic granular sludge has been studied with various immunological techniques, but the distribution of acetogenic bacteria, especially in this sludge, could not be investigated due to the absence of identification methods allowing a differentiation between the groups of bacteria (Harmsen et al., 1996).

The remarkable breakthrough in rRNA-based phylogenetic analysis has provided means of developing tools for the examination of microbial communities are examined. Fluorescently labeled rRNA-targeted oligonucleotides, a variety of microensors, real-time image analysis, and confocal microscopy have been developed and have provided in situ monitoring of populations by noninvasive means. These techniques have been used to identify and quantify specific populations within a variety of complex microbial mixtures (Davey and O'Toole, 2000).

Since an unexpectedly large number of organisms with unique phenotypes belong almost exclusively to the members of the domain *Archaea*, a systematic study on whole-cell hybridization of *Archaea* should be performed. It can not be granted that methods optimized for the members of Bacteria will also be appropriate for *Archaea*. It is necessary to evaluate and modify existing protocols to assure permability of the cell periphery and maintenance of cell integrity (Burggraf et al., 1994).

Methanogenic consortia can be studied effectively by rRNA-based techniques and studies indicated that these techniques were well suited because methanogens can be

identified and quantified at different levels of specificity (phylogenetic depth). The classification of methanogens was based on comparative rRNA sequencing, and the oligonucleotide probes that were designed. Species-specific probes can be designed for more specific applications. Besides, methanogenic consortia can be studied intact by whole-cell hybridization which allows the identification and enumeration of individual community members in their natural spatial positions. Especially, in the studies like the colonization of surfaces, attached-film reactors, and the formation of granular microbial consortia such as UASB reactors; the characterization of spatial relationships should be particularly useful (Raskin et al., 1994).

In addition, by using in situ probes and confocal scanning laser microscopy (CSLM), the potential for high microdiversity in a natural microbial community can be investigated without the selective bias of cultivation, extraction, or amplification (Davey and O'Toole, 2000).

2.1.2.1 FISH technique combined with CSLM

FISH technique is defined as a process, which vividly paints chromosomes or portions of chromosomes with fluorescent molecules. A better understanding of microbiology and ecology of *Archaea* in biomass is an important part of improving process performance and control in anaerobic systems. FISH technique has been used to examine the spatial distributions of various microbial activities in biomass and has led to new experimental findings (Jang et al., 2003). FISH technique is used to localize organisms belonging to the bacterial domain and various types of methanogens in sludge granules (Davey and O'Toole, 2000). FISH technique with oligonucleotide probes targeting the 16S rRNA of microorganisms can be used to evaluate the abundance and distribution of specific phylogenetic groups in their natural microhabitats (Wu et al., 2001). The most commonly used target molecule for FISH technique is 16S rRNA in microbiology due to its genetic stability, domain structure with conserved and variable regions, and high copy numbers. Although there are so many databases for sequence, 16S rRNA gene offers the greatest ease and accuracy in identification for most microorganisms which is especially important during the identification of mixed bacterial populations or unculturable organisms. FISH technique allows nucleic acid sequences to be examined inside the cells

without altering the cell's morphology or the integrity of its various compartments. Fluorescent probes are safe and give better resolution. They also do not need additional detection steps. Besides, fluorescent probes can be labeled with dyes of different emission wavelength thus enabling detection of various target sequences within a single hybridization step **(Moter and Göbel, 2000)**.

Besides, the accuracy and reliability of the FISH technique is highly dependent on the specificity of the oligonucleotide probe that necessitates careful design and critical evaluation of new probes. It is possible to get low signal intensities which may be the result of the fact that probe penetration into the bacterial cell is not sufficient depending on the structure of the bacterial cell wall. Low physiological activity can result in low signal intensity or false negative results. Bright fluorescent dyes like Cy3 are usually recommended for slow-growing species to be detected. Usually no significant problem arises with gram-negative organisms because of their permeability even for poly-ribonucleotide probes **(Moter and Göbel, 2000)**.

Surface-attached biofilms as well as sludge granules readily form in anaerobic reactors. rRNA probes have been used to identify and quantify phylogenetically defined populations of organisms in sludge granules. Methanogens in anaerobic systems catalyzed final stages in the anaerobic mineralization of organic matter, and depend on other microorganisms (fermentative bacteria) to convert complex organic matter to simpler compounds, such as hydrogen and acetate, which in turn could serve as their substrates. The diversity and spatial distribution of populations within the community were studied by identification of cells hybridized in situ with fluorescent rRNA-targeted probes **(Davey and O'Toole, 2000)**.

The choice of target site and design of probes should be done with greatest care and specificity, sensitivity and ease of tissue penetration must be considered. There are different ways of labeling. The most commonly used and the cheapest, fastest, and easiest way of labeling is the direct fluorescent labeling due to the fact that it does not need any further detection steps after hybridization. Fluorescent labeled oligonucleotides can be purchased and commercially available such as Fluorescein-Isothiocyanate (FITC), 5-(-6-)carboxyfluorescein-*N*-hydroxysuccinimide-ester- (FluoX)) and rhodamine-derivates (Tetramethyl-Rhodamine-Isothiocyanate (TRITC),

Texas Red), and more recently cyanine dyes like Cy3 and Cy5. It was reported that cyanine dyes (Cy3 and Cy5) were superior to the classical dyes due to their stability to photobleaching and their capability in providing significant brighter staining. Aromatic diamidines like 4',6-diamidino-2-phenylidole dihydrochloride (DAPI) can be used for blue fluorescent counterstaining which non-intercalatively binds to DNA with great affinity (Moter and Göbel, 2000).

Methanogens can be readily identified under light microscopes by their emitted fluorescence under epi-fluorescent excitations at either 350 nm or 420 nm because of the fact that all methanogens, and only methanogens, have both the F₃₅₀ and the F₄₂₀ cofactors (Fang et al., 1994). Acetate is commonly converted to methane and carbondioxide by acetoclastic methanogens, i.e. *Methanosarcina* spp. and *Methanosaeta* spp. *Methanosaeta* spp. appear to be predominant and play an important role in most anaerobic reactors treating different types of wastewaters due to their high affinity and low threshold value for acetate compared to *Methanosarcina* spp. (Domingues et al., 2002).

Euryarchaeota is a physiologically diverse kingdom in the domain *Archaea* and there are three major phyla of *Euryarchaeota*: methanogens, halophiles and thermoacidophiles (URL 2.2). *Euryarchaeota* is a phylum of domain *Archaea* containing extremely halophilic organisms, methanogens and some extremely thermophilic aerobes and anaerobes (URL 2.3). It was indicated that the oligonucleotide probe EURY498 can be used for most members of the kingdom *Euryarchaeota*. However, due to the greater phylogenetic diversity in this archaeal kingdom, there are a few exceptions, that tend to be members of highly diverged lineages (the extreme halophiles and seven members of the orders *Methanomicrobiales* and *Methanobacteriales*). Those exceptions could be included without decreasing the probe's specificity by introducing degeneracies in the sequence of probe EURY498 (Burggraf et al., 1994).

This modern molecular biological technique provides the in-situ examination of the spatial distribution of methanogens in anaerobic ecosystems. Finally, in situ hybridization with fluorescently labeled oligonucleotide probes based on rRNA sequences has proven to be a powerful tool to identify and localize microorganisms

in their natural habitats (**Harmsen et al., 1996**). Phylogenetic, ecologic, diagnostic, and environmental studies in microbiology have been sensitively and quickly conducted with FISH technique over the last decade (**Moter and Göbel, 2000**).

In the last few years, knowledge of microbial cultures, their structures and functions has changed significantly due to advances in light microscopy. It was recognized, however that alternative transmission light microscopy techniques could provide limited but useful information about biomass structure such as measurement of depth. Many scientists ignored warnings that the necessary sample dehydration procedures might generate artifacts, particularly in biological specimens, which created several incorrect dogmas (e.g., biofilms are flat confluent films). These microscopy problems were not adequately resolved until the advent of Confocal Scanning Laser Microscopy (CSLM) with computer rendered 3-D images (**Keevil, 2003**).

Electron microscopy was the method of choice for investigating microbial biofilms under high resolution. However, sample preparation for electron microscopy causes dehydrated samples and thus, resulting in a deceptively simplistic view of biofilms due to the fact that the biofilm collapsed when water was removed. On the other hand, CSLM allows the visualization of fully hydrated samples and has revealed the elaborate three-dimensional structure of biofilms. CSLM has been used very effectively for the observation of the microbial development without disrupting the community (**Davey and O'Toole, 2000**).

CSLM was an important invention of the 80's, since it offers observation of thin optical sections in thick, intact specimens. In conventional fluorescence microscopy, out-of-focus fluorescence, evoked by the excitation light, tends to overwhelm details in the actual image plane. CLSM uses a tiny laser spot, focused in a defined image plane, to excite fluorescence. This spot is scanned in lines across the field of view resembling image formation by an electron beam in a TV-screen. Detection of fluorescence is by a photomultiplier with high sensitivity. Out-of-focus fluorescence is a minor problem here, because the intensity of the laser beam decreases with the third potency above and below the focal plane. Moreover, a special aperture in front of the photomultiplier excludes any remaining out-of-focus fluorescence. The light

source is a laser that produces high-intensity, coherent light of a defined wavelength. Between laser and beam splitter, where the light is mirrored into the objective, an excitation aperture (called pinhole) produces a sufficiently thin laser beam. The objective projects (focuses) this pinhole into the focal plane within the specimen. If fluorescence is here excited, it forms one image point (pixel; acronym for picture element) out of a raster of usually 512 by 512 pixels. After straight transmission of the beam splitter only this pixel is detected by the photomultiplier, since the emission pinhole is in a confocal position (conjugate) to the excitation pinhole.

CLSM depends on a small spot of high intensity with a known wavelength. Although other techniques are possible, laser light is ideally suited for this demand. The principle of a Laser (light amplification by stimulated emission of radiation) is based on the fact that excited atoms or molecules when impinged by quanta of a certain energy content will radiate photons of a wavelength which are perfectly coherent; i.e. the waves are in step (in the same phase) and all of the identical wavelength. The exciting electromagnetic radiation (e.g. by “optical pumping” with light flashes) has to carry the energy of the energy difference between the excited and the non-excited state of the core substance.

The scanning unit is responsible to illuminate and record the entire field. This unit “writes” the laser spot along columns and lines into the image plane. Usually are two galvanometers used to move a mirror that shifts the laser spot in the x-axis up to the end of a line, then one line down (one step in the y-axis) and again along the x-axis, etc. In CLSM, images are not directly visible in the ocular. The different intensities recorded by the photomultiplier (PMT) over time have to be converted back into x/y information by the computer controlling the CLSM. During scanning, the PMT-readings are depicted on the CLSM monitor. The user can thus control the intensity by increasing or decreasing the voltage of the photomultiplier. It is also possible to change the offset of the PMT. These changes soften or harden the contrast in a CLSM-image. The aim of voltage and offset changes is to produce an image with a black background and sufficiently outstanding fluorescence. If the fluorescence is weak, the photomultiplier has to work at its limits. This involves that the background gets spotted with white pixels caused by spontaneous electron delivery by thermic processes in the PMT. These are randomly distributed. Improvement of the image

quality in this case is by “averaging” several images. Primary image information is for each pixel an intensity scale of 0-255 grey values, 0 being black and 255 white. For the ease to discriminate slight intensity differences, these grey values can be transformed in different colour palettes, the “glow scale” being the most common used palette, reaching from black over dark red, red, orange, yellow to white. These colour palettes are not to be confused with the fluorescence wavelength. The CLSM does not record the wavelength of the fluorescence. The emission wavelengths of different fluorochromes can, however, indirectly be analysed with multichannel detection. By use of several channels, each provided with an emission filter and a photomultiplier, different fluorochromes can be separated from each other and/or from autofluorescence of the tissue and the colours separately analysed (URL 2.4).

Advances in non-destructive methods of microscopic analysis using CSLM have led to more detailed information regarding biological processes. The method allows us to obtain quantitative information and to make three-dimensional reconstructions of cell volumes. CSLM is the method of choice for monitoring structure formation of live biofilms in lab-scale reactors. As a result of its non-invasiveness and non-destructive character, it enables the *in vivo* reconstitution of the 3-D structure of microbial biomass in their naturally hydrated form and biomass formation can be observed in detail by scanning the same site repeatedly (Xavier et al., 2003).

Conventional concepts concerning the internal structure of biofilms assume a rather homogeneous layer of cells. Due to advances in CSLM, this view has been investigated by observing intact and undisturbed biofilms. CSLM helps to detect heterogeneous spatial structures consisting of clusters of bacteria as well as voids and channels. Microbial populations and biological activity should be observed directly for providing exact information on cluster and population dynamics, metabolic processes, resistance to antimicrobial agents, or predation within an organized functional biofilm structure. Acquiring numerical information about biofilm morphology is one of the primary interests and is done by digital image processing. It is used in microbiology, especially in combination with CSLM. By using CSLM, the percentage of the area covered by microorganisms in relation to a lens-dependent reference area, microbial distribution may be determined for each confocal optical section (Kuehn et al., 1998).

2.2 High-rate Anaerobic Treatment Systems

High-rate anaerobic treatment has emerged as a viable alternative for the treatment of many industrial and municipal wastewaters (Hickey et al., 1991). All the high-rate processes have the ability to separate hydraulic and solid retention times effectively. Relatively low hydraulic retention times are allowed due to the accumulation of a high biomass concentration in the system (Hickey et al., 1991). Besides, low construction cost and small land requirements are other benefits of high rate systems generally at temperatures above 20°C and high loading rates can be applied. Operation and maintenance costs are lower as well as energy consumption and equipment requirement. As compared to aerobic and physical-chemical treatment processes, lower sludge production occurs and the biogas produced can be used for energy production (Elmitwalli, 2000).

On the other hand, shorter start-up period, low pathogen removal, post treatment requirement for reaching the effluent standards, low removal efficiency of particulate organic material at low temperatures and odor nuisance risk from sulfate reduction to sulfide are the main drawbacks of high rate anaerobic systems (Elmitwalli, 2000). Especially at low temperatures, a considerable amount of CH₄, CO₂, H₂ and H₂S will remain in the effluent because the solubility of gaseous compounds present in biogas increases with a decrease in temperature. Thus, discharged reactor effluent may release considerable amounts of dissolved odorous gases necessitating a sufficient post-treatment (Lens et al., 2001). Besides, an aerobic polishing (post-treatment) step is also required after the anaerobic system (pre-treatment) to meet effluent quality standards, such as for effluent nitrogen and phosphorus levels (Angenent et al., 2001).

Three fundamental aspects on which the concept of high-rate anaerobic reactors are based can be summarized as follows (Iza, 1991);

- 1) Biomass can accumulate within the reactor by means of settling, attachment to solids (fixed or mobile) or by recirculation. Slowly growing microorganisms can be retained by ensuring that the mean solids retention time becomes much longer than the mean hydraulic retention time.

- 2) Contact between biomass and wastewater is improved that overcomes problems of diffusion of substrates and products from the bulk liquid to biofilms or granules.
- 3) Owing to adaptation and growth, the activity of the biomass is enhanced.

Not only can efficient operation primarily be determined by allowing the retention of high biomass concentration within the reactor, but by effective separation of the biomass from the liquid phase (Iza, 1991). In high-rate anaerobic treatment technologies, optimum contact between the retained biomass and the waste undergoing treatment should also be maintained. The contact between biomass and wastewater is adversely affected by; channeling, dead-zone formation, and clogging of poorly designed distribution systems. The formation of preferential paths through the reactor is termed as channeling whereas sludge compaction or clogging of matrix interstitial spaces by solid may cause dead-zone formation (Iza, 1991). There is no common indicator that shows the activity of the biomass in anaerobic systems because of the fact that, the anaerobic process is performed by various bacterial trophic groups with respective anaerobic pathways. Thus, characterization of biomass and analysis of its activity should be estimated by the application of metabolic activity assays using batch tests such as; biogas production rate, specific methane production rate or specific COD removal rate based on general substrates and volatile suspended solids amount, specific substrate conversion rate (acetate, H_2 - CO_2 , formate, propionate, butyrate, glucose, etc.). Besides, characterization of biomass and analysis of its activity may also be estimated by the application of microbial population assay such as; most probable number (MPN) enumeration, immunological assay (especially for identification of methanogenic species), and microscopic and electronic microscopic observation. If methane, carbon dioxide, H_2 , H_2S , etc. content of the total biogas can be determined individually, the activity would be evaluated more precisely (Iza, 1991). Anaerobic digester performance failure is often accompanied by a build-up of the VFAs. Acetate, propionate and butyrate are the more likely VFA that accumulate initially. Thus, an accurate control of operational conditions is needed and complete breakdown of fatty acids has to be realized in the system (Rebac, 1998).

Typical high-rate anaerobic reactors are classified as contact reactor, anaerobic filter reactor, downflow stationary fixed film reactor, fluidized bed reactor, upflow anaerobic sludge bed reactor, and hybrid reactor **(Iza, 1991)**.

Anaerobic wastewater treatment concept merely offers benefits in the light of very desirable development and implementation of sustainable and non-vulnerable methods and technologies compared to conventional aerobic methods **(Lettinga, 1996)**.

2.2.1 Upflow anaerobic sludge bed (UASB) reactor

The UASB reactor system was developed by Lettinga and his coworkers in 1970's has received widespread acceptance and been used successfully in the treatment of several types of wastewaters **(Hickey et al., 1991)**. As UASB processes have become more popular, extensive research work has been conducted to understand physicochemical and biological characteristics of the granular sludge and the mechanism of its formation **(Yoda and Nishimura, 1997)**. UASB reactor has a capability to retain a high concentration of active suspended biomass and explains the great success of this reactor **(Elmitwalli, 2000)**. Biomass recirculation is not required in the UASB reactor **(Patel and Madamwar, 2002)**. Compared to other anaerobic reactor configurations, UASB reactors have the advantage of avoiding any support media for biomass attached growth, an important characteristic in the treatment of wastewaters consisting of high influent suspended solids **(De Sousa and Foresti, 1996)**. A biological reaction zone and a sedimentation zone are the two main zones found in a typical UASB reactor. The organic compounds in the influent are converted to methane and carbon dioxide in the reaction zone during the passage of the flow upward through a bed of highly active sludge. The gas-solid-liquid separator at the top of the reactor helps the gas produced and the sludge buoyed by entrapped or attached gas bubbles to be separated from the liquid effluent **(Hickey et al., 1991)**.

The wastewater passes through the anaerobic biomass in which dissolved substrate is digested and the particulate matter firstly is captured and next digested in high rate systems **(Elmitwalli, 2000)**. Modern 'high rate' anaerobic treatment systems are based on sludge immobilization retaining as much viable sludge as possible in the

reactor **(Rebac, 1998)**. Thus, much greater Solids Retention Time (SRT) than the Hydraulic Retention Time (HRT) can be provided **(Banik et al., 1997)**. A microbial balance between the fast-growing acidogens and the slow-growing methanogens needs to be achieved in a successful anaerobic treatment. The slow growth rate of methanogens necessitates an efficient biomass retention that can not be achieved in conventional suspended growth completely mixed anaerobic reactors **(Ndon and Dague, 1997)**. Since biomass is retained at high concentrations in an UASB reactor, high removal efficiencies can be achieved at high volumetric COD loading rates can be achieved in the process. The anaerobic sludge developed has good settling property and is often granular in nature, if the UASB reactors are operated well **(Hickey et al., 1991)**. The granular sludge formed in UASB reactor has a high methanogenic activity and it has better settleability than flocculant sludge. Thus, maximum loading rate of the UASB reactor system is improved **(Elmitwalli, 2000)**.

The microcolonies that involve the biofilm can be composed of single-species populations or multimember communities of bacteria, depending on the environmental parameters under which they are formed. In biofilms formed in UASB reactors (continuous-flow systems comprising multiple microbial species, where the flow occurs from bottom to top of the reactor), aggregates consisting of complex bacterial communities (referred to as flocs or sludge granules) predominate. This is primarily due to the fact that the degradation of organic materials to methane and carbon dioxide is a community-level process that is driven by the close contact of multiple guilds interacting in a food web. Besides, the bacteria are advantageously positioned in these reactors through aggregation. Because there is no surface area for attachment except for the walls in this type of reactor, the formation of granular sludge acts as a mechanism by which the biofilm communities settle to the bottom of the reactor and this prevents their being washed out of the system. In addition, the biomass is more readily exposed to the continuous supply of nutrients being pumped into the bottom of the reactor through granular sedimentation. Hence, not only does microbial biology affect biofilm structure, but also environmental parameters affect the formation and the structure of biofilm **(Davey and O'Toole, 2000)**.

The success of the UASB process relies on the formation of active and settleable sludge granules. Granulation mechanism as well as the microbial composition of the

granules have been studied by several researchers and the microbiology of methanogens has been investigated. These also provided a full understanding of the syntrophic associations between the hydrogen-producing acetogens and the hydrogen-consuming methanogens (Fang et al., 1994).

Determination of the potential loading capacity of the UASB reactors can be done by specific methanogenic activity (SMA) tests, thus resulting in the use of suitable OLR's. By SMA test, any deterioration in the performance of the system under different operating conditions can be assessed and reactor stability can be established (Ince et al., 2001).

2.3 Anaerobic Treatment of Low Strength Wastewaters

Anaerobic pretreatment for low-strength wastewaters have advantages over aerobic treatment, which include decreased sludge production and lower energy requirements and decreased operating costs (Angenent et al., 2001). Low strength wastewaters can be defined as those having an organic pollution below 1500 mg/l which generally consist of a variety of biodegradable compounds such as simple short-chain VFAs, alcohols, and carbohydrates. However, they frequently also consist of proteins, suspended solids of different origin, fats or long-chain fatty acids. Their dissolved oxygen (DO) concentration may be found up to saturation level (10-12 mg O₂/l at 4°C) inside the reactor (Rebac, 1998).

Modern 'high rate' anaerobic treatment systems are typically applied to the treatment of high-strength wastewaters at temperatures in the mesophilic range (25 to 40°C) (Banik and Dague, 1997). In former years it was reported that anaerobic pre-treatment was appropriate for wastewater having a COD higher than 5000 mg/l. However, in recent years anaerobic treatment of wastewaters having low COD concentrations around 1000 mg/l could be also efficient especially with high-rate reactors such as UASB reactor and fluidized bed reactors (Austermann-Haun and Rosenwinkel, 1997). Many industrial wastewaters such as alcoholic and soft drink bottling industries, paper recycling and paper making mills, fruit and vegetable canneries etc. and domestic wastewaters were reported to have COD values below 1000 mg/l (Banik and Dague, 1997). Owing to the presence of large amounts of

biocatalyst (anaerobic sludge), the COD removal capacity of anaerobic high-rate reactors, in principle, is sufficient, despite the eventually low operational temperature of the treatment plant (**van Lier et al., 1997a**). For full-scale treatment of low-strength wastewaters, the UASB reactor was widely used (**Angenent et al., 2001**). Achievement of the higher biological conversion rates is the main key factor which is particularly the case for low-strength wastewaters, especially, at lower temperatures (**Rebac et al., 1999**).

According to Monod kinetics, the specific sludge activity depends on the substrate concentration. The affinity of the bacteria for the substrate is indicated by the saturation constant (K_s). Turbulence was achieved by the high biogas production per unit of influent during the treatment of high strength wastewaters. In a UASB reactor, the mixing intensity should not too excessive in order to maintain a high retention of biomass regardless of wastewater strength (**Kato et al., 1997**).

Low concentrations of substrates were present in the reactor under dilute wastewater operation, which will produce a low bacterial activity. In a traditional UASB reactor this might have resulted in channeling of the wastewater through the bed and therefore in a poor water-sludge contact (**Jeison and Chamy, 1999**).

Low substrate concentration occurring inside the reactor, the possible presence of DO, and lower temperatures were the main problems related with low strength wastewaters. Low substrate levels inside anaerobic bioreactors occur due to low influent COD concentrations which may result in lower effective concentrations inside the granules than in the bulk liquid when the consumption of substrate by bacteria is faster than the transport of substrate into biofilms (**Kato et al., 1997**). Since low COD concentration of the influent results in very low substrate levels, low biogas productions will occur inside the reactor as well. Thus, HRT should determine the reactor volume of the reactor when treating low strength wastewater because of the fact that severe hydraulic wash out of the sludge might occur at the maximum possible OLR with respect to the maximum COD conversion capacity of the sludge (**Rebac, 1998**). The possible presence of DO is the other serious problem in anaerobic systems treating dilute wastewaters. Oxygen is a toxic compound

especially for the methanogens which are usually considered as strict anaerobes (Kato et al., 1997).

Anaerobic wastewater treatment represents a very suitable method for low strength and soluble wastewaters. However, also for the treatment of a partially soluble low strength wastewater like domestic sewage the feasibility of the system has been proven (Lettinga, 1996).

Domestic sewage composition changes significantly in time and from one place to another. It is apparent that when flow, concentration and composition fluctuate greatly, anaerobic treatment efficiency will decrease considerably (Elmitwalli, 2000). The concentration of organic matter can vary by a factor of 2-10 in a few hours and the flow rate can fluctuate by a factor of 4 which depends mainly on the size of population (the larger the population, the smaller the fluctuation) and the type of sewer. Due to rain and run-off water, combined sewers have much higher fluctuations (Lens et al., 2001). Suspended Solids (SS) represent the major COD fraction in domestic wastewater and thus, high removal of total COD (Tot-COD) will be achieved by high removal of SS which occurs by settling and by filtration through the sludge bed and/or filter media. Entrapment, sorption, and straining are three mechanisms of filtration and influent concentration, temperature, reactor height, HRT, upflow velocity and particle size and density are the main factors affecting the physical removal of SS in anaerobic high rate systems. SS and colloidal particles represent around 70%-80% and 30%-20% of particulate matter respectively in domestic sewage (Elmitwalli, 2000).

The high fraction of particulate COD, moderate biodegradability of various COD fractions, low strength character with various concentrations and its relatively low temperature make sewage to be categorized as a 'complex' wastewater (Lettinga et al., 2001). Presence of fatty compounds, proteins, and detergents, among other barely known compounds, make domestic sewage quite complex and impose limitations to anaerobic process with respect to COD removal efficiency, and also in terms of the maximum OLRs and HLR's to be applied. Generally, the requirement of post-treatment in many situations, the special attention in designing for avoiding hydraulic overloading and odour problem are to be imposed (Foresti, 2001).

Since imbalance in the production and consumption of VFA is the main intrinsic factor affecting process stability in the treatment of high-strength wastewaters, process collapse in anaerobic reactors treating domestic sewage has also to be taken into consideration. It was reported that VFA production is not significant in low-strength wastewater treatment, and VFA concentrations were always below the inhibitory limits permitting the methanogenic process to be progressively established (Foresti, 2001).

The most appropriate anaerobic system to treat domestic wastewater has been considered as the UASB reactor because of its simplicity, low investment and operation costs and the long favorable experience in the treatment of a wide range of industrial wastewaters (Elmitwalli, 2000). First application of UASB reactors for domestic sewage treatment gave contradictory results. The performance was not as high as expected and bad odours produced in some plants made its application undesired (Foresti, 2001). Although it was reported that more than 70% COD_{tot} removal could be obtained in raw domestic sewage treatment, more energy is needed for mixing due to high liquid viscosity at low temperature. Besides, sludge bed reactors are less easily mixed, particularly at low biogas production rates. Also particles will settle slower because of a decreased liquid-solids separation at low temperatures (Rebac, 1998).

Particulate organics are physically removed due to settling, adsorption and entrapment which is the first step in the anaerobic treatment and conversion of domestic sewage. The rate-limiting step in the overall digestion process is the hydrolysis of retained particulates which need relatively long retention times, depending on the applied process temperature (Lettinga et al., 2001). Particularly, UASB reactors treating wastewaters with a high SS concentration, such as alcohol distillery wastewater (very concentrated) and domestic sewage (very dilute) are generally operated with a flocculent type of biomass and the actual SRT is the most crucial parameter in the entire wastewater treatment system. Sludge bed systems might be equipped with a filter section (hybrid reactor) or a lamella separator for enhancing SS removal (van Lier et al., 2001).

There are factors that might affect the anaerobic treatment of domestic sewage such as; flow and strength variation, sulphate (SO_4^{2-}), suspended solids and temperature. Temperature effects might cause slow growth of bacteria, low methanogenic activity, slow hydrolysis, increased gas solubility and inhibition by high acetate concentration in the anaerobic treatment of low strength wastewater. However, the relatively low levels of acetate in domestic sewage make it unlikely that inhibition by acetate will occur (**Lens et al., 2001**). Discharge standards can be rarely complied by the effluents from anaerobic reactors treating domestic sewage. Nutrients and pathogens are the main important constituents or components, which deserve attention as well as the remaining fraction of particulate and soluble organic matter (**Foresti, 2001**). The results reported in the literature for conventional UASB reactors treating domestic sewage usually indicate COD removal efficiencies below 70% and SS in the final effluent around 60-100 mg/l (**Chernicharo and Cardoso, 1999**).

2.4 Anaerobic Treatment at Different Temperatures

An anaerobic digester is a microbial community in which the same four major metabolic groups of microorganisms require to interact for the efficient conversion of organic matter into methane. A microbial community structure having unique characteristics is required for an effective conversion of organic matter into methane which may depend on operating conditions (e.g., substrate, temperature, retention time, and other operating parameters) (**Raskin et al., 1994**). Operating temperature is one of the most important parameters for anaerobic treatment that selects the dominant bacterial flora and determines growth rate (**Patel and Madamwar, 2002**). Anaerobic digestion is considered to be possible in three temperature ranges termed as psychrophilic, mesophilic and thermophilic (**Lens et al., 2001**). Anaerobic processes are commonly known to be sensitive to sudden environmental changes (**Lau and Fang, 1997**). The particulate COD in the influent is virtually reduced by means of the entrapment effect of the sludge bed (**Uemura and Harada, 2000**). Numbers and activities of most bacteria are strongly dependent on the temperature and just after the temperature changes, the activities of bacteria are significantly suppressed resulting in VFAs accumulation until the bacteria recovery from temperature shock (**Cha et al., 1997**). The anaerobic bacteria are generally divided

into three thermal groups; psychrophiles, mesophiles, and thermophiles with optimum temperatures at $<20^{\circ}\text{C}$, $25\text{--}40^{\circ}\text{C}$, and $>45^{\circ}\text{C}$, respectively (**van Lier et al., 1997a**). The bacterial growth rates of methanogenic thermophiles and mesophiles are quite well determined (**Rebac, 1998**). The temperature, exposure time and bacterial composition of sludge are the main important factors in anaerobic systems. Unstable performance and in some extreme cases complete failure of the system could result when exposed to temperature shock in anaerobic reactors operated under mesophilic conditions. Thus, temperature should be carefully controlled for anaerobic treatment of wastewaters (**Lau and Fang, 1997**). Thermophilic anaerobic digestion has many disadvantages such as the reduced process stability, reduced dewatering properties of the fermented sludge, and the requirement for large energy amounts for heating. However, one of its biggest advantages is considered to be the thermal destruction of pathogenic bacteria at elevated temperatures (**Gallert and Winter, 1997**). Since temperature is reported to significantly affect the anaerobic conversion process, changes to the conventional design are likely needed if high-rate reactor systems are going to be applied at 'sub-optimal' temperatures, i.e. at temperatures higher than 45°C and/or lower than 20°C (**van Lier et al., 1997a**). Full-scale applications of anaerobic treatment are restricted to wastewaters with a temperature exceeding 18°C . Temperatures of many wastewaters are lower than the mesophilic range ($<25^{\circ}\text{C}$) and normally they have to be heated for efficient treatment (**Banik and Dague, 1997**). **Duran (1996)** showed that, the conventional method of using mesophilic (30°C to 40°C) bacteria produces a better quality effluent than the thermophilic (50°C to 55°C) bacteria. However, if substrate utilization rates are considered, not only do thermophilic bacteria indicate higher rates but also they are capable of treating higher organic loading rates. Although the same metabolism is exhibited by the existing bacteria in thermophilic and mesophilic conditions, it was revealed that the former have a shorter doubling time as compared to the latter. Thus, longer start-up periods and possible system failure is expected when important parameters such as OLR, HRT, pH and temperature are unstable (**Matainaho, 2000**).

At extreme temperatures, syntrophic acetate oxidation is probably the preferential pathway for acetate conversion in which acetate oxidizers and hydrogen utilizing methanogens are playing important roles. On the other hand, at lower temperatures,

the acetoclastic reaction is the favored mechanism. Although acetate is readily methanized at extreme temperatures (80°C), the limiting step is the propionate degradation. Thus, low effluent quality is observed at such high temperatures (Lepistö and Rintala, 1997).

The effects of thermophilic conditions explained above brought the importance of low temperature effects into consideration in anaerobic processes. However, many recent researches conducted at all temperature conditions revealed that temperature is not a limiting factor in anaerobic treatment applications if the appropriate process design is chosen (van Lier et al., 1997a). Anaerobic wastewater treatment has been also reported in recent studies as a feasible system for treating cold wastewaters at loading rates higher than 10 kgCOD/m³.d at temperatures down to 10°C and HRT less than 1.5 h (Lettinga, 1996). Biological reaction rates are expected to drop by a half for a 10°C drop based on the Arrhenius relationship (Nachaiyasit and Stuckey, 1997). Generally, lowering the operational temperature causes the maximum specific growth and substrate utilization rates decrease as well as an increase in net biomass yield (g biomass/g substrate converted) of methanogenic population or acidogenic sludge. Hydrolysis of proteins and lipids decreases significantly with decreasing temperature, especially when approaching 15°C (Lettinga et al., 2001). At lower temperatures, slow-growing microorganisms are more affected than the faster growing microorganisms (Nachaiyasit and Stuckey, 1997).

It was reported that, anaerobic process was no longer limited to temperatures in the mesophilic range. Anaerobic digestion was not a fragile process and could withstand temperature and organic loading shocks (Mergaert et al., 1992). It is reported that if the organisms are acclimatized to desired temperatures, the reactors can be operated even under psychrophilic and thermophilic conditions (Patel and Madamwar, 2002).

2.4.1 Anaerobic treatment of low strength wastewaters at low temperatures

Psychrophilic anaerobic treatment is an attractive option for wastewaters which are discharged at moderate to low temperatures (Lettinga et al., 2001). The optimal temperature for psychrophilic microorganisms is around 17°C. Most of the reactions

yield less energy at low temperatures except for a few reactions (hydrogenotrophic sulphate reduction, hydrogenotrophic methane production and acetate formation from hydrogen and bicarbonate) (Lens et al., 2001). Temperature affects the performance of all biological treatment processes (Banik and Dague, 1997) and low temperatures in anaerobic treatment have always been associated with low reactor performance (Kato et al., 1997). High-rate anaerobic systems are generally applied in the temperature range between 25–40°C. When they are operated in lower temperature ranges such as 5–20°C, various adaptations of the conventional high-rate reactor design are needed. The methanogenic biomass and the wastewater should be in sufficient contact, a condition which can be achieved by increased liquid upflow velocities (van Lier et al., 1997b). If conventional UASB type high-rate reactors are applied for low strength wastewaters, then process failure may result from channeling of the wastewater through the sludge bed. Although the channeling may be prevented by increasing the superficial liquid upward velocities, an expansion of the sludge bed may occur. The wastewater characteristics and the settling properties of the sludge granules are the main factors on which the needed superficial liquid upward velocity and the degree of expansion are dependent (van Lier et al., 1997a). It was also reported that the specific biogas production rate is relatively low under psychrophilic conditions (van Lier et al., 1997b). Gas production increased with a decrease in HRT and decreased with a decrease in temperatures. As the temperature decreases, the methane percentage in the total biogas increases (Singh and Viraraghavan, 2001). Lower rates of substrate removal and biomass growth occur at lower temperatures. Besides, the possibility of increasing the biomass concentration in the reactor, offsetting the reduced specific substrate removal rates is offered because of the fact that, endogenous decay rates are also lower at reduced temperatures (Banik and Dague, 1997). The possibility of increasing the biomass concentration in the reactor is also reported in another study indicating that lowering the operational temperature may lead to an increased biomass yield (g biomass/d substrate converted) of methanogenic population (Rebac, 1998).

VFAs accumulation in the reactor might be the first indicator that shows that the system is not operating at optimal conditions. High VFAs concentrations in the effluent result in an immediate decrease in reactor pH as well as elevated effluent SS.

This may be attributed to the fact that methanogens are more sensitive to temperature reduction than acetogens (Nachaiyasit and Stuckey, 1997; Langenhoff and Stuckey, 2000).

In accordance with Monod kinetics, lower substrate concentrations in the reactor result in lower removal rates. Overall, a deterioration of process performance occurs due to lower substrate concentrations as well as reduced temperatures as compared to the case of high substrate concentration and higher temperatures (Banik and Dague, 1997). A non-desirable accumulation of non-or partially degradable organic matter under psychrophilic conditions are the other impact of low temperature (van Lier et al., 1997a). The accumulation of SS occurs because of the slow hydrolysis resulting in deterioration of the methanogenic activity of the sludge and this may be prevented by allowing sewage settle prior to the anaerobic treatment (Elmitwalli, 2000).

Lipids and/or proteins are adsorbed in or/and on the aggregates may cause sludge flotation particularly in treating very low strength wastewaters under high hydraulic loading rates ($HRT < 1-2$ h). This necessitates a complete retention of the viable sludge as well as the erosion of granular sludge should remain at a very low level (Lettinga, 1996).

Characteristics of wastewaters, the biodegradability of pollutants, and the settleability of particulate matter are, of course, very important to achieve successful treatment apart from temperature and the loading conditions of the system (Banik and Dague, 1997). A considerable amount of CH_4 , CO_2 , H_2 and H_2S will remain in the effluent of reactors operating at low temperatures because the solubility of gaseous compounds present in biogas increases with a decrease in temperature (Lens et al., 2001). Slow growth rate of methanogens results in low bicarbonate alkalinity under psychrophilic conditions. Besides, an imbalance between the ratio of methanogenic organisms and OLR occurs (Patel and Madamwar, 2002).

On the other hand, low-strength wastewaters such as domestic and food-processing wastewater are often discharged at ambient temperatures (15 to 20°C) and heating the wastewater to maintain mesophilic conditions (35°C) for anaerobic treatment necessitates high energy (Angenent et al., 2001).

The activity of two important methanogenic genera (*Methanosaeta* and *Methanosarcina*) mainly responsible for methane production from acetate is seriously affected by temperature. The 35-40°C range is the optimal temperature for both *Archaea*. The *Methanothrix* (now renamed as *Methanosaeta*) *soengii* can only be seen if the temperature is at a minimum of 10-15°C. It was reported that the activity at 10-15°C will be 10 to 20 times lower than at 35°C (Lens et al., 2001).

A decrease in removal efficiency; an increase in effluent VFAs; elevated effluent SS; a decrease in pH; a retardation in gas production; and, a reduction in methane content owing to the lack of conversion of VFAs to methane are the main drawbacks of reduction in operating temperature (Nachaiyasit and Stuckey, 1997). On the other hand, despite of the draw backs above, successful anaerobic treatment of many dilute streams, which are also low in temperature, without the addition of heat would be a significant achievement in reducing the cost and energy needs for wastewater treatment (Banik and Dague, 1997).

Applications of anaerobic processes are now being extended to the treatment of dilute industrial wastewaters, and even anaerobic sewage treatment is thought to be achievable under certain conditions (Raskin et al., 1994). Although some industrial wastewaters are warm (>35°C), others, including domestic sewage, are quite cool (5-15°C) (Nachaiyasit and Stuckey, 1997). Since domestic sewage has a temperature lower than 35°C, heating is required during the mesophilic anaerobic treatment. Thus, anaerobic treatment systems allow substantially lower treatment costs due to their ability to operate at low temperatures (10-20°C) (Langenhoff and Stuckey, 2000). The 'complex' character of sewage makes anaerobic sewage treatment a real challenge in moderate climates. Several studies reported that the anaerobic treatment of domestic sewage might also present an attractive and feasible option for moderate climates. However, more sophisticated reactors are required at the lower temperatures that prevail in the winter (Lettinga et al., 2001). Direct anaerobic treatment of domestic sewage is generally not applied because of the fact that the high SS concentration in sewage causes considerable difficulties. Under low temperature conditions, the SS are hydrolysed very slowly and tend to accumulate in the reactor. Thus, the reactor volume available for the active biomass sludge

decreases and a low COD conversion may be the result. Adequate activation of the anaerobic sludge can be succeeded at a COD_{sol}/VSS ratio above 10, which lies around 1 in domestic sewage. It was indicated by mathematical modelling that the reactor volume tended to fill up with inactive SS rather than with active microbial biocatalyst (Lens et al., 2001). It is reported that in order to guarantee an efficient treatment of domestic sewage under low temperature conditions, at least part of the SS present in the wastewater should be removed before feeding the wastewater to a sludge bed reactor. At low temperatures, the increase of CO_2 dissolution in water might cause a decrease in the pH of the reactor because the solubility of gaseous compounds present in biogas increases with decrease in temperature; yet sewage generally has adequate buffer capacity (Lens et al., 2001).

2.5 Previous Studies on the Related Subject

Langenhoff and Stuckey (2000) reported that removal efficiencies ranging from 0 to 90% could be observed in UASB reactors at temperatures as low as 5°C.

A decrease in temperature from 35°C to 25°C did not result in any significant drop in COD removal. However, the growth rate of the methanogens and syntrophs was severely affected and VFAs in the effluent increased when the temperature was decreased to 15°C (Nachaiyasit and Stuckey, 1997).

Cha et al. (1997) reported that when the temperature was dropped from 30°C to 25°C, 20°C, 15°C sequentially, the numbers of bacteria were slowly reduced without showing temperature shock. The acidogenesis, on the other hand, was severely affected after each temperature drop. Besides, abrupt changes in gas production rates were observed due to shock temperature decrease.

Kettunen and Rintala (1998) studied the anaerobic treatment of municipal landfill leachate in a pilot-scale UASB reactor at low temperatures (13-23°C). Since leachate temperature decreased in winter, the reactor operated successfully by decreasing the process temperature. 50-55% COD and up to 72% BOD_7 removals were achieved with OLRs of 1.4-2.0 kg COD/m³.d at 13-14°C.

At 11°C and at an HRT of 6 h, 70 to 90% COD removal was achieved in UASB reactor. When the operating temperature decreased to 6°C, the reactor performance was not very adequate with an average COD removal of 40%. They reported that UASB system could be successfully applied for the pre-treatment/treatment of municipal wastewater under low-temperature conditions (Singh and Viraraghavan, 2001).

COD removals ranging from 65 to 80% have been presented at ambient temperatures and at an HRT= 6 to 10 h and OLRs lower than 3.0 kgCOD/m³.d in UASB reactor. Results on the use of UASB reactor in the treatment of pre-settled sewage were also presented. COD removals higher than 50% with a mean effluent COD concentration of 72 mg/l were obtained having the mean monthly sewage temperature ranging from 16°C (winter) to 25°C (summer) (Foresti, 2001).

Elmitwalli et al. (2001) reported that suspended and dissolved COD optimization could be obtained at an HRT of 4+4 h, while an HRT of 4+8 h is required to achieve 60% COD_{col} removal at 13°C in a two-step anaerobic filter (AF)+anaerobic hybrid (AH) system.

COD reduction could be achieved as 45% in a temperature range of 8-10°C with an HRT of 8 h in a pilot-scale UASB reactor treating municipal wastewater (Mergaert et al., 1992).

Uemura and Harada (2000) investigated the feasibility of the UASB process for the treatment of sewage at a fixed HRT of 4.7 h and at temperatures ranging from 25 to 13°C and they could achieve a total COD removal of 69.4% based on total influent and soluble effluent, on average, throughout the entire experiment.

Davey and O'Toole (2000) used FISH technique to localize organisms belonging to the bacterial domain and various types of methanogens in sludge granules and showed that the outer layers of the granules were populated with a variety of bacterial colonies most likely involved in hydrolyzing complex organic matter, while the interior of the granule consisted of methanogenic microcolonies. In addition, the syntrophic strains that need low hydrogen partial pressures in order to oxidize propionate were found to be tightly associated with the methanogens in

microcolonies. A layered microbial architecture in sludge granules where the bacteria on the surface of the granule hydrolyze complex organic materials, thereby providing the anaerobic bacteria in the interior of the biofilm with an energy source is proved by this study. Besides, CSLM was used to identify the specific starting strains and transconjugants in the community by using 16S rRNA hybridization probes and expression of green fluorescence.

Wu et al. (2001) have used a full-scale UASB reactor treating terephthalate-acid wastewater and they have applied FISH technique to identify syntrophic propionate-oxidizing bacteria and to localize methanogens in anaerobic granular sludge systems. The distribution of archaeal and bacterial populations in the granular consortium was determined using *in situ* hybridization with specific probes of *Archaea* (ARC915) and *Bacteria* (EUB338), respectively. Quantitative FISH analysis indicated that the acetoclastic *Methanosaeta* (MX825 probe) was more abundant than the hydrogenotrophic methanogens which correlated with the cloning result that *Methanosaeta*-related clones were the most predominant archaeal population (more than 70% of total *Archaea* clones). They also investigated microbial community by electron microscopy and found out that the bamboo-shaped cells with electron-opaque bodies and a sheath-like structure strongly resembled acetoclastic *Methanosaeta*-like organisms and formed dense clusters throughout the granule.

Use of molecular methods for describing microbial populations indicated that a diverse group of *Bacteria* and *Archaea* was involved in a study about an anaerobic baffled reactor treating an industrial dye effluent. A combination of FISH probing and analysis of 98 archaeal 16S rDNA clone revealed that a methanogenic population dominated by *Methanosaeta* species and consisting of species of *Methanobacterium* and *Methanospirillum* and relatively unstudied methanogen, *Methanomethylovorans hollandic*, contributed to successful anaerobic treatment of the industrial waste (Plumb et al., 2001).

Jianrong et al. (1997) reported that *Methanosaeta* appeared in a type of single cell other than long filaments that was considered to improve the specific surface as well as to favor the contact and metabolism of substrate. *Methanococcus* spp. was also seen in section of granule. They also observed that *Methanosaeta* were the

predominant acetoclastic methanogen which existed in bundles of filaments with two forms of active and empty cells.

Akarsubasi et al. (2005) also reported the predominance of *Methanosaeta concillii*-like sequences by archaeal DGGE profiles in the seed sludge obtained from an UASB reactor of an alcohol distillery treatment plant.

Fang et al. (1994) reported that both UASB granules treating sucrose and brewery wastewaters indicated a layered structure. The layered structure seems to be limited to granules treating soluble carbohydrates such as sucrose, glucose and brewery wastewaters. On the other hand, no layered structure was observed for granules degrading non-carbohydrate substrates such as glutamate.

Araujo et al. (2000) used the FISH technique combined with CSLM for monitoring the development of anaerobic biofilms. They observed that *Methanobacterium formicicum*, *Methanosaeta concilli* and *Methanosarcina barkeri* could colonize on different types of surfaces and determined a *Methanobacterium*-like cell as the predominant *Methanoarchaea*.

3. EXPERIMENTAL WORK

3.1 Aim of the Experimental Work

The possibility of anaerobic treatment of low-strength wastewaters under psychrophilic conditions was discussed and evaluated by simultaneous comparison of the differences between the efficiencies of the two UASB reactors operated at mesophilic ($35\pm 2^{\circ}\text{C}$) and at psychrophilic ($\leq 17^{\circ}\text{C}$) temperatures. Besides, not only did this thesis help to assess the anaerobic treatment of low strength wastewaters at low temperatures, but it also helped to evaluate the possibility of achieving a more feasible system without heating requirement.

In the first part of the study, a synthetic wastewater characterizing a domestic wastewater was fed into both UASB reactors in order to determine the optimum OLR and the optimum operating temperature. The mesophilic UASB reactor was operated at $35\pm 2^{\circ}\text{C}$ whereas the second UASB reactor was operated at sub-mesophilic conditions at the beginning. The temperature in the sub-mesophilic reactor was reduced step by step to quite low values ($10\pm 1^{\circ}\text{C}$) for maintaining psychrophilic conditions at the end of the first part of the study. In the second part, both reactors were fed with raw domestic wastewater (sewage) and the psychrophilic reactor was operated at $13\pm 2^{\circ}\text{C}$ at an OLR of around $0.7 \text{ kgCOD/m}^3\cdot\text{day}$.

Both reactors were daily monitored in order to evaluate their performances. Temperature, pH, alkalinity, and total biogas production were daily measured whereas COD analyses were done 3 times per week. Total solids and total volatile solids of the granular sludges were measured when the organic loadings changed inside the reactors. The methane content of the total biogas was also measured at each organic loading rate. Nutrient concentrations were also determined twice in a month.

Besides, FISH analyses were also done at several operation times for both synthetic and raw domestic sewage for investigating the temperature effects on microbial diversity. In this study, the FISH technique was used in combination with CSLM to visualize the locations of several microorganisms of particular interest in both mesophilic and psychrophilic granular sludges. Oligonucleotide probes specific to bacteria and several phylogenetic groups of methanogens were used to characterize and compare the overall microbial communities in both types of granules. The samples for FISH experiments were taken from both UASB reactors at several operation times during synthetic and raw sewage feeding.

3.2 Materials and Methods

3.2.1 Mesophilic and psychrophilic UASB reactors

Two identical lab-scale anaerobic continuous-flow UASB reactors, each with an effective volume of 6.45 L, were set up for anaerobic treatability tests. The reactors consisted of plexiglass columns of 1.0 m in height and 90 mm in diameter, on the top of which special gas-solids-liquid separators were installed. The mesophilic UASB reactor was operated at $35^{\circ}\text{C} \pm 2.0^{\circ}\text{C}$ (Figures 3.1.a and 3.2a) during the study. On the other hand, the psychrophilic reactor was operated at room temperature at sub-mesophilic conditions during the start-up and the temperature inside the reactor was then reduced to around 10°C at the end of the first part of the study for the estimation of process performance of a UASB reactor when treating sewage under relatively moderate to low temperature conditions. The temperature in the sub-mesophilic reactor varied between $21.5\text{--}26^{\circ}\text{C}$ during summer, however the temperature was reduced to around $17\text{--}19^{\circ}\text{C}$ during autumn. The experiments were carried on at different temperatures in later studies, reducing from sub-mesophilic to psychrophilic region in small decrements by the hose-pipe in which cooled tap water traveled and the psychrophilic conditions could be maintained (Figures 3.1b and 3.2b). The reactors were inoculated (20%) by the granular sludge taken from Pasabahce Tekel Raki Industry in Istanbul having a VSS/SS ratio of 91%. In both reactors, around 21.16 g VSS was added per liter of reactor as a seed. A crucial step for a stable operation of anaerobic reactors at the designed OLR is the start-up period. High quality methanogenic sludges are required as inoculums (Foresti, 2001). It was

reported that the start-up of anaerobic sludge bed systems was quick, within a few days with granular seed sludges (Lettinga, 1996). Mesophilic sludge is used for the start-up of the reactors, after which time the temperature was slowly decreased (Langenhoff and Stuckey, 2000). The characterization of the seed used in the study is given in Table 3.1.

Table 3.1: The characterization of the seed used in the study

Parameter	Unit (g/l)
Total Solids	128
Total Volatile Solids	112
Total Suspended Solids	115
Total Volatile Suspended Solids	105

3.2.2 Wastewater source

3.2.2.1 Synthetic domestic wastewater

The composition of raw domestic sewage varies considerably in time and from one place to another. Since big fluctuations in flow, concentration and composition of domestic sewage may make the comparison between the performances of the mesophilic and psychrophilic UASB reactors difficult, a synthetic wastewater was prepared in this study which characterized domestic sewage. This wastewater was fed into both reactors in the first part of the study. The synthetic sewage was prepared according to International Organization for Standardization (ISO). The COD_{tot} concentration of the synthetic sewage was measured as 300 mg/l, which represented a very low strength sewage. Thus, the amounts given in Table 3.2 were doubled in the preparation of the synthetic sewage in order to represent a medium strength domestic sewage.

Table 3.2: The composition of synthetic domestic sewage (pH=7.5±0.5) (URL 3.1)

Components	Amount (mg/l)
Peptone	160
Meat Extract	110
Urea	30
NaCl	7
CaCl ₂ .7H ₂ O	4
MgSO ₄ .7H ₂ O	2
K ₂ HPO ₄	28

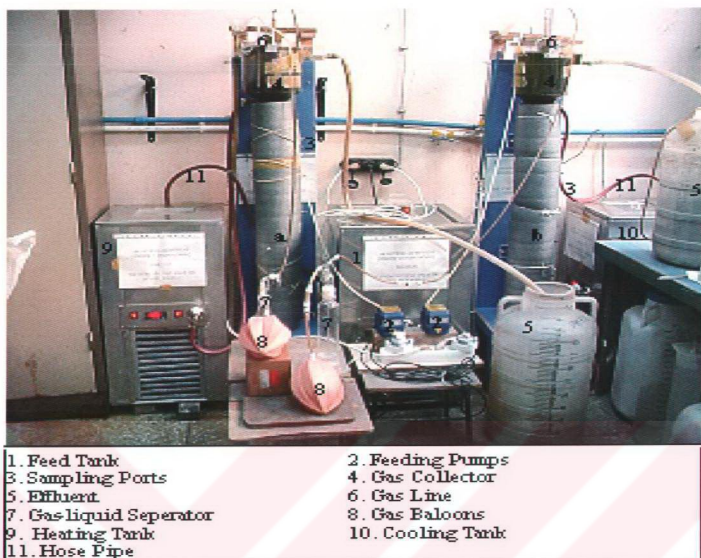


Figure 3.1: (a) Mesophilic UASB reactor (b) Psychrophilic UASB reactor

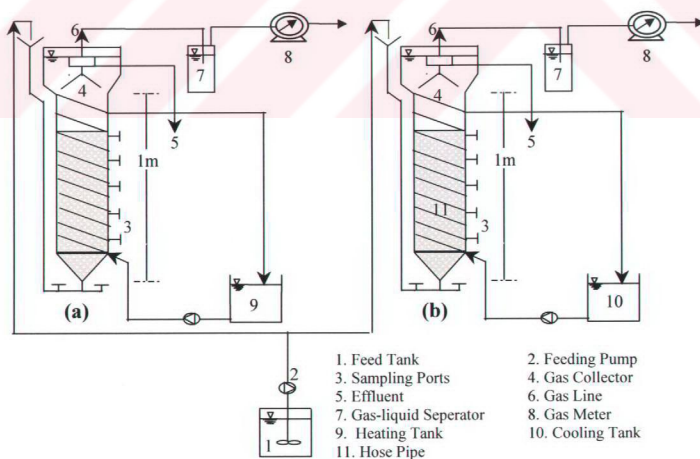


Figure 3.2: Schematic views of UASB reactors (a) Mesophilic (b) Psychrophilic

3.2.2.2 Raw domestic wastewater

Raw domestic sewage was taken from the outlet of the grit chamber of the Baltalimanı Preliminary Treatment Plant located in Istanbul. In the Goldenhorn Project, the wastewater was collected at Baltalimanı by open trenches and tunnel collectors with diameters of 2200-3600 mm. After pretreatment, the wastewater was discharged with 2200 mm diameter pipes to Bosphorus under 70 m depth, from the sea surface. The construction of the plant was completed in 1996. Baltalimanı pre-treatment plant is located on an area of 20,000 m² for the purpose of future extensions. The plant is planned to treat the domestic wastewater in flow rate capacities of 400,000 m³/day (equivalent to one million residents) in the first stage and 760,000 m³/day (equivalent to two million residents) in the second stage. There is a coarse screen building, pumping station, a pretreatment plant, an administrative and a workshop building, electricity supply system and a transformer building in the plant. The coarse screen building is in a cylindrical shape with 16 m diameter and 30 m depth and includes 4 screens and 2 penstocks. The bar openings are 100 mm. The wastewater from the coarse screen is elevated to the level of 20.35 m from -11.34 m by 4 main and 1 standby pumps, with the capacities 3.62 m³/s each, and transferred to pretreatment by the gravity. The pumping station is designed to pump the wastewater with a peak capacity of 1,250,000 m³/day. The wastewater from 2 separate pipes of 2x1400 mm in diameter from the open discharge channel comes to the screen having 10 mm bar openings. After that the wastewater passes through several treatment processes such as grit chamber, oil skimmers and scan removal and is transferred to the sea outfall with 2200 mm diameter pipes following the flow measurement channel. This plant has been planned to serve for future expansion. The biological treatment plant project is in consideration and will be integrated from this point onwards. Baltalimanı pre-treatment plant can be considered as a major step in plant chains to solve environmental problems of Istanbul city (İSKİ, 1995). It was reported that one of the most convenient methods for leachate control is to treat landfill leachates with domestic wastewaters and mixing 2% of landfill leachates by volume with domestic wastewaters does not lead to important operational problems in municipal treatment plants (Yangin et al., 2002). Very small percentages of leachate are added into sewerage and pretreated in the Baltalimanı pretreatment

plant. The characterization of the pre-settled and mixed raw sewage taken at different sampling dates is presented in Table 3.3.

Table 3.3: The characterization of raw domestic sewage

Parameter	Unit	Value
Pre-settled (Sampling Date: June 2003)		
COD _{tot}	mg/l	260
COD _{sol}	mg/l	140
COD _{sol} /COD	%	56
TKN	mg/l	43
NH ₃ -N	mg/l	32
TP	mg/l	9.5
PO ₄ ²⁻	mg/l	6.8
pH	-	7.4
Alkalinity	mg CaCO ₃ /l	355
Conductivity	mmho/cm	5000
Salinity	ppt	3.9
Mixed (Sampling Date: July 2003)		
COD _{tot}	mg/l	220
COD _{sol}	mg/l	85
COD _{sol} /COD	%	42
TKN	mg/l	35
NH ₃ -N	mg/l	27
TP	mg/l	-
PO ₄ ²⁻	mg/l	-
pH	-	7.6
Alkalinity	mg CaCO ₃ /l	300
Conductivity	mmho/cm	6140
Salinity	ppt	5.0

The raw sewage used in this study presented a weak characterization. This was due to the fact that sea water was added into the sewerage system during the summer for preventing putrefaction and cleaning the pipes. This could be seen from high conductivity and salinity values (Table 3.3). Lower concentrations (except pH, conductivity and salinity) in the mixed sewage compared to the values in the pre-settled sewage might have been due to different sampling dates. It was obvious that, as weather temperature increased more sea water was added to the sewerage system which caused more dilution and thus lower influent concentrations.

3.2.3 Microbiological experiments

3.2.3.1 Confocal scanning laser microscopy, LSM 510 META

Every LSM 510 META (Figure 3.3) is based on one of the high-performance research microscopes of Zeiss. Depending on the specific applications, one of the Axioplan 2 imaging MOT, Axiovert 200 M and Axioskop 2 FS MOT can be chosen. All of them are equipped with ICS optics, which are unsurpassed for image quality, flexibility and optical perfection. The motorized microscope models are interchangeable and fully supported by the LSM software. The software automatically identifies the microscope settings and the objectives used, and controls all movements and measurements carried out by the system with high precision. In the way it implements the confocal principle, the design of the LSM 510 META system is unmatched. It allows multi fluorescence images to be collected without compromising resolution and efficiency. The unique scanning module is the core of the LSM 510 META. It contains motorized collimators, scanning mirrors, individually adjustable and positionable pinholes, and highly sensitive detectors including the META detector. All these components are arranged to ensure optimum specimen illumination and efficient collection of reflected or emitted light. A highly efficient optical grating provides an innovative way of separating the fluorescence emissions in the META detector ([URL 3.2](#)).

The LSM 510 is controlled via a standard high-end Pentium PC. Linking to the electronic control system is made via an ultrafast SCS interface. The PC comes with the 32-bit WINDOWS NT 4.0 or WINDOWS operating system. The software has two levels. On the simple operator interface level, a result will be achieved after a few prompts; graphical prompting of the user in conjunction with automatic setting of many parameters is an ideal tool for daily routine jobs. The expert level offers perfect facilities for individual settings of functions and parameters.

Conversion of light signals into a digital image is affected by means of four 12-bit A/D converters, each of that can generate 4096 brightness levels. The software provides an enormously wide range of image processing functions, including all standard 2D/3D (stereo, projection) functions identical to sophisticated 3D reconstruction capabilities (surface and alpha rendering), digital processing of voxels

and 3D measurement functions (surface areas, volumes). As all files and images are recorded in MS Access databases, elegant image database editing is just as easy as transferring the records to other programs. These information is taken from the Operating Manual Rel. 3.0 of LSM 510 and LSM 510 META Laser Scanning Microscopes.

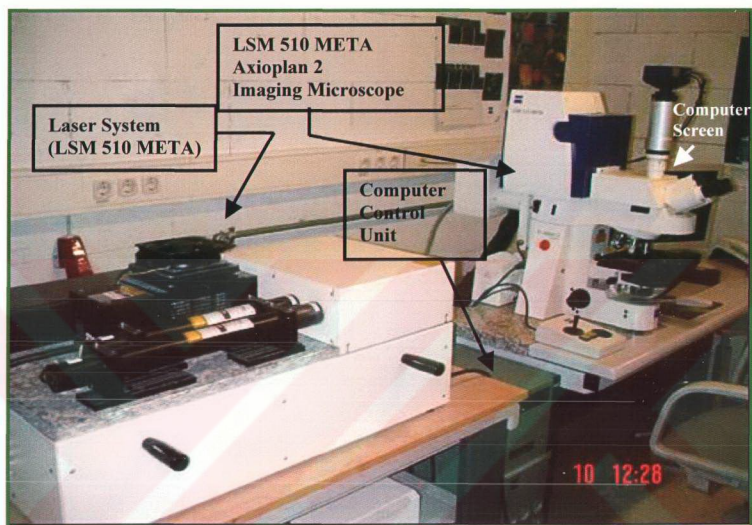


Figure 3.3: LSM 510 META Laser Scanning Microscope

3.2.3.2 FISH with homogenized granules

FISH was carried out in three steps; fixation, hybridization and identification. For fixation, the sludge granules were fixed in pure ethanol solution containing NaCl and stored at -20°C. The sludge granules were homogenized for about 5 minutes by RW 20 DZM, Janke and Kunkel type homogenization apparatus. For homogenization 3 granules were homogenized in 5 ml of double distilled water on ice. The homogenized granules were transferred to Eppendorf tubes and resuspended at a vortex mixer each time before transferring to the slides. The immobilization of the homogenized fixed sample suspensions was done on slides. Gelatin-coated (0.1% gelatin and 0.01% $\text{KCr}(\text{SO}_4)_2$) multiwell glass slides were used for the fixed cells to be spotted. Each slide consisted of 10 wells and on each well 5-10 μl of sample was

put. The slides were then allowed to dry at room temperature. The samples were then dehydrated by successive passages through 50, 80 and 99% ethanol washes for 3 minutes for each.

The oligonucleotide probes and their target groups which are relevant for this study are listed in Table 3.4 (Raskin et al., 1994). The best-suited concentration of formamide in the hybridization buffer was taken from probeBase.net to acquire the best hybridization intensity (URL 3.3). Cy-3 and Cy-5 cyanine dyes were used for labeling all the oligonucleotide probes used in this study according to target microorganisms. The chemical components used in the hybridization and washing buffers and their required amounts are presented in Table 3.5 according to the optimum formamide percentages for each oligonucleotide probes. 8 μ l of hybridization buffer was placed onto the wells. A moisture chamber was prepared by folding a piece of tissue and by soaking it with the rest of the hybridization buffer. The moisture chamber was equilibrated for several minutes at the hybridization temperature at 46°C. 1 μ l of probe was spreaded (concentration 30 or 50 ng/ μ l) without scratching the surface. Then the slides were transferred into the moisture chamber and the hybridization was performed in the hybridization oven at 46°C for about two hours. Then, a washing buffer was used to rinse and remove the unbound oligonucleotides after hybridization.

The hybridization solution was then removed from the slides by washing them with washing buffer and then by soaking and incubating them into the washing buffer in a preheated water bath at 48°C for about 20 min. After incubation with washing buffer, the slides were rinsed with double distilled water to remove the washing buffer avoiding the removal of the samples from the wells. The slides were then immediately dried in an air stream. 4,6-diamidino-2-phenylindole (DAPI) was added to the wash buffer (2 μ l DAPI of 10 μ g per ml) to detect all DNA in the dark. 15 min incubation at 4°C was applied to the slides with DAPI and than rinsed briefly with double distilled water and immediately air-dried. The slides were embedded with Citifluor, Glycerol/PBS medium and cover slips were put before identification. The slides were investigated with a Zeiss, Jena, Germany confocal (LSM510-META) (Figure 3.3) microscope and digital images of the slides were taken with Axioplan 2 imaging digital camera.

3.2.3.3 Z-stacking with non-homogenized granules

Whole granulation study was done using the advanced image acquisition Z-stacking module prevailed at LSM 510 META Laser Scanning Microscopy. For Z-stacking, non-homogenized granules were used. 2-3 granules of each sample, having approximately the same diameter, were put in an Eppendorf tube and dried at 46°C for about 10 minutes. The granules in the tube were then dehydrated by successive passages through 50, 80 and 99% ethanol washes for 3 minutes for each. Ethanol amounts were taken such that all the granules were covered by the ethanol solution and then removed by pipette. For hybridization, the granules were hybridized by a hybridization buffer having the final concentrations of 20% formamide for *Archaea* (ARC915) and bacteria (EUB338). 6 µl of probe and 16 µl of hybridization buffer was added into the Eppendorf tube. Then the hybridization was performed in GFL3032 type shaker in which the temperature was fixed at 46°C. Hybridization was performed overnight while shaking at the same time at a speed of 120 min⁻¹. The hybridization solution was removed from the Eppendorf tubes by pipette next day. Then, washing buffer was added twice into the Eppendorf tubes and discarded by pipette and the granules in the washing buffer were incubated in a preheated water bath at 48°C for about 20 min. After incubation with washing buffer, the granules were rinsed with double distilled water and then immediately dried in an air stream. No DAPI was used for detecting all DNA in the dark.

Table 3.4: Oligonucleotide probes and relevant characteristics of each target group used in this study

Probe	Target Group	Relevant characteristic(s) of microorganisms
MX825	<i>Methanosaeta</i>	Use only acetate
MS821-SARC1645	<i>Methanosarcina</i>	Use only acetate and other substrates (H ₂ -CO ₂ , methanol and methylamines)
MC1109	<i>Methanococcales</i>	Mostly use H ₂ -CO ₂ and formate
MB310	<i>Methanobacteriales</i>	Mostly use H ₂ -CO ₂ , some use H ₂ -CO ₂ and formate
MG1200	<i>Methanogenium</i> relatives	Mostly use H ₂ -CO ₂ and formate
EURY498	Most Euryarchaeota	
ARC915	<i>Archaea</i>	
EUB338	Bacteria	

Table 3.5: Optimal hybridization conditions for the oligonucleotide probes used in this study

Oligonucleotide Probes		MB310 (Cy3)	MX825 (Cy3)	MC1109 (Cy3)	MG1200 (Cy3)	ARC915 (Cy3)	EUB338 (Cy3)	SARCI645 (Cy3)	EURY498 (Cy3)
Formamide (%)		35	20	20	20	20	20	15	0
Hybridization Buffer (2 hr at T=46°C)	5 M NaCl (μ l)	360	360	360	360	360	360	360	360
	Tris/HCl pH8 (μ l)	40	40	40	40	40	40	40	40
	Incubated Distilled H ₂ O (μ l)	900	1200	1200	1200	1200	1200	1300	1600
	Formamide (μ l)	700	400	400	400	400	400	300	0
	10%-ige SDS (μ l)	2	2	2	2	2	2	2	2
Washing Buffer (20 min at T=48°C)	5 M NaCl (μ l)	700	2150	2150	2150	2150	2150	3180	9000
	Tris/HCl pH8 (μ l)	1000	1000	1000	1000	1000	1000	1000	1000
	10%-ige SDS (μ l)	50	50	50	50	50	50	50	50
	EDTA (μ l)	500	500	500	500	500	500	0	0
	Incubated Distilled H ₂ O (μ l)	50	50	50	50	50	50	50	50

3.2.4 Analytical methods

The analyses were performed according to the Standard Methods for the Examination of Water and Wastewater (**Standard Methods, 1998**). The COD_{tot} and COD_{sol} analyses were performed by the dichromate closed reflux Colorimetric Method using Hach DR/2010 Model spectrophotometer. The samples were filtered through Cellulose Nitrate Filters having pore sizes of 0.45 μ m for COD_{sol} analyses. For TSS and VSS analyses of the biomass, fresh samples were taken and were centrifuged by the apparatus Janetzki T32A model for 20 minutes for the separation of soluble and suspended solids. The pellets were used for TSS and VSS analyses.

The pellets after centrifugation were transferred into crucibles and dried overnight at 105°C for TSS determinations. VSS concentrations were determined by ignition at 600°C for 30 minutes. Total biogas was measured by Ritter model gas meter. Its methane and carbon dioxide content were measured by HP 4890A Model GC. The pH values in the samples were measured by Orion 520A Model pH meter. Conductivity was measured with Hach 2511 Model Conductivity meter.

4. RESULTS AND DISCUSSION

4.1 Experiments with Synthetic Sewage

4.1.1 Start-up at mesophilic and sub-mesophilic UASB reactors

During the start-up, the first UASB reactor was operated as a mesophilic reactor at $35\pm 2^{\circ}\text{C}$ whereas the second UASB reactor was operated as a sub-mesophilic reactor at around $22\pm 2^{\circ}\text{C}$. Both reactors were operated at an average HRT of 29 hr for two weeks. The food to microorganism (F/M) ratio was around $0.022 \text{ kgCOD/kgMLVSS.d}^{-1}$. After two weeks, the mesophilic and sub-mesophilic UASB reactors were operated at an average HRT of 21 hr for about four months. In the first three months, there was no cooling equipment in the feed tank and due to high room temperatures, especially in summer; it was very difficult to keep the influent COD concentration constant (Figure 4.1a). Thus after three months of operation, cooling equipment was installed inside the feed tank and the temperature inside the tank was kept at 4°C , which significantly prevented the fluctuations in COD concentrations. However, it was still difficult to maintain a constant influent characteristic because of the fact that the ultimate dilution had important effects. Influent COD concentrations fluctuated between 450-600 mg/l during the study.

Total COD (COD_{tot}) removals of 71% and 69% were obtained for the mesophilic and sub-mesophilic reactors, respectively at an OLR of $0.36 \text{ kgCOD/m}^3\cdot\text{day}$ ($\text{HRT}=29 \text{ hr}$). COD_{tot} removals were obtained as 86% and 82% for the mesophilic and sub-mesophilic reactors when both reactors were operated at an OLR of $0.56 \text{ kgCOD/m}^3\cdot\text{day}$ ($\text{HRT}=21 \text{ hr}$) (Figure 4.1b). At $\text{HRT}=21 \text{ hr}$, the temperature in the mesophilic UASB reactor was $35\pm 2^{\circ}\text{C}$ whereas the temperature in the sub-mesophilic UASB reactor increased to around $26\pm 2^{\circ}\text{C}$ in summer. Results indicated almost the same COD_{tot} removals at both UASB reactors treating synthetic sewage

during the start-up. Soluble COD (COD_{sol}) removals were obtained as 91% and 87% for the mesophilic and sub-mesophilic reactors at an HRT=21 hr, respectively.

4.1.2 Steady-state in mesophilic and sub-mesophilic UASB reactors

After stable COD_{tot} removals were obtained, the average HRT was reduced to 13.5 hr in mesophilic and sub-mesophilic UASB reactors. Average COD_{tot} removals were obtained as 90% and 83% for the mesophilic and sub-mesophilic UASB reactors at an average OLR of 0.93 kgCOD/m³.day, respectively. Results did not indicate any important effect of increasing OLR on COD_{tot} removals at both UASB reactors. Since room temperature shifted to fall condition, the temperature in the sub-mesophilic UASB reactor decreased to around 21±2°C. On the other hand, the temperature in the mesophilic UASB reactor was still kept at 35±2°C. COD_{sol} removals at this HRT were obtained as 87% and 86% for the mesophilic and sub-mesophilic UASB reactors, respectively.

After HRT was reduced to around 11 hr, very slight decrease in COD_{tot} removals was observed in the mesophilic and sub-mesophilic reactors (Figure 4.1b). Results indicated at this HRT that COD_{tot} removals were 87% and 76% for the mesophilic and sub-mesophilic reactors (OLR=1.14 kgCOD/m³.day), respectively. The temperature in the sub-mesophilic reactor was 19±2°C.

The mesophilic and the sub-mesophilic UASB reactors were then operated at an average OLR of 1.4 kgCOD/m³.day (HRT of 8.65 hr) for about one month keeping the mesophilic UASB reactor at 35±2°C whereas the temperature in the sub-mesophilic UASB reactor was still at 19±2°C. Results indicated COD_{tot} removal improvement in the sub-mesophilic UASB reactor at this OLR value. The COD_{tot} removal could be obtained as the same in the mesophilic UASB reactor. At this OLR value, COD_{tot} removals were 87% and 84% for the mesophilic and sub-mesophilic reactors, respectively.

COD_{tot} removals were obtained as 86% and 78% when the average HRT was reduced to around 5.5 hr in the mesophilic and sub-mesophilic reactors, respectively. The average OLR at this HRT was around 2.4 kgCOD/m³.day. At this OLR value, the temperature in the mesophilic UASB reactor was 35±2°C and the temperature in

the sub-mesophilic UASB reactor decreased to around $16\pm 2^{\circ}\text{C}$. Results showed no decrease in the mesophilic UASB reactor in the case of COD_{tot} removal. The slight COD_{tot} removal decrease in the sub-mesophilic UASB reactor could be due to the decrease in the temperature of the reactor.

Results showed that increasing OLR did not cause any significant effect in mesophilic and sub-mesophilic UASB reactors in terms of COD_{tot} removal. This might have been due to the fact that the COD_{tot} concentration of the influent was not high. **Vieira and Garcia (1992)** also did not observe any significant change in organic removal efficiency at an UASB reactor operated with HRT values between 5-15 hours at room temperature. Although the average COD_{tot} removal in the mesophilic reactor was higher than in the sub-mesophilic reactor, sub-mesophilic reactor could tolerate such low HRT values (Figure 4.1b). The temperature of the reactor decreased to around $16\pm 2^{\circ}\text{C}$ at the end of the study with the sub-mesophilic UASB reactor.

4.1.3 Steady-state in mesophilic and psychrophilic UASB reactors

After finalizing the study for the evaluation of the performance of UASB reactor at sub-mesophilic condition, the temperature of the reactor was decreased and was kept at $10\pm 2^{\circ}\text{C}$ starting with an HRT of 12.5 hr. The average OLR at this HRT was around $1.05 \text{ kgCOD}/\text{m}^3\cdot\text{day}$. It was reported that the optimal temperature of psychrophilic microorganisms is around 17°C (**Lens et al., 2001**). The aim of decreasing the temperature to such low values was to find out whether the UASB reactor could tolerate this. In the first week, the COD_{tot} removal decreased significantly to less than 68% in the psychrophilic reactor whereas under the same conditions, up to 89% COD_{tot} removal was observed in the mesophilic reactor (Figure 4.2a). However, COD_{tot} removal improved in the following days indicating that the microorganisms could well adapt to such low temperatures. COD_{tot} removal up to 82% could be measured at an HRT of 12.5 hr at $10\pm 1^{\circ}\text{C}$ (Figure 4.2b). At the same HRT, COD_{tot} reached up to 92% in the mesophilic reactor. After the average HRT value decreased to around 8.0 hr and 6.0 hr, average COD_{tot} removals were 90% and 82% for the mesophilic and psychrophilic ($10\pm 1^{\circ}\text{C}$) UASB reactors at both

HRT values, respectively. The average OLR values at these HRT values were around 1.61 and 2.05 kgCOD/m³.day, respectively.

Although the average COD_{tot} removal in the mesophilic reactor was higher than in the psychrophilic reactor, the psychrophilic reactor could well tolerate such low temperature values which indicated that no heating was required for the treatment of synthetic sewage.

F/M ratios, specific substrate utilization rates (U), OLR's and SRT's at different HRT's and temperature values are given in Table 4.1 where S₀ and S₁ represent influent and effluent COD_{sol} concentrations, respectively. X values represent the average VSS concentration in the reactors.

Table 4.1: Operational parameters at different HRT's and temperatures in UASB reactors treating synthetic domestic sewage

Mesophilic								
HRT _{avr} (hr)	T _{avr} (°C)	S ₀ (mg/l)	S ₁ (mg/l)	X (mg/l)	F/M (day ⁻¹)	U (day ⁻¹)	OLR (kgCOD/m ³ .d)	SRT (day)
21.0	35	495	49	8891	0.06	0.06	0.56	158
13.5	35	518	46	9473	0.10	0.09	0.93	106
11.0	35	508	46	9666	0.12	0.11	1.14	87
8.65	35	501	51	10141	0.14	0.12	1.40	73
5.50	35	539	52	10827	0.22	0.20	2.37	47
6.50	34	544	40	10219	0.20	0.18	2.05	58
Sub-mesophilic								
21.0	26	494	53	11925	0.05	0.04	0.56	212
13.5	20	521	63	11606	0.08	0.07	0.93	130
11.0	19	509	64	11499	0.10	0.09	1.14	103
8.65	19	499	51	12356	0.11	0.10	1.40	89
5.50	16	539	70	13595	0.17	0.15	2.37	63
Psychrophilic								
6.50	10.86	534	60	12341	0.16	0.14	2.05	67

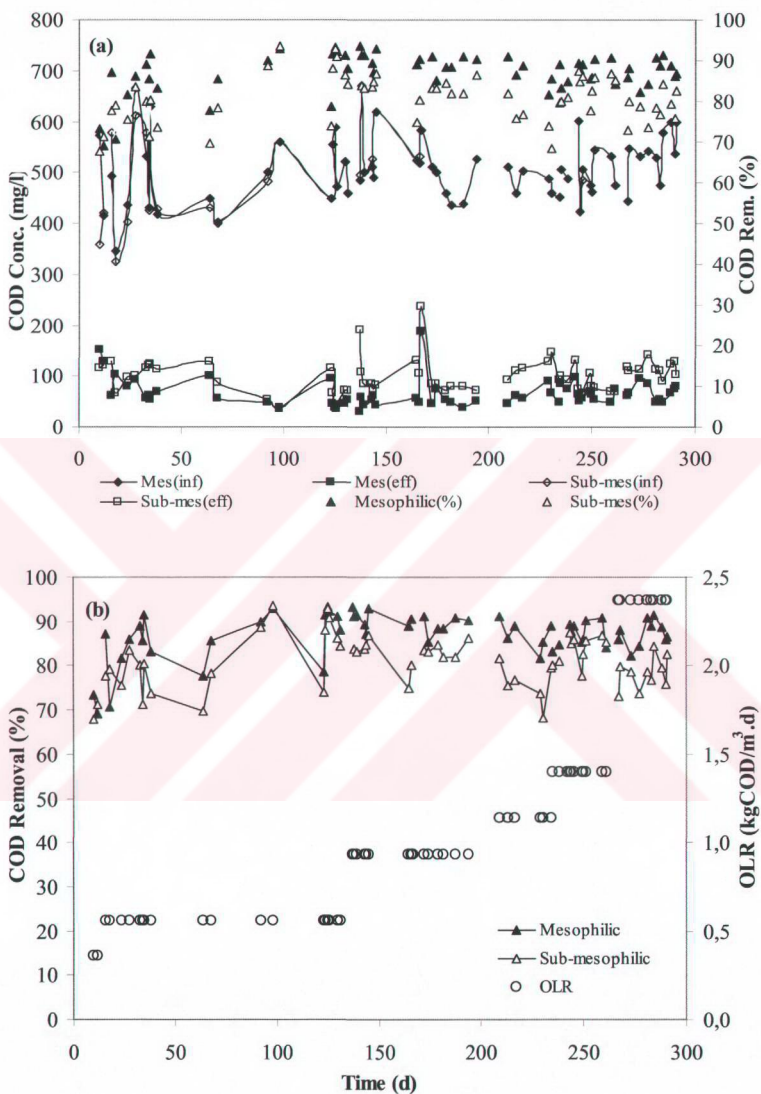


Figure 4.1: (a) COD_{tot} values and (b) COD_{tot} removals at increased OLR's in mesophilic and sub-mesophilic UASB reactors treating synthetic wastewater

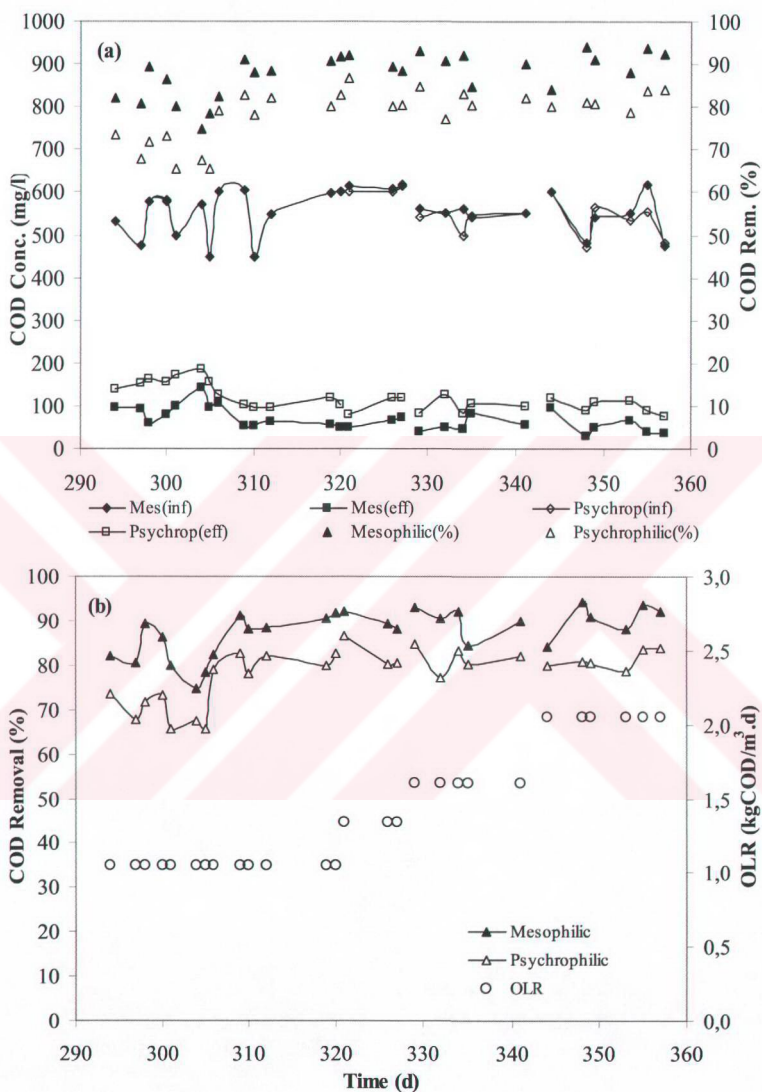


Figure 4.2: (a) COD_{tot} values and (b) COD_{tot} removals at increased OLR's in mesophilic and psychrophilic UASB reactors treating synthetic wastewater

4.1.4 Biogas production in the UASB reactors treating synthetic sewage

Total biogas production could not be measured efficiently in mesophilic and sub-mesophilic UASB reactors for about four months at the beginning of the operation. After 4 months, total biogas produced from both reactors were collected in balloons and measured by two gas meters daily and the produced biogas per COD removed (biogas yield) was shown in Figure 4.3. Since, biogas productions were measured at different temperatures; all the measured values in Figure 4.3 indicate the standard conditions using the ideal gas equation. Daily biogas productions and biogas yields at different HRT's and OLR's in both UASB reactors treating synthetic sewage are summarized in Table 4.2.

When the temperatures in the sub-mesophilic reactor were above 15°C, approximately the same biogas yields were obtained as in the mesophilic reactor (Table 4.2). On the other hand, gas productions as well as biogas yields reduced to a great extent when the temperature of the reactor was reduced to 10±1°C at an OLR of around 1.35 kgCOD/m³.day (Table 4.2). Although biogas yield improved as operation continued at 10±1°C, biogas yield at mesophilic UASB reactor indicated around twice higher values compared to psychrophilic UASB reactor.

The CO₂ content of the sub-mesophilic and the psychrophilic UASB reactors indicated lower values compared to the mesophilic UASB reactor at temperatures below 20°C (data not shown). Since solubility of gases increases as temperature decreases, more CO₂ was dissolved in the effluent which decreased the CO₂ content in the produced biogas. **Elmitwalli (2000)** also reported that the solubility of gases increased at declining temperatures below 20°C.

Besides, results indicated that as HRT decreased, daily biogas production increased (Table 4.2). **Singh and Viraraghavan (2001)** also found similar results in their study. They reported that gas production increased with a decrease in HRT and decreased with a decrease in temperature. The high values of HRT at the beginning of operation produced low up-flow velocities, which in turn enabled the presence of poorly mixed zones in the reactor. As a consequence of this fact the contact between active biomass and substrate was not optimal and the biogas production was minimal. However, as the HRT was gradually decreased the up-flow velocities were increased producing a better contact between biomass and substrate and leading to an

improvement of COD_{sol} removal and the corresponding increase in biogas production (Rodriguez et al., 2001). It was also reported that as the temperature decreased, the methane percentage in the total biogas increased as also seen in this study (data not shown).

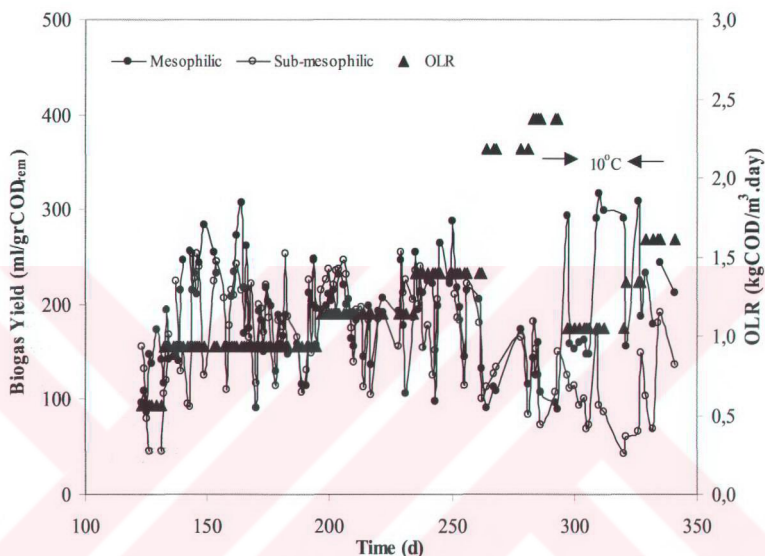


Figure 4.3: Biogas yields in both UASB reactors at increased OLR's

Table 4.2: Daily biogas productions and biogas yields at different OLR's and temperatures in UASB reactors treating synthetic domestic sewage

HRT (hour)	Mesophilic				Sub-mesophilic		
	OLR (kgCOD/m ³ .day)	T _{avr} (°C)	Q _{gas} (l/day)	Biogas yield (ml/gCOD _{rem})	T _{avr} (°C)	Q _{gas} (l/day)	Biogas yield (ml/gCOD _{rem})
21.0	0.56	35	0.42	127	26	0.23	92
13.5	0.93	35	1.00	194	20	0.83	182
11.0	1.14	35	1.16	190	19	1.06	200
8.65	1.40	35	1.73	208	19	1.60	182
5.50	2.37	34	1.66	120	16	1.59	128
HRT (hour)	Mesophilic				Psychrophilic		
	OLR (kgCOD/m ³ .day)	T _{avr} (°C)	Q _{gas} (l/day)	Biogas yield (ml/gCOD _{rem})	T _{avr} (°C)	Q _{gas} (l/day)	Biogas yield (ml/gCOD _{rem})
12.5	1.05	33	1.26	220	11	0.61	98
11.0	1.35	33	1.40	217	10	0.58	92
8.15	1.61	32	2.40	217	10	0.57	136

4.1.5 pH and alkalinity

The pH values in the effluent of the mesophilic UASB reactor were slightly higher compared to sub-mesophilic (Figure 4.4a) and psychrophilic (Figure 4.4b) temperatures. This also matched with the lower carbon dioxide content of the biogas produced at the sub-mesophilic and the psychrophilic UASB reactors at temperatures below 20°C compared to the mesophilic UASB reactor. Since solubility of gases increases as temperature decreases, more CO₂ was dissolved which decreased the pH value of the effluent of UASB reactor operated at lower temperatures.

Alkalinity played an important factor during the study. Alkalinity in the effluent of psychrophilic reactor was reduced as well as the COD_{tot} removal after the temperature of the reactor reduced to 10±1°C. However, an increase in the effluent alkalinity of the psychrophilic UASB reactor was observed after the reactor adapted to low temperature. Results also indicated that after this adaptation time, an improvement in COD_{tot} removal could also be observed. Lower pH values at the sub-mesophilic and the psychrophilic temperatures might have also been due to higher VFAs concentrations in the effluent that resulted a decrease in reactor pH. It is reported that methanogens were more sensitive to temperature reduction than acetogens and accumulation of VFAs occurred more in psychrophilic reactors. This might also be an indicator that the system was not operating at optimal conditions (Nachaiyasit and Stuckey, 1997; Langenhoff and Stuckey, 2000).

4.1.6 Sludge concentrations

Sludge samples were taken from the bottom ports of the reactors as OLR increased. Table 4.3 shows an apparent biomass loss (reduction of TSS and VSS) during the start-up. Despite of this, there was a substantial improvement in biogas production in both UASB reactors. TSS and VSS concentrations of the available sludge in both reactors might have decreased significantly after around 130 days due to unavoidable sludge wash-out during the start-up. However, this decrease occurred much more in the mesophilic UASB reactor. TSS and VSS concentrations of the biomass increased as time passed. Although TSS and VSS concentrations of the biomass in both reactors decreased compared to the initial values, VSS/TSS ratios remained almost the same during the study (Table 4.3). Besides, sludge samples were also taken from all of the ports installed at different heights of the UASB reactors, but almost no TSS

and VSS concentrations at higher ports of the reactors could be observed. This could be attributed to the fact that the settleability of the biomass could be well provided in both UASB reactors. It is reported that in reactors where granular sludge is used as inoculum, the biomass has high settleability character and could be well retained inside the reactors.

Table 4.3: TSS and VSS in both UASB reactors treating synthetic domestic sewage

Time (day)	Mesophilic			Psychrophilic		
	TSS (g/l)	VSS (g/l)	VSS/TSS (%)	TSS (g/l)	VSS (g/l)	VSS/TSS (%)
0	115	105	91	115	105	91
131	57	50	88	75	67	90
234	63	55	87	72	65	90
288	69	61	88	85	77	90
357	66	58	87	79	70	88

4.2 Experiments with Raw Sewage

4.2.1 Experiments with pre-settled domestic sewage

After finalizing the study with synthetic sewage, the study was carried on by feeding both reactors with raw domestic sewage. The mesophilic reactor was still operated at $35\pm 2^{\circ}\text{C}$ whereas the psychrophilic reactor was operated at $13\pm 2^{\circ}\text{C}$. Both reactors were fed with pre-settled domestic sewage for about 45 days and operated at an average HRT of around 9.0 hr.

The composition of domestic sewage changes in time and from one place to another. It is apparent that when flow, concentration and composition fluctuate greatly, anaerobic treatment efficiency will decrease considerably (Elmitwalli, 2000). Since high amounts of sea water were mixed with raw domestic wastewater in the sewerage system during sampling periods; the influent was much diluted and the character of domestic sewage changed significantly in the study. Not only did sea water addition cause high dilution in the influent, but it also increased salinity and thus conductivity at a great extent (Figure 4.5). Influent COD concentrations fluctuated between 180-315 mg/l which reflected a weak sewage.

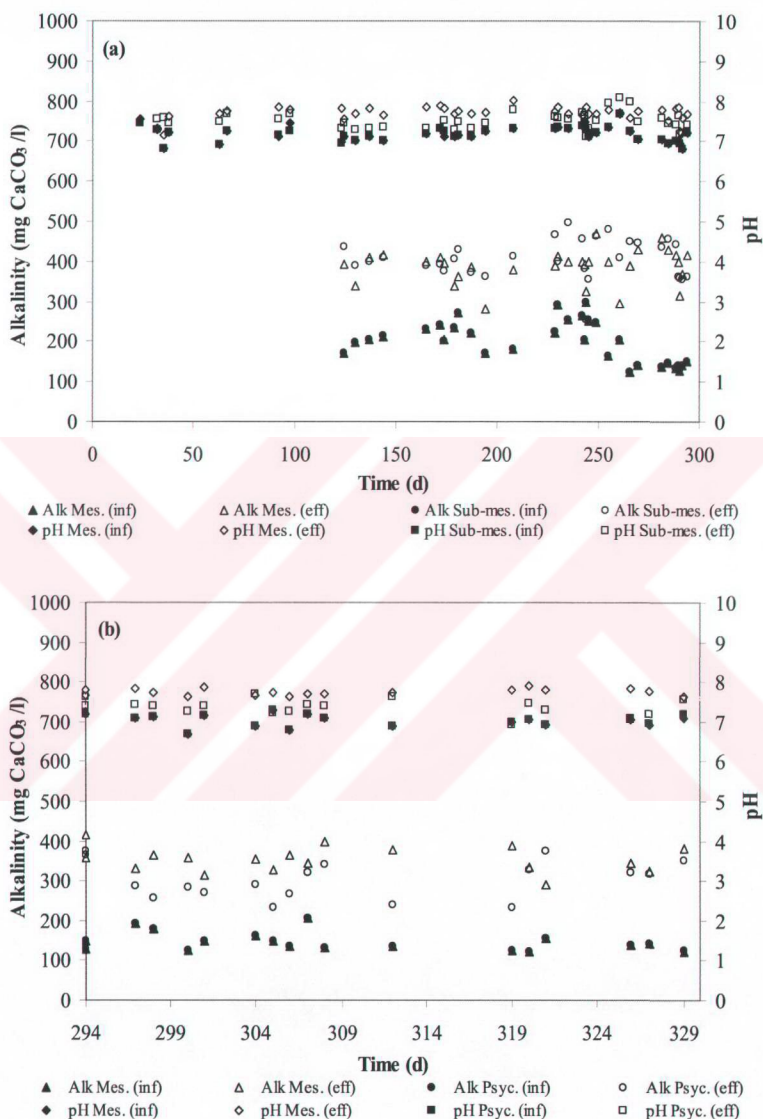


Figure 4.4: pH and alkalinity versus time in UASB reactors treating synthetic sewage (a) mesophilic and sub-mesophilic (b) mesophilic and psychrophilic

Average COD_{tot} removals were obtained as 38-71% (average 58%) and 23-50% (average 39%) for the mesophilic and psychrophilic reactors at an $HRT=9.25$ hr ($OLR=0.7$ kgCOD/m³.day), respectively (Figure 4.6a-b). On the other hand, average COD_{sol} removals were obtained as 10-55% (average 38%) and 5-46% (average 30%) for the mesophilic and psychrophilic reactors, respectively when both reactors operated at the same HRT. Effluent COD_{sol} concentrations were observed higher in psychrophilic reactor than in the mesophilic UASB reactor. Pre-sedimentation of domestic sewage is recommended by several researchers because of the fact that SS accumulation in the sludge bed is prevented at low temperatures and the formation of granular sludge may be promoted. However, the SS concentration in the pre-settled sewage was still high for achieving a stable performance during anaerobic treatment (Elmitwalli, 2000).

4.2.2 Experiments with mixed domestic sewage

The study was carried on by feeding both reactors with raw domestic sewage without pre-sedimentation. The mesophilic reactor was operated at $35\pm2^{\circ}C$ whereas the psychrophilic reactor was operated at $13\pm2^{\circ}C$. Both reactors were operated at an average HRT of 7.5 hr ($OLR\approx 0.7$ kgCOD/m³.day). Results also did not indicate any stability due to variable characteristics of domestic sewage. Influent COD concentrations fluctuated between 165-270 mg/l and reflected a weak domestic sewage. It was very surprising to see lower COD_{tot} and COD_{sol} concentrations in the influent of mixed raw sewage that pre-settled raw sewage. It was due to the different sampling dates. Since influent conductivity values increased at mixed raw sewage feeding, this indicated that sea water addition had also increased in the sewerage system. Thus, more dilution occurred and influent COD concentrations decreased compared with the influent COD concentrations during pre-settled sewage feeding. COD_{tot} removals were obtained as 39-64% (average 49%) and 24-54% (average 35%) for the mesophilic and psychrophilic reactors, respectively (Figure 4.6a-b). COD_{sol} removals decreased compared with COD_{sol} removals from pre-settled sewage and average COD_{sol} removals were obtained as 14% and 11% for the mesophilic and psychrophilic reactors, respectively. It was apparent that pre-settlement increased COD_{sol} removals at a great extent.

As SS represents the major COD fraction in domestic sewage, high removal of SS will lead to a high removal of COD_{tot} . The removal of large particles in the SS fraction is dependent mainly on drag forces and settling characteristics. In an anaerobic reactor the removal of SS occurs by settling and filtration through the sludge bed and/or filter media. The filtration mechanism includes entrapment, sorption and straining (Metcalf and Eddy, 1991). Lower COD_{tot} removals at psychrophilic UASB reactor could be due to higher SS concentrations in the effluent. It was reported that the removal of SS in UASB reactors with both flocculent and granular sludge declined when the temperature of the domestic sewage decreased. The removal of dissolved matter is mainly a biological process, some physical aspects are also included (Elmitwalli, 2000). It could be concluded that COD_{tot} removals in this study were because of entrapment of the solids and filtration through the sludge bed. COD_{sol} removals, on the other hand, were due to the removal of the easily degradable part of the raw sewage. Due to low biodegradability, COD_{sol} removals were very low at UASB reactors treating raw sewage. It was also reported that the rate of hydrolysis for domestic sewage particulate decreased at declining temperature that caused accumulation of SS in the anaerobic reactor, e.g. protein and lipids. The design HRT of the anaerobic reactors treating domestic sewage was dependent on both biological and physical factors, like the removal of SS and the extent of hydrolysis. It was observed that when either hydrolysis was lower or COD removal by SS was higher, a longer HRT was required at a certain sludge retention time (SRT). At temperatures below 15°C, SRT should have exceeded 100 day in order to retain sufficient methanogenic activity (Elmitwalli, 2000). HRT applied was obviously not long enough for the hydrolysis to be realized and thus low COD removals were obtained in this study. In anaerobic treatment processes which do not treat pre-settled domestic sewage, HRT should be chosen longer for more effective hydrolysis of the particulate solids. Low COD_{tot} and COD_{sol} removals at UASB reactors could be also due to deterioration of the methanogenic activity of the sludges.

Uemura and Harada (2000) mentioned that although granular sludge had a higher methanogenic activity and a distinctly better settleability than flocculant sludge, the treatment of domestic sewage at low temperatures at one step granular sludge UASB

reactor lead to accumulation of SS due to slow hydrolysis resulting in deterioration of the methanogenic activity of the sludge.

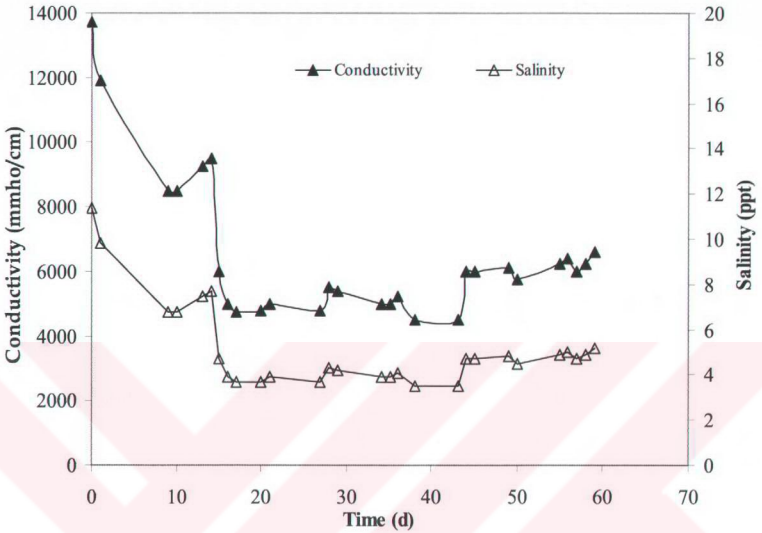


Figure 4.5: Conductivity and salinity versus time in the influents of mesophilic and psychrophilic UASB reactors

4.2.3 Biogas production

No significant total biogas productions were observed in both reactors during anaerobic treatment of either pre-settled or mixed raw domestic sewage. **Castilla et al. (2000)** also detected no apparent biogas during anaerobic treatment of municipal wastewater with a removal efficiency of 44% at an HRT of 0.6 hr. No significant biogas production could be due to the fact that an important fraction was dissolved in the liquid phase. Gas production was rather limited in another study and calculations suggested that more than 50% of the total CH_4 produced was lost due to its dissolution in the effluent (**Noyola et al., 1988**). In this study, a fraction of methane could have left the reactor with the effluent due to the raw sewage having low concentration.

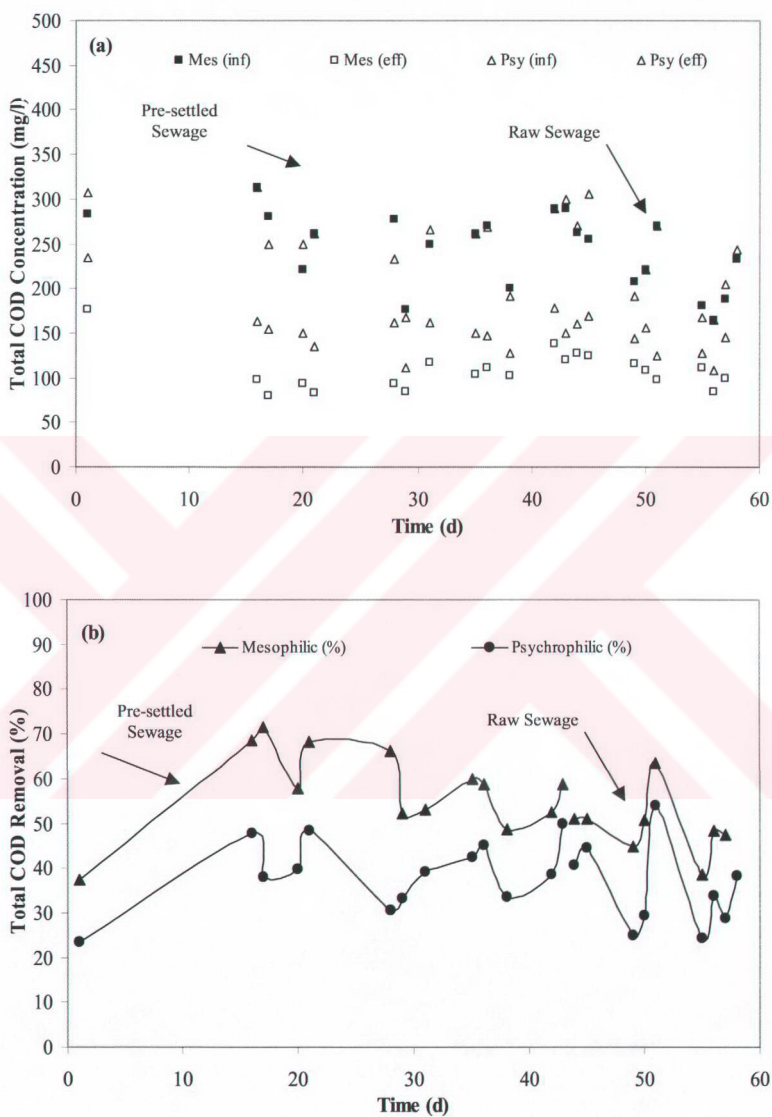


Figure 4.6: (a) COD_{tot} concentrations and (b) COD_{tot} removals in mesophilic and psychrophilic UASB reactors treating pre-settled and mixed domestic sewage

4.2.4 pH and alkalinity

The pH in the effluent of mesophilic reactor gave slightly higher values compared to psychrophilic reactor (Figure 4.7). Alkalinity was monitored on a regular basis and found to be sufficient (ranged from 230-400 and 210-495 mg CaCO_3/l at mesophilic and psychrophilic UASB reactors, respectively) for maintaining required pH levels. Influent alkalinity values fluctuated significantly and did not indicate any stability (Figure 4.7). Lower pH values in the psychrophilic UASB reactor might be indications of slightly higher VFAs concentrations in the effluent that led to a slight decrease in reactor pH. However, negative effects produced by accumulation of acidity were not observed as pH values at both UASB reactors were kept within the optimum range. Besides, the high increase of solubility of CO_2 indicates that a slightly lower reactor pH might prevail under psychrophilic conditions (Lettinga et al., 2001)

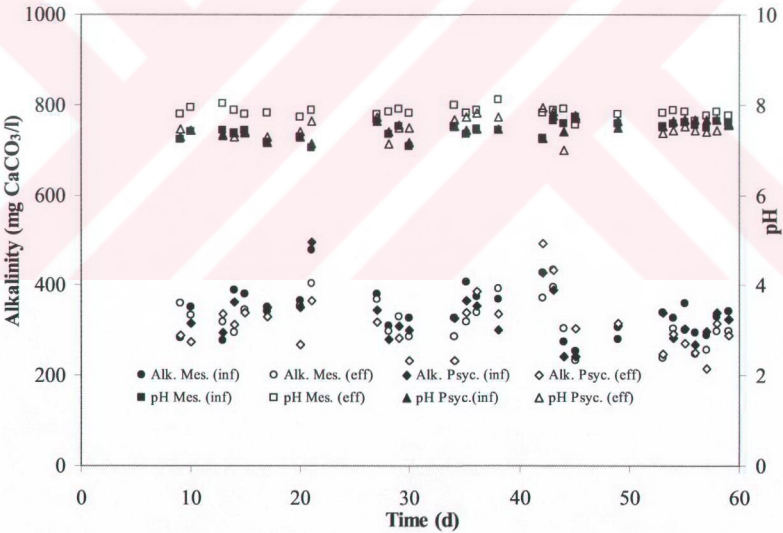


Figure 4.7: pH and alkalinity versus time in mesophilic and psychrophilic UASB reactors treating raw domestic sewage

4.2.5 Sludge concentrations

Sludge samples were taken from the bottom ports of the reactors during the study with pre-settled and raw domestic sewage. TSS and VSS concentrations of the available sludge in mesophilic reactor decreased whereas TSS and VSS concentrations of the available sludge in psychrophilic reactor did not change significantly. The VSS/TSS ratios remained the same in the psychrophilic reactor whereas the VSS/TSS ratio decreased in mesophilic reactor compared to the results with synthetic sewage (Table 4.4).

Table 4.4: TSS and VSS in both UASB reactors treating raw domestic sewage

Time (day)	Mesophilic			Psychrophilic		
	TSS (g/l)	VSS (g/l)	VSS/TSS (%)	TSS (g/l)	VSS (g/l)	VSS/TSS (%)
22	47	39	84	81	75	92
43	52	43	84	74	65	89
58	55	46	82	79	69	87

4.2.6 Ammonia and TKN

NH₄-N and TKN were measured in the influent and the effluent of the mesophilic and the psychrophilic UASB reactors. As sea water addition increased in the sewerage system, the influent NH₄-N and TKN concentrations decreased (Table 4.5 and Table 4.6). NH₄/TKN ratios of the influent pre-settled and mixed raw sewage could be calculated as 74% and 79%, respectively. It could be concluded that NH₄-N concentrations in the effluent of the mesophilic and the psychrophilic UASB reactors increased which was expected at all anaerobic systems.

Anaerobic treatment is effective in removing biodegradable organic compounds, leaving mineralized compounds like ammonium, phosphate and sulfur in the solution. Therefore, these compounds have to be removed by an additional post-treatment step to meet sufficiently the criteria for a sustainable environmental protection. Besides it was reported that no pathogen removal could be achieved at low temperatures. Since high ammonia and TKN concentrations were found in the effluents of the mesophilic and the psychrophilic UASB reactors, post-treatment was required. Aerobic polishing ponds appear to be the most commonly used method for the treatment of UASB reactors effluents treating domestic wastewater to meet

effluent quality standards (Tare et al., 1997). After post-treatment step, effluents may be used for irrigation purposes. Due to regional water shortage problems in Turkey, direct usage of sewage is still preferred for agricultural irrigation purposes. Sewage provides a good source of nutrients (N, P) for the irrigated crops. However, this uncontrolled usage negatively affects public health (Arslan et al., 2005). Thus, effluents should be monitored carefully in order to meet the criteria given in Part 7 'Usage of treated wastewaters for irrigation' in the Technical Bulletin of Turkish Water Pollution Control Regulation as written in Article 28 (WPCR, 1991).

Table 4.5: NH₄-N in both UASB reactors treating raw domestic sewage

Time (day)	Mesophilic		Psychrophilic	
	NH ₄ -N influent (mg/l)	NH ₄ -N effluent (mg/l)	NH ₄ -N influent (mg/l)	NH ₄ -N effluent (mg/l)
Pre-settled				
0	43	-	43	-
27	33	41	33	38
29	26	30	26	31
38	36	40	36	39
Mixed				
45	27	30	27	30
55	28	28	28	29

Table 4.6: TKN-N in both UASB reactors treating raw domestic sewage

Time (day)	Mesophilic		Psychrophilic	
	TKN-N influent (mg/l)	TKN-N effluent (mg/l)	TKN-N influent (mg/l)	TKN-N effluent (mg/l)
Pre-settled				
0	55	-	55	-
27	42	44	42	44
38	44	42	44	45
Mixed				
45	36	40	36	37
55	33	32	33	32

4.3 FISH Results Combined with CSLM

Methanogens belong to the kingdom of Euryarchaeota in the domain of *Archaea*. The *Archaea* differ from bacteria in many aspects such as; cell wall composition, sensitivity to antibiotics, translation and transcription machinery, and very strict demands to anaerobic culture conditions (Lange and Ahring, 2001). Fluorescent in situ hybridization (FISH) technique combined with confocal scanning laser microscopy (CSLM) was used to analyze the spatial distribution of specially labeled target cells present in mesophilic and psychrophilic UASB reactors by using oligonucleotide probes. The aim was to evaluate how both granule structures have differed, depending on temperature, operational parameters and wastewater composition. The sludge samples were investigated by using the two general probes ARC915 and EUB338 which were complementary to conserved regions within the archaeal and most bacterial domains, respectively. The sludge samples were also investigated by using specific oligonucleotide probes targeting different groups of methanogens. Phylogenetically, the methanogens are divided into five orders, the *Methanobacteriales*, the *Methanococcales*, the *Methanosarcinales*, the *Methanopyrales*, and the *Methanomicrobiales*. The methanogens are closer to Eukarya than to Bacteria in some aspects of their molecular biology such as their transcription apparatus. On the other hand, other features of methanogens (and of the *Archaea* in general) are more similar to Bacteria than to Eukarya, since they are indeed prokaryotes (Lange and Ahring, 2001).

The oligonucleotide probes and their target groups relevant for this study are listed in Table 3.4. Formamide is added to significantly enhance the stringency of hybridization but is also reported in some cases to enhance the hybridization intensity (Amann et al., 1992). The formamide concentration used in the hybridization buffer for each oligonucleotide probe was given in Table 3.5. By using these specific probes, the major methanogenic *Archaea* present in both reactors at all sampling times was investigated. Which of the species was intensively dominant was identified comparatively in mesophilic and psychrophilic granular sludge samples. All probes specific to a group of methanogens were used in combination with ARC915 as control. The probe specific to most Euryarchaeota (EURY498) was

successfully used for the determination of phylum Euryarchaeota of domain *Archaea* more.

4.3.1 FISH results with the granular seed

The mesophilic and psychrophilic UASB reactors were inoculated with the granular sludge taken from a full scale UASB reactor treating effluents of an alcohol distillation industry. The granular seed sludge had a considerably high VSS/SS ratio of about 91%. To identify the microorganisms in seed sludge, DAPI staining and FISH with Cy3 (red) labeled Arch915 (ARC915) (*Archaea* Domain) and Fluorescein (green) labeled Eub338 (Bacteria Domain) probes were applied to granules dispersed in a homogenizer. To investigate the acetoclastic methanogens, Cy3 (red) labeled MX825 (*Methanosaeta* spp.) and MS821 (*Methanosarcina* spp.) probes were used. Photomicrographs of DAPI staining and hybridization results are presented in Figure 4.8a-f. Results indicated that methanogenic *Archaea* (Arch915) were intensively present in seed sludge and acetoclastic *Methanosaeta* was the abundant methanogen (Figure 4.8a, b, d, and e). Only very few signals were obtained with Eub338 as an indication of inconsiderable bacterial amount in seed sludge (Figure 4.8c and f) (Gomec et al., 2004).

4.3.2 FISH results with synthetic domestic sewage

In-situ hybridization results showed that archaeal cells representing the methanogens (ARC915) were intensively dominant in the bottom sampling ports of both mesophilic and psychrophilic UASB reactors treating synthetic sewage. The results of dual probing with general probe for *Archaea* ARC915 (Cy3 labeled) and bacteria EUB338 (Cy5 labeled) indicated that archaeal and bacterial cells could be detected at significant amounts. Besides, good signals with these two general probes ARC915 and EUB338 could be obtained in mesophilic (Figure 4.9a-b-c) and psychrophilic (Figure 4.10a-b-c, Figure 4.11a-b-c, Figure 4.12a-b-c, 4.13a-b-c) UASB reactors. Figures showed that archaeal and bacterial cells lived together in the granules at both temperatures. On the other hand, since *Methanosarcina* and *Methanosaeta* species are the genera of *Archaea*, *Methanosarcina* (Figures 4.11b and 4.13b) and *Methanosaeta* (Figures 4.9b, 4.10b, 4.11b, and 4.12b) like species could be detected using the general probe for *Archaea* (ARC915).

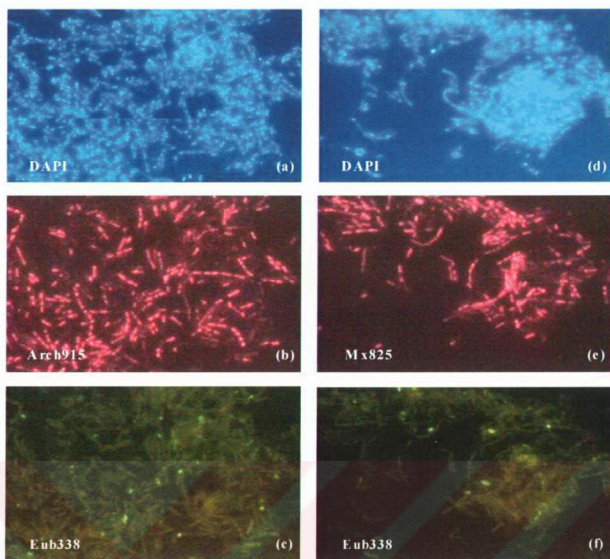


Figure 4.8: Fluorescent in-situ hybridization and DAPI staining photomicrographs of seed sludge (1000x); samples were (a-d) stained with DAPI (all microorganisms) and hybridized with (b-e) Cy3 (red) labeled Arch915 (Methanogenic *Archaea*) and Mx825 (*Methanosaeta*) probes and (c-f) Fluorescent (green) labeled Eub338 (bacteria) probe

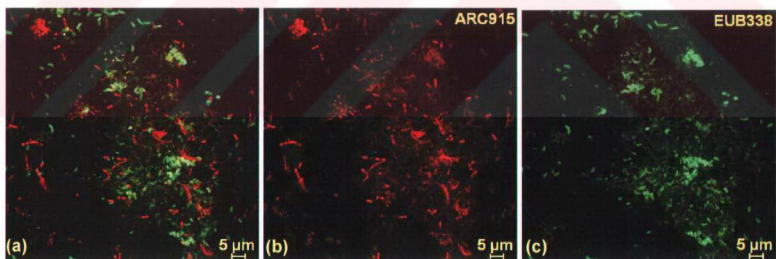


Figure 4.9: FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (a) dual probing with Cy3 (red) labelled *Archaea* (ARC915) and Cy5 (green) labelled bacteria (EUB338) (b) only signals of the ARC915 probe from the same field in panel a (c) only signals of the EUB338 probe from the same field in panel a

Archaeal cells detected with probe ARC915 were found in very high numbers in both granules taken from the mesophilic and the psychrophilic UASB reactors treating synthetic sewage. Not only were *Methanosaeta* and *Methanosarcina* like species observed, but also *Methanobacterium* species could be detected with ARC915 probe. It was also concluded that *Methanosaeta* spp. had longer filamentous forms in the psychrophilic granules and their filamentous shapes were more apparent compared to the mesophilic granules. Higher amounts of *Methanosarcina* spp. in the psychrophilic granules could be observed by using ARC915. In Figure 4.13a and Figure 4.13b, FISH photomicrographs indicating *Methanosarcina* spp. in psychrophilic UASB reactor treating synthetic sewage at a temperature of around 11°C having a HRT of around 6.5 hr are shown.

Dual probing with ARC915 (Cy3 labelled) and EUB338 (Cy5 labelled) was applied and it was observed that *Methanosarcina* like species were very obvious at such low temperatures and they could be successfully detected with the general *Archaea* probe ARC915. In Figure 4.13c, the presence of bacteria at around 11°C could also be seen.

FISH experiments were repeated with dual probing of two general probes EUB338 and ARC915. However, this time EUB338 was labeled with Cy3 (red) whereas ARC915 was labeled with Cy5. Results also showed significant amounts of bacterial and archaeal cells living together in both UASB reactors treating synthetic sewage at mesophilic (Figure 4.14a-b-c and Figure 4.15a-b-c) and psychrophilic (Figure 4.16a-b-c) temperatures.

Specific probes MS821 and SARCI645 for *Methanosarcina* like species were used. For hybridization, each Cy3 labelled probe was hybridized by buffers having 0%, 15%, 20%, and 35% formamide concentrations for ensuring the optimal hybridization stringency. Results indicated that formamide concentrations higher than 15% in the hybridization buffer did not have any effect on obtaining positive signals. However, hybridization buffer containing 0% formamide concentration gave relatively low signals. The best suited formamide concentration for SARCI645 probe targeting genus *Methanosarcina* was given as 15% in probeBase which proved the findings.

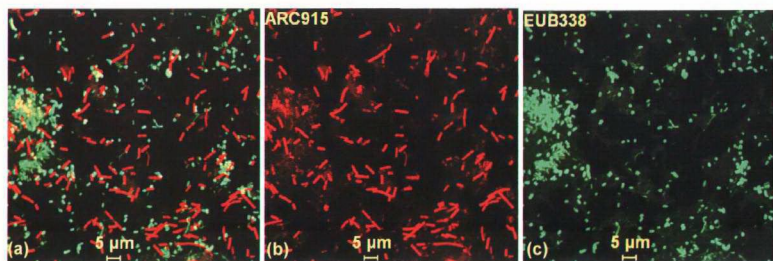


Figure 4.10: FISH results in psychrophilic UASB reactor treating synthetic sewage (T=16°C, HRT=4.8 hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

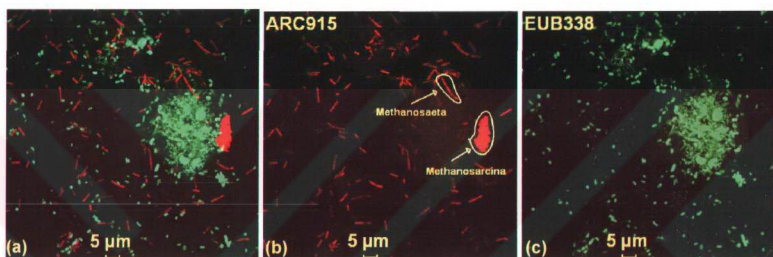


Figure 4.11: FISH results in psychrophilic UASB reactor treating synthetic sewage (T=16°C, HRT=4.8 hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

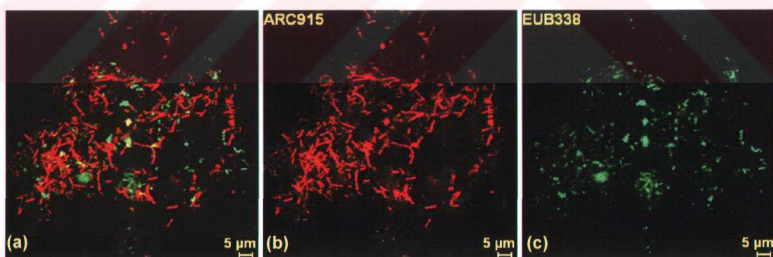


Figure 4.12: FISH results in psychrophilic UASB reactor (T=16°C, HRT=4.8 hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

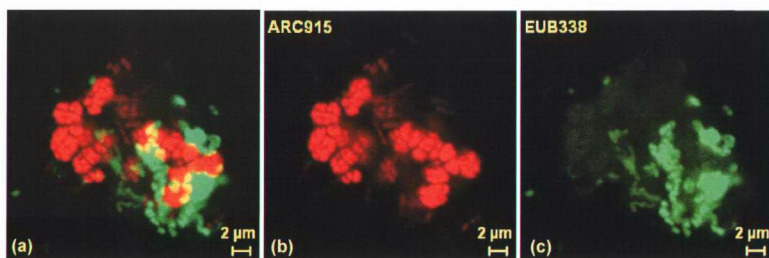


Figure 4.13: FISH results in psychrophilic UASB reactor treating synthetic sewage (T=11°C, HRT=6.25 hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

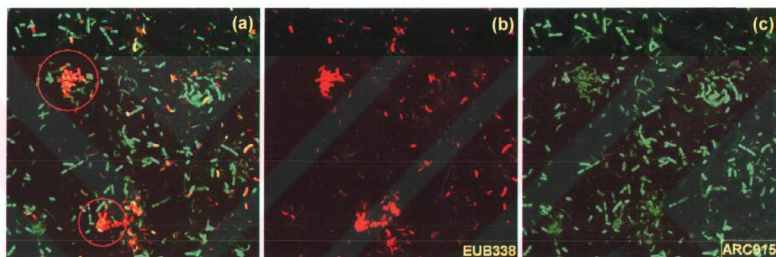


Figure 4.14: FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

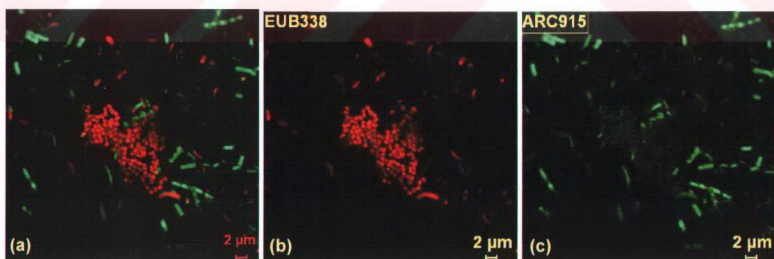


Figure 4.15: FISH results in mesophilic UASB reactor (HRT=4.8 hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

Positive control was applied to the specific probes MS821 and SARCI645 by using pure cultures (DSM800) for *Methanosarcina barkeri* in Figure 4.17a-b-c and Figure 4.18a-b-c, respectively. DSM is the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [Braunschweig, Germany] (Burggraf et al., 1994). *Methanosarcina* cells were normally found in clusters and individual small and/or large clumps. Results indicated that both specific probes worked well with the pure culture DSM800. However, the specific probe MS821 gave relatively weak hybridization signals with the sludge samples used in this study whereas hybridization signals were good with SARCI645. Thus, SARCI645 was used in rest of the study. An important step in developing in situ hybridization protocols is to ensure that probes have access to the rRNA (Sorensen et al., 1997). Thus it could be concluded that SARCI645 probe could enter the cells and was hybridized to the ribosomes better.

MS821 and SARCI645 specific probes were used in combination with ARC915 in Figure 4.19a-b-c-d, 4.20a-b-c, and 4.21a-b-c-d for the identification of *Methanosarcina* spp. in the mesophilic UASB reactor. SARCI645 specific probe was also used in combination with ARC915 for the identification of *Methanosarcina* spp. in the psychrophilic UASB reactor in Figure 4.22a-b-c. High amounts of *Methanosarcina* spp. could be observed in both UASB reactors treating synthetic sewage at operation day 294. However, the density in psychrophilic granules was much higher compared to mesophilic granules. Results also indicated that the signals with SARCI645 probe were higher in psychrophilic granules compared to mesophilic granules. Although *Methanosarcina* spp. could be detected in mesophilic granules, their signals had very low intensity. Although only *Methanosaeta* spp. were identified and no signals of *Methanosarcina* spp. could be observed in seed sludge, high amounts of *Methanosarcina* spp. were detected as operation continued. This could be attributed to the fact that the number of *Methanosarcina* spp. could have increased due to the operation conditions. *Methanosarcina* spp. might have been found as single cells instead of clusters and they could have been found at non-detectable levels in the seed sludge.

Methanosaeta abundant in seed sludge were identified as the major methanogenic *Archaea* in both reactors treating synthetic sewage at all sampling days.

Methanosaeta and *Methanospirillum* have resistant fibrillar sheet layers, made up by protein and glycoprotein, surrounding an inner wall (Lange and Ahring, 2001).

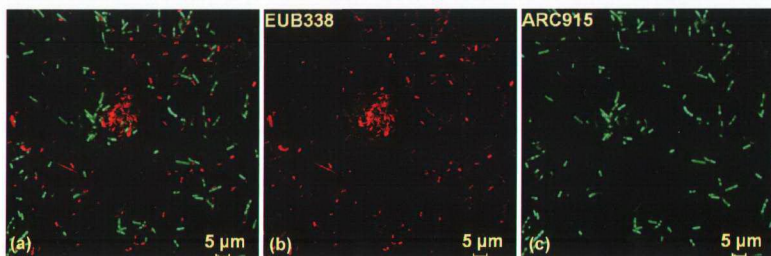


Figure 4.16: FISH results in psychrophilic UASB reactor (T=11°C, HRT=6.25 hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

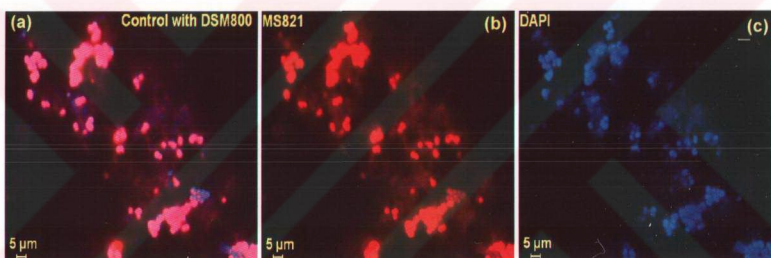


Figure 4.17: (a) Positive control with DSM800 for *Methanosarcina* specific probe MS821 (b) MS821 probe (Cy3-red) (c) stained with DAPI (blue)

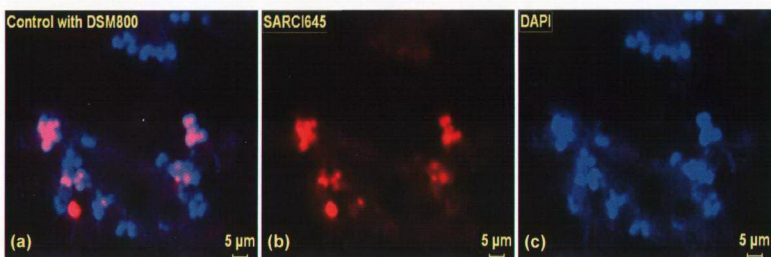


Figure 4.18: (a) Positive control with DSM800 for *Methanosarcina* specific probe SARC1645 (b) SARC1645 probe (Cy3-red) (c) stained with DAPI (blue)

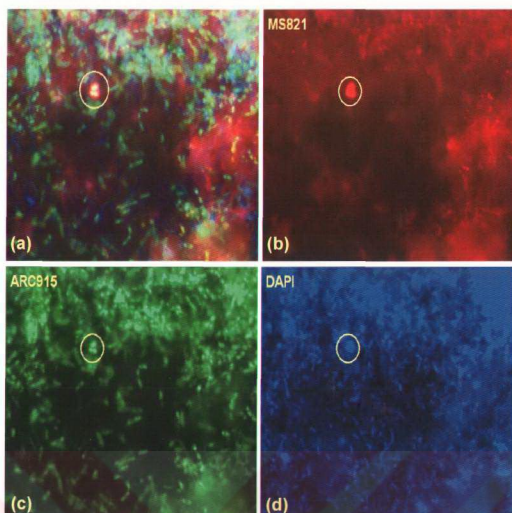


Figure 4.19: DAPI staining and FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (a) dual probing with MS821 (Cy3-red) and ARC915 (Cy5-green) (b) only MS821 signals (c) only ARC915 signals (d) all microorganisms (DAPI) (blue)

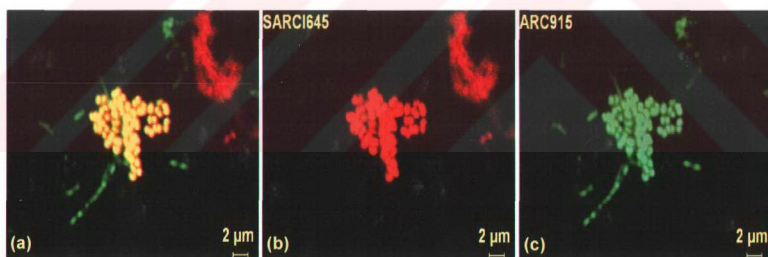


Figure 4.20: FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (a) dual probing with SARC1645 (Cy3-red) and ARC915 (Cy5-green) (b) only SARC1645 signals (c) only ARC915 signals

Methanosaeta-specific oligonucleotide probe MX825 was used and this probe had 100% homology to *M. concilii* and *Methanoxthrix soehngenii* 16S ribosomal gene sequences (Rocheleau et al., 1999). Since, there is only one mesophilic *Methanosaeta* species (*Methanosaeta concilii*) identified (Patel and Sprott, 1990), and morphologies of cells in seed sludge and in all samples taken from the

psychrophilic reactor are exceptionally comparable, temperature adaptation can be the single explanation of this circumstance. In a similar study, **van Lier et al. (1997b)** also found an intensive *Methanosaeta* population in a two stage expanded granular sludge blanket (EGSB) reactor treating partly acidified wastewater at 8°C.

Although *Methanosaeta* is known as a sensitive and slowly growing methanogen (**Jetten et al., 1990**), it is obvious that *Methanosaeta* species in the seed sludge of the mesophilic reactor did not loose their activities even though they were exposed to very low temperatures and HRT's. Dual probing with *Methanosaeta* (MX825) and *Archaea* (ARC915) from the mesophilic reactor with HRT's of around 4.8 and 6.25 hr are shown in Figure 4.23a-b-c and 4.24a-b-c, respectively. Dual probing with *Methanosaeta* (MX825) and *Archaea* (ARC915) at psychrophilic reactor with HRT's of around 4.8 hr is shown in Figure 4.25a-b-c. Acetoclastic *Methanosaeta* was the abundant methanogen and they were observed at high amounts at the operation day 294 at both mesophilic and psychrophilic UASB reactors treating synthetic sewage (Figure 4.23a-b-c and Figure 4.25a-b-c). *Methanosaeta* spp. indicated either short or long filamentous-shaped structures and they intended to come together as shown in Figure 4.24b. Although the density of *Methanosaeta* spp. in mesophilic and psychrophilic UASB reactors were the same at day 294, it was observed that *Methanosaeta* spp. were found in longer filamentous morphologies in the psychrophilic granules than in the mesophilic granules. Moreover in psychrophilic granules, a higher tendency in *Methanosaeta* to come together was observed.

At day 362, *Methanosaeta* cells decreased in both UASB reactors. However, their tendency to shift to shorter filaments was more apparent in mesophilic UASB reactor consequently losing their aggregating property. On the other hand, although the density of *Methanosaeta* spp. decreased in the psychrophilic UASB reactor at day 362, they still kept their long filamentous forms and cluster characteristics. *Methanosaeta* spp. may show two morphological forms: a filamentous type consisting of long multicellular rod-shaped microorganisms and a rod-shaped microorganisms consisting of fragments of up to five cells. It is reported that *Methanosaeta* spp. typically grow in the filamentous form under substrate limitation and results in sludge bulking and hence wash out from the system (**Schmidt and Ahring, 1999**).

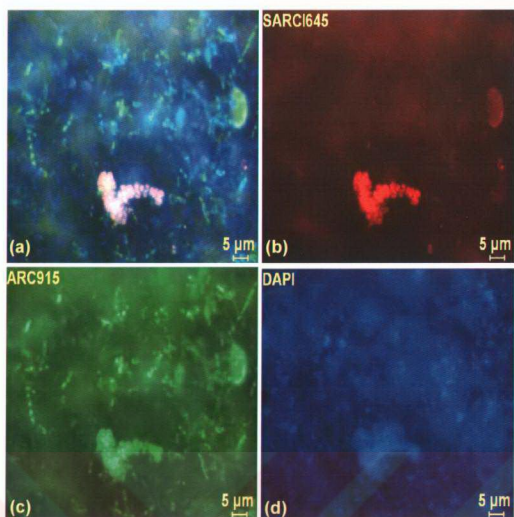


Figure 4.21:DAPI staining and FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (a) dual probing with SARCI645 (Cy3- red) and ARC915 (Cy5-green) (b) only SARCI645 signals (c) only ARC915 signals (d) all microorganisms (DAPI) (blue)

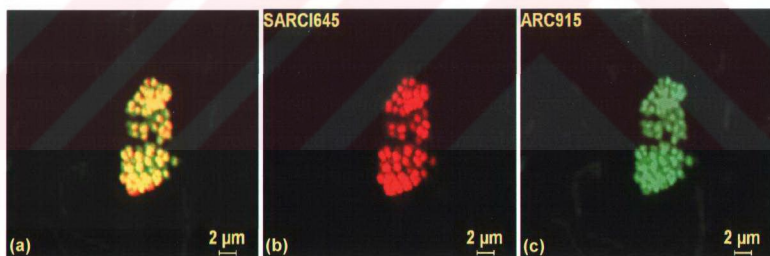


Figure 4.22: FISH results in psychrophilic UASB reactor treating synthetic sewage (T=16°C, HRT=4.8 hr) (a) dual probing with SARCI645 (Cy3-red) and ARC915 (Cy5-green) (b) only SARCI645 signals (c) only ARC915 signals

Schmidt and Ahring (1999) reported that the immobilization patterns of the two methanogens, namely *Methanosaeta* and *Methanosarcina* spp., were significantly different, indicating that the roles of the two methanogens are different in UASB granules. They also reported that *Methanosaeta concilii* grew only on existing support material in the reactor and did not self-immobilize. They can solely be immobilized if existing inert material or clumps of other microorganisms are present.

Methanosarcina mazeii, on the other hand, indicated a different immobilization pattern: in the bottom of the reactor where the acetate concentration was high, this organisms formed large clumps. These results show that the immobilization patterns of the two methanogens depend on the location in the granular layer and the availability of inert support material (Schmidt and Ahring, 1999).

Acetate is the precursor of approximately two-thirds of the methane produced in anaerobic bioreactors. Only two genera of methanogenic *Archaea* are known to use acetate as sole energy source: *Methanosarcina* and *Methanotherix* (recently known as *Methanosaeta*). *Methanosarcina* appears to be a generalist with a high growth rate, but low affinity for acetate. *Methanosaeta* is a specialist having a high affinity for acetate, but low growth rate (Jetten et al., 1992).

On the other hand, the specific probe MB310 for the identification of *Methanobacterium* like species (*Methanobacterium* spp., *Methanobrevibacter* spp., *Methanosphaera* spp.) was used. Domain *Methanobacterium* is a genus of anaerobic, rod-shaped *Methanobacteriaceae*. They are non-motile and use ammonia as the sole source of nitrogen. These methanogens are found in aquatic sediments, soil, sewage, and the gastrointestinal tract of animals. *Methanobacterium* and *Methanobrevibacter* belong to the family *Methanobacteriales* (Lange and Ahring, 2001). Positive control was applied to the specific probe MB310 by using pure culture DSM862 for *Methanobacterium bryantii* (Figure 4.26a-b-c) and MB310 probe worked well with the pure culture. Results indicated that *Methanobacterium* species could be found in very high numbers in both UASB reactors treating synthetic sewage at both temperatures (Figure 4.27a-b-c, Figure 4.28a-b-c, and Figure 4.29a-b-c-d).

The probe specific to most members of the kingdom Euryarchaeota (EURY498) was also used for the determination of *Methanosarcina* spp., *Methanobacterium* spp. and *Methanosaeta* spp. more precisely. The oligonucleotide EURY498 probe is complementary to almost all euryarchaeal sequences in the region between 498 and 510. It was reported that no addition of formamide was necessary to achieve optimal stringency (Burggraf et al., 1994).

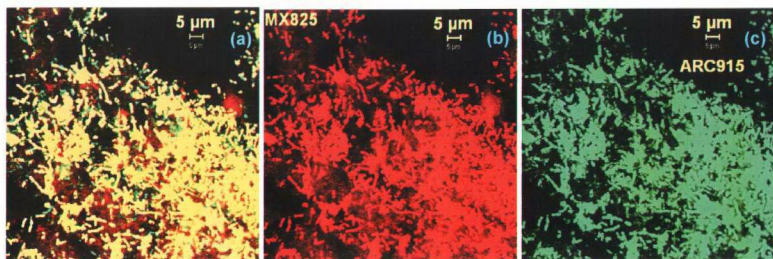


Figure 4.23: FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

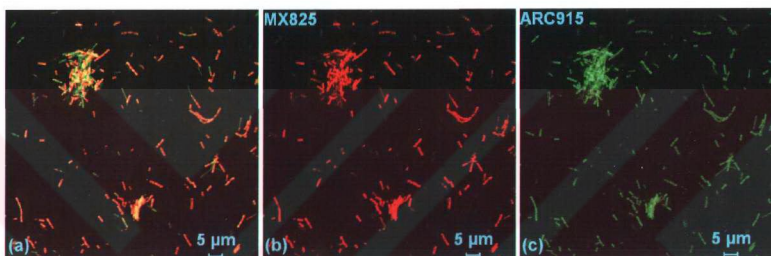


Figure 4.24: FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=6.25 hr) (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

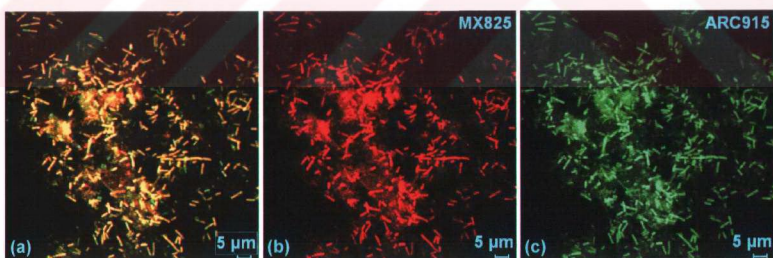


Figure 4.25: FISH results in psychrophilic UASB reactor (T=16°C, HRT=4.8 hr) (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

Oligonucleotide probes EURY498 in combination with ARC915 were used as dual probing and it was concluded that similar cells with EURY498 could be observed as with ARC915 as shown in Figure 4.30a-b-c. However, when the ARC915 probe was labelled with Cy5, the signals were very weak and it was very difficult to obtain

good photomicrographs. This might be attributed to the fact that 0% formamide ratio in the hybridization buffer could not give signals having strong intensities. Again *Methanosaeta* and *Mertanosarcina* like species could be observed by using dual probing with EURY (Cy3 labelled) and ARC915 (Cy5 labelled). Although *Mertanosarcina* spp. could be detected in mesophilic sludge samples, they were found in higher numbers in the psychrophilic sludge samples with EURY498.

Dual probing with SARC1645 (Cy3 labelled) and EURY498 (Cy5 labelled) was also applied. *Mertanosarcina* spp. were observed both at mesophilic (Figure 4.31a-b-c-d) and psychrophilic (Figure 4.32a-b-c) sludge samples treating synthetic sewage. Results indicated that *Mertanosarcina* spp. were more dense at psychrophilic UASB reactor compared to mesophilic UASB reactor. Higher amounts of *Mertanosarcina* spp. in the psychrophilic granules were also found out by using SARC1645 and ARC915 probes and FISH by EURY498 indicated similar results. However, it was observed that positive signals with SARC1645 were very weak which might be attributed to 0% formamide ratio in the hybridization buffer. Thus, it is recommended that since EURY498 probe gave best intensities at 0% formamide, this probe should be used individually instead of dual probing with other probes.

Specific probes MC1109 and MG1200 were also used for the identification of *Methanococcales* and *Methanogenium* relatives (*Methanomicrobium* spp., *Methanogenium* spp., *Methanoculleus* spp., *Methanospirillum* spp., *Methanocorpusculum* spp., *Methanoplanus* spp.), respectively. No positive hybridization signals were found with either MC1109 or with MG1200 probes. **Diaz et al. (2003)**, **Araujo et al. (2000)**, and **Montenegro et al. (2003)** have also reported negative hybridization results using both probes in biofilms developed using granular sludges from a brewery full scale UASB reactor, from a domestic sewage treatment plant, and from an anaerobic hybrid reactor treating pentachlorophenol with a mixture of fatty acids as inoculums, respectively. **Liu et al. (2002)** found only 1% *Methanococcales*. MC1109 is a specific probe for *Methanococcales* that have, until now only been isolated from marine environments. MG1200 is specific for methanogens that belong to the families *Methanomicrobiaceae*, *Methanocorpusculaceae* and *Methanoplanaceae* (included in the order *Methanomicrobiales*) limited to the use of H_2 and formate as substrates. Most of the members require a relatively high sodium ion concentration (**Diaz et al., 2003**).

Thus, it was expected not to detect these types of methanobacteria in the granular sludges used in this study.

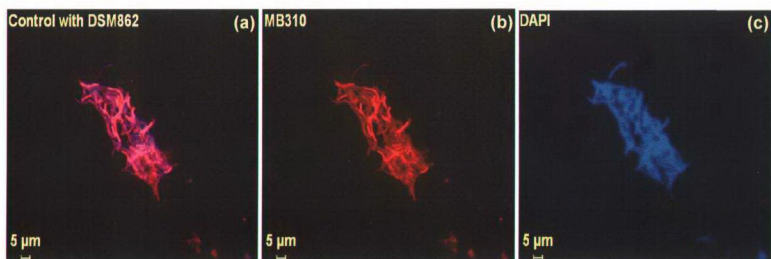


Figure 4.26: (a) Positive control with DSM862 for *Methanobacterium* specific probe MB310 (b) MB310 probe (Cy3-red) (c) stained with DAPI (blue)

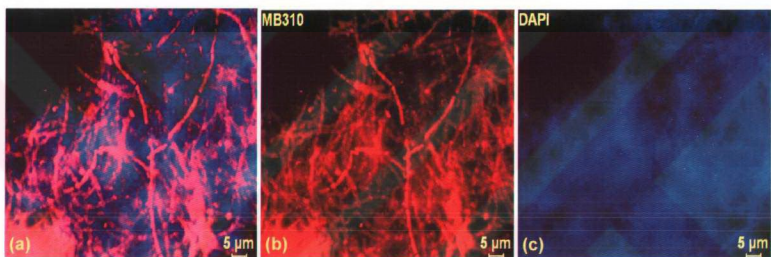


Figure 4.27: (a) DAPI staining and FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (b) only MB310 signals (c) all microorganisms (DAPI) (blue)

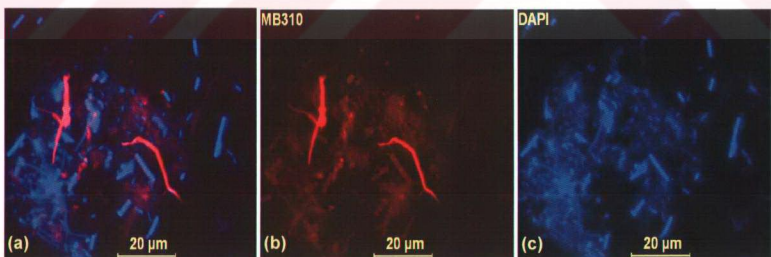


Figure 4.28: (a) DAPI staining and FISH results in mesophilic UASB reactor treating synthetic sewage (HRT=4.8 hr) (b) only MB310 signals (c) all microorganisms (DAPI) (blue)

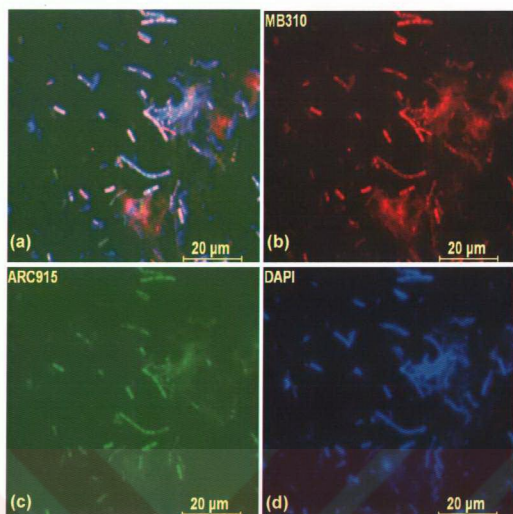


Figure 4.29: DAPI staining and FISH results in psychrophilic UASB reactor treating synthetic sewage ($T=11^{\circ}\text{C}$, $\text{HRT}=6.25$ hr) (a) dual probing with MB310 (Cy3- red) and ARC915 (Cy5-green) (b) only MB310 signals (c) only ARC915 signals (d) all microorganisms (DAPI) (blue)

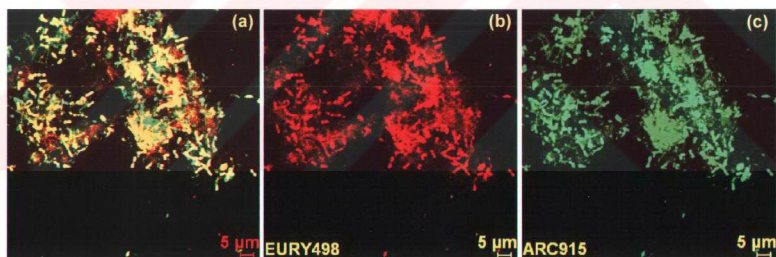


Figure 4.30: FISH results in mesophilic UASB reactor treating synthetic sewage ($\text{HRT}=4.8$ hr) (a) dual probing with EURY498 (Cy3-red) and ARC915 (Cy5-green) (b) only EURY498 signals (c) only ARC915 signals

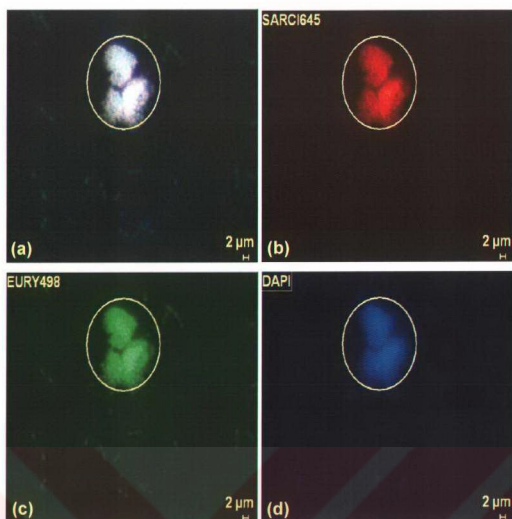


Figure 4.31: DAPI staining and FISH results in mesophilic UASB reactor (HRT=4.8 hr) (a) dual probing with SARC1645 (Cy3- red) and EURY498 (Cy5-green) (b) only SARC1645 signals (c) only EURY498 signals (d) all microorganisms (DAPI) (blue)

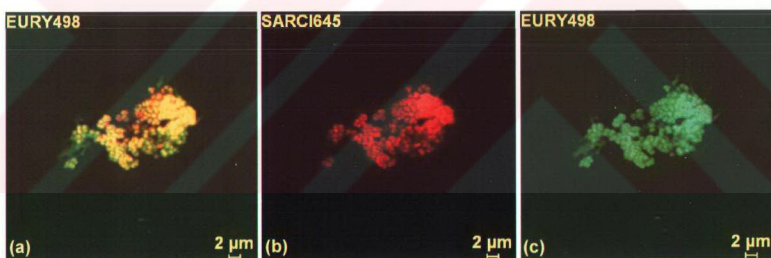


Figure 4.32: FISH results in psychrophilic UASB reactor (T=16°C, HRT=4.8 hr) (a) dual probing with SARC1645 (Cy3- red) and EURY498 (Cy5-green) (b) only SARC1645 signals (c) only EURY498 signals

4.3.3 FISH results with raw domestic sewage

In-situ hybridization results showed that archaeal cells representing the methanogens were also intensively dominant in the bottom sampling ports of both mesophilic and psychrophilic UASB reactors treating raw sewage. The results of dual probing with the general probes for bacteria (EUB338-Cy3 labeled) and *Archaea* (ARC915-Cy5 labeled) indicated that bacterial and archaeal cells could be detected and good signals could be obtained with these two probes in mesophilic UASB reactor at HRT values of 9.25 hr and 7.7 hr, respectively (Figure 4.33a-b-c and Figure 4.34a-b-c). Dual probing with ARC915 (Cy3 labeled) and EUB338 (Cy5 labeled) was also applied to psychrophilic sludge samples and FISH photomicrographs are shown in Figure 4.35a-b-c and Figure 4.36a-b-c, respectively. These photomicrographs indicated that *Methanosarcina* (Figure 4.35b) and *Methanosaeta* (Figure 4.36b) like species could be apparently detected with ARC915 probe in psychrophilic granules. In some cases, the length of archaeal and bacterial cells could be observed as 15 μm (Figure 4.36b) and 20 μm (Figure 4.36c), respectively. Bacterial cells indicated either short and/or long filamentous or coccus (Figure 4.36c) forms and some of the coccus-shaped bacterial cells were found as cocci. Besides vibrio shaped bacterial cells could also be observed. Results indicated that bacterial cells were denser in the psychrophilic sludge than in the mesophilic sludge treating raw domestic sewage. The bacterial cells decreased at a great extent in the mesophilic UASB reactor and positive signals with EUB338 probe were not observed at high numbers. Overall, bacterial cells in mesophilic granules treating raw sewage were detected in much lower numbers compared to mesophilic granules treating synthetic sewage. This proved that wastewater composition significantly affected the microbial cells.

The decrease in bacterial cells was also observed in psychrophilic granules when pre-settled raw sewage was fed into the reactor. However, this decrease was not as drastic as in the case of mesophilic granules. It was also observed that more vibrio shaped bacterial cells were found as clusters in the psychrophilic UASB reactor. Although the density of the bacterial cells in the psychrophilic granules decreased after the substrate was changed to pre-settled raw sewage, the cells could still be detected in high numbers compared to the bacterial cells in the mesophilic granules. Figure 4.37a-b-c indicated that significant amounts of bacterial and archaeal cells

still prevailed in psychrophilic granules. When dual probing with EUB338 and ARC915 was applied to psychrophilic sludge (Figure 4.38a), it was observed that bacterial cells came together forming clusters (Figure 4.38b). *Methanosaeta* like species were very obvious at a temperature of 14°C (HRT=9.25 hr) (Figure 4.38c).

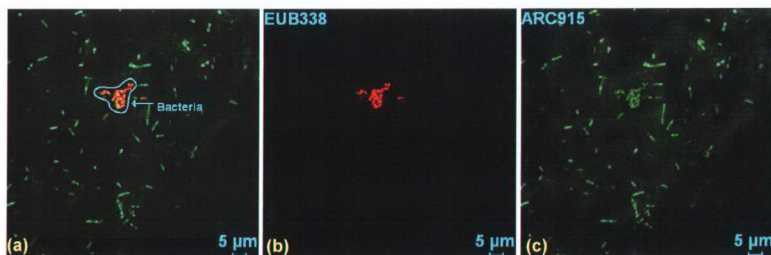


Figure 4.33: FISH results in mesophilic UASB reactor treating raw sewage (HRT=9.25 hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

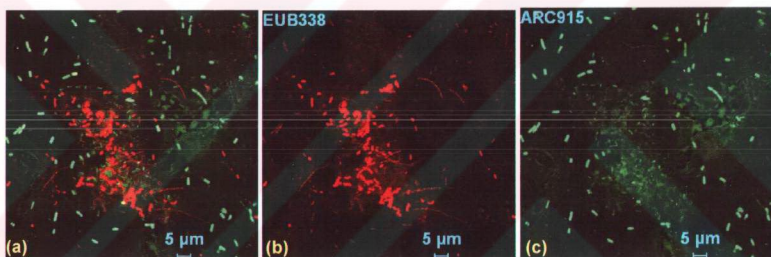


Figure 4.34: FISH results in mesophilic UASB reactor treating raw sewage (HRT=7.7 hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

Dual probing with ARC915 and EUB338 indicated that *Methanosaeta* like species intended to come together to form clusters in psychrophilic granules (Figure 4.39a-b) after fed with mixed raw sewage. Figure 4.39c presented that bacterial cells having different morphological structures could be seen at a temperature of 13.5°C. Besides, *Methanosaeta* and *Methanosarcina* like species could also be detected using ARC915 probe (Figure 4.40a-b) and significant amounts of bacterial cells could be observed (Figure 4.40c).

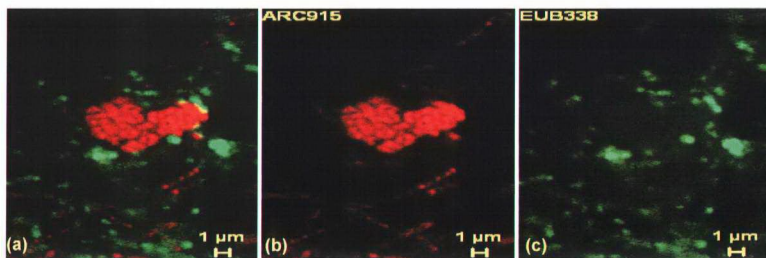


Figure 4.35: FISH results in psychrophilic UASB reactor treating pre-settled raw sewage ($T=14^{\circ}\text{C}$, $\text{HRT}=9.25$ hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

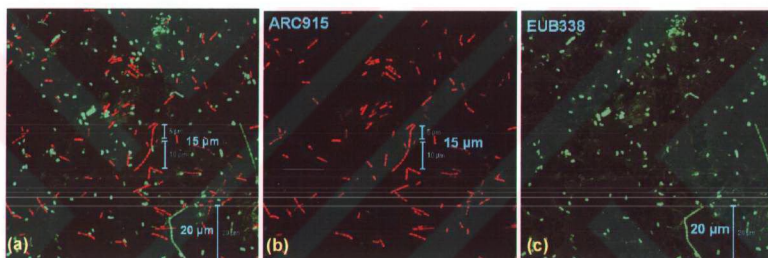


Figure 4.36: FISH results in psychrophilic UASB reactor treating pre-settled raw sewage ($T=14^{\circ}\text{C}$, $\text{HRT}=9.25$ hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

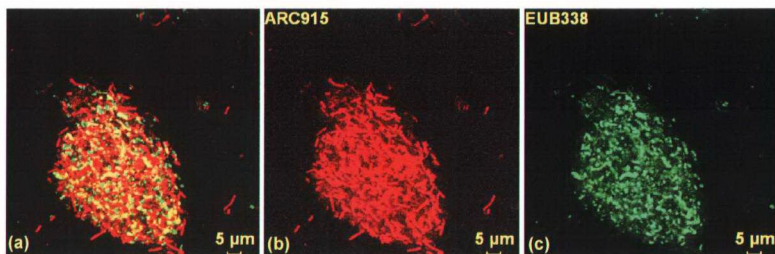


Figure 4.37: FISH results in psychrophilic UASB reactor treating pre-settled raw sewage ($T=14^{\circ}\text{C}$, $\text{HRT}=9.25$ hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

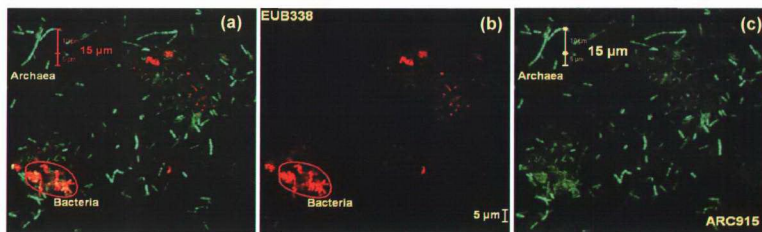


Figure 4.38: FISH results in psychrophilic UASBR treating pre-settled raw sewage ($T=14^{\circ}\text{C}$, $\text{HRT}=9.25$ hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

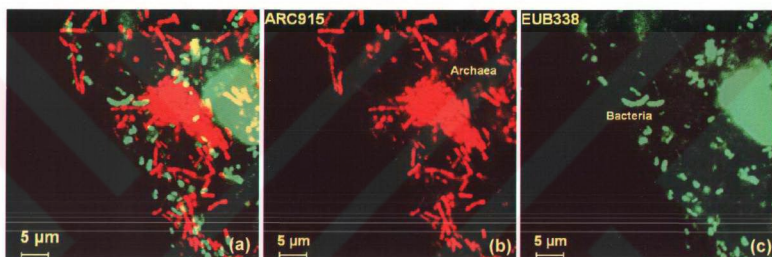


Figure 4.39: FISH results in psychrophilic UASBR treating raw sewage ($T=13.5^{\circ}\text{C}$, $\text{HRT}=7.7$ hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

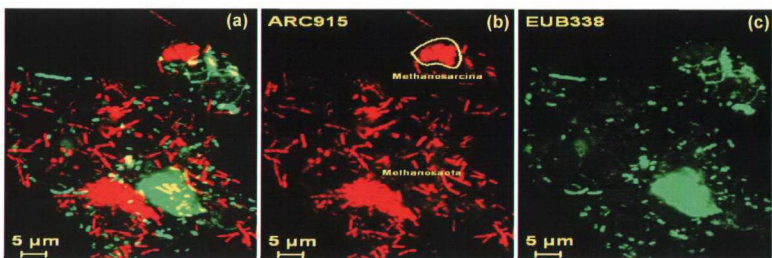


Figure 4.40: FISH results in psychrophilic UASBR treating raw sewage ($T=13.5^{\circ}\text{C}$, $\text{HRT}=7.7$ hr) (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals

Dual probing with EUB338 (Cy3 labelled) and ARC915 (Cy5 labelled) in psychrophilic granules also indicated several bacterial cells coming together as clusters (Figure 4.41a-b) and *Methanosaeta* like species could be detected at such low temperature as shown in Figure 4.41c. Besides, although the psychrophilic UASB reactor was not operated for about 5 months and started to be operated afterwards, significant amounts of archaeal and bacterial cells could still be detected (Figure 4.42a-b-c) in psychrophilic granules. However, almost no microbial cells could be observed in the mesophilic UASB reactor (figure not shown).

On the other hand, specific probe SARC1645 for *Methanosarcina* like species was used for the granules treating raw sewage. For hybridization, each Cy3 labelled probe was hybridized by hybridization buffers having 0%, 15%, 20%, and 35% formamide concentrations for ensuring the optimal hybridization stringency. It was observed that formamide concentration did not give any significant effect on FISH results. SARC1645 specific probe was used in combination with ARC915 in Figure 4.43a-b-c and Figure 4.44a-b-c at HRT values of 9.25 hr and 7.7 hr in psychrophilic UASB reactor treating pre-settled and mixed domestic sewage, respectively. Although the density of *Methanosarcina* spp. decreased after raw sewage was fed into both UASB reactors, the decrease in the psychrophilic UASB reactor was not as drastic as in the mesophilic UASB reactor. Almost no *Methanosarcina* clumps could be detected in mesophilic granules fed with pre-settled raw sewage. On the other hand, *Methanosarcina* spp. clusters could still be observed in psychrophilic granules treating both pre-settled and mixed raw sewage. It could be concluded that morphology of *Methanosarcina* spp. might have been changed from clumps to single cells in mesophilic granules after raw sewage feeding. It was reported that changes in morphology from clumps to single cells of *Methanosarcina* spp. cause the granules to disintegrate (Schmidt and Ahring, 1999).

Methanosaeta abundant in seed sludge were also identified as the major methanogenic *Archaea* in both reactors treating raw sewage at all sampling days. *Methanosaeta*-specific oligonucleotide probe MX825 was used. Although *Methanosaeta* species in granular sludge taken from the mesophilic UASB reactor treating synthetic sewage were detected in high numbers, their density decreased after fed with pre-settled raw sewage (Figure 4.45a-b-c).

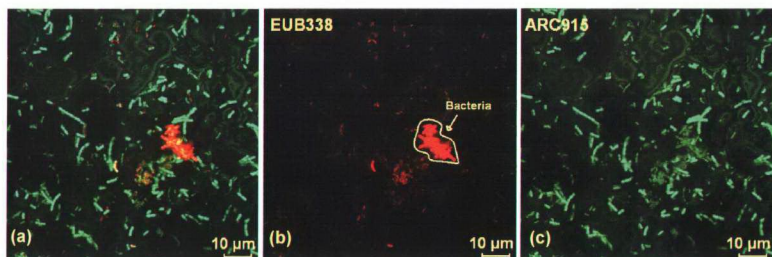


Figure 4.41: FISH results in psychrophilic UASB reactor treating raw sewage (T=13.5°C, HRT=7.7 hr) (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

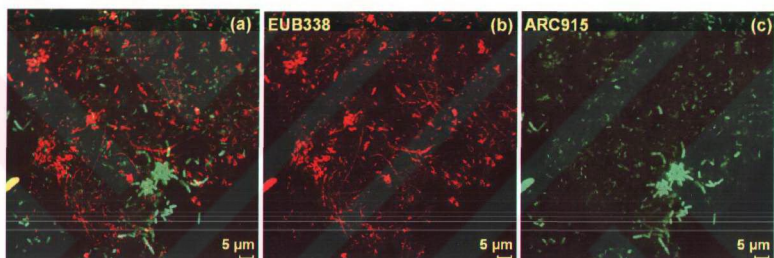


Figure 4.42: FISH results in psychrophilic UASB reactor treating raw sewage (a) dual probing with EUB338 (Cy3-red) and ARC915 (Cy5-green) (b) only EUB338 signals (c) only ARC915 signals

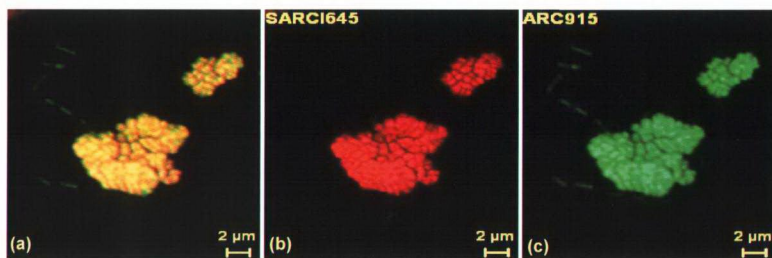


Figure 4.43: FISH results in psychrophilic UASB reactor treating pre-settled raw sewage (T=14°C, HRT=9.25 hr) (a) dual probing with SARCI645 (Cy3-red) and ARC915 (Cy5-green) (b) only SARCI645 signals (c) only ARC915 signals

Methanosaeta related species prevailing in seed sludge have noticeably lost their long filamentous forms and they deteriorated when raw sewage was fed into the reactors. When pre-settled raw sewage was fed into UASB reactors, the microbial structures were not affected very seriously. However, when completely mixed raw sewage was fed into UASB reactors, the long filamentous forms of *Methanosaeta* shifted to shorter forms and their densities decreased apparently (Figure 4.46a-b-c). The decrease in the long filamentous form and density of *Methanosaeta* spp. was observed much more and more apparently in the mesophilic granules compared to psychrophilic granules. Figure 4.46a-b-c and Figure 4.47a-b-c indicated the difference between the mesophilic and psychrophilic granules taken at the same experimental time and conditions.

Both UASB reactors were not operated for about 5 months and started to be fed with raw pre-settled sewage after this period. They were operated for about 2 months afterwards. When FISH technique was applied to the granular sludge sample taken from the mesophilic UASB reactor, it was observed that almost no *Methanosaeta* spp. were present (figure not shown). On the other hand, although their density decreased at a great extent, there was still *Methanosaeta* spp. present in the psychrophilic UASB reactor (Figure 4.48a-b-c). However, their densities and cluster forms decreased and the long filamentous forms deteriorated and shifted to shorter filamentous forms. This concluded that instead of temperature, the morphology of cells was affected very much due to the lack of a stable operation. After two months of operation with raw sewage, it was observed that the density of *Methanosaeta* spp. in the mesophilic granules decreased much more compared to the *Methanosaeta* spp. in the psychrophilic granules. In the psychrophilic granule, still long and cluster forms of *Methanosaeta* spp. could be observed, whereas in the mesophilic granule their filamentous forms were shorter which indicated deterioration. This could be attributed to the fact that psychrophilic granules were more tolerant to unstable conditions.

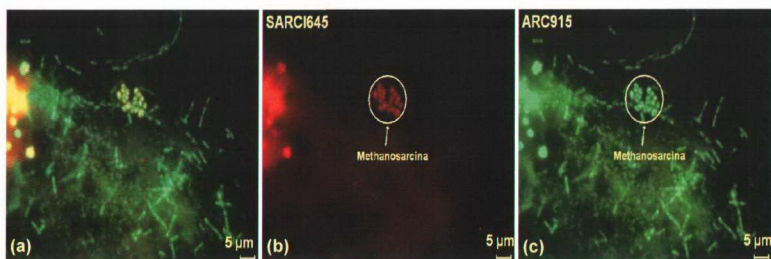


Figure 4.44: FISH results in psychrophilic UASB reactor treating raw sewage (T=13.5°C, HRT=7.7 hr) (a) dual probing with SARC1645 (Cy3-red) and ARC915 (Cy5-green) (b) only SARC1645 signals (c) only ARC915 signals

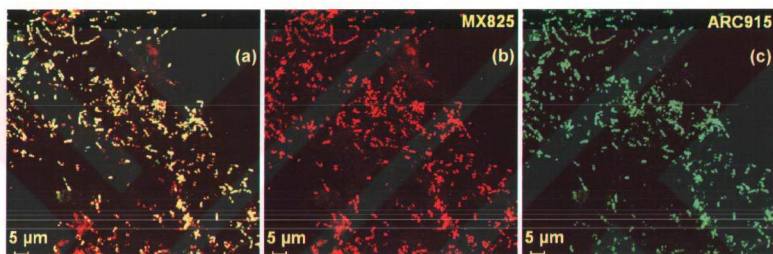


Figure 4.45: FISH results in mesophilic UASB reactor treating pre-settled raw sewage (HRT=9.25 hr) (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

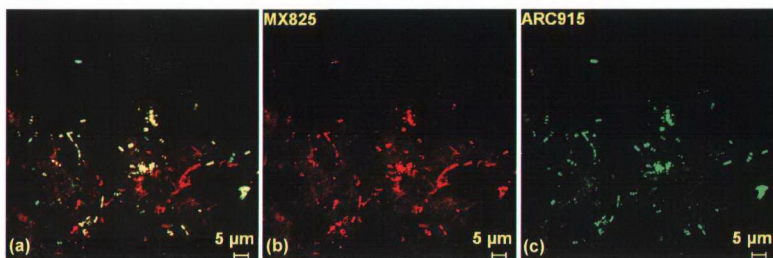


Figure 4.46: FISH results in mesophilic UASB reactor treating raw sewage (HRT=7.7 hr) (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

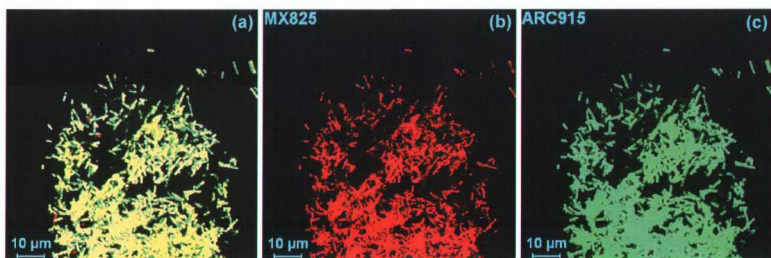


Figure 4.47: FISH results in psychrophilic UASB reactor treating raw sewage (T=13.5°C, HRT=7.7 hr) (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

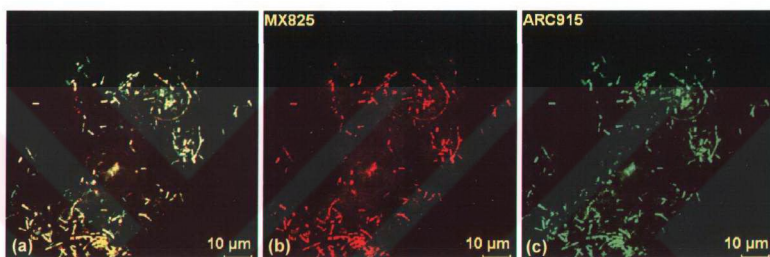


Figure 4.48: FISH results in psychrophilic UASB reactor treating raw sewage (a) dual probing with MX825 (Cy3-red) and ARC915 (Cy5-green) (b) only MX825 signals (c) only ARC915 signals

On the other hand, the specific probe MB310 was used for *Methanobacterium* species in the sludge samples treating raw sewage. Results indicated that *Methanobacterium* species could be detected at high amounts in the mesophilic (Figure 4.49a-b-c and Figure 4.50a-b-c) and psychrophilic UASB reactors (figure not shown) treating raw sewage. Results indicated at all sampling times that *Methanobacterium* spp. could be detected at high amounts in both UASB reactors. Although their densities decreased in mesophilic granules, *Methanobacterium* species could still be detected at higher amounts compared to other microbial species such as *Methanosaeta* and *Methanosarcina* species.

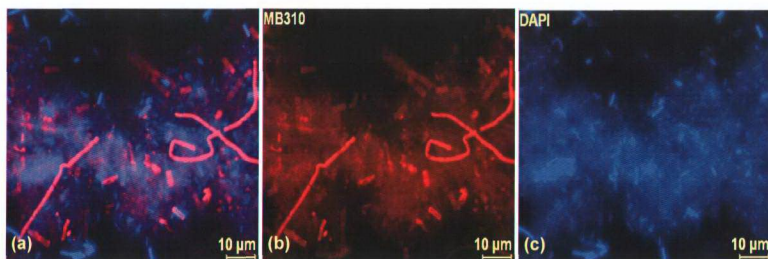


Figure 4.49: (a) DAPI staining and FISH results in mesophilic UASB reactor treating pre-settled raw sewage (HRT=9.25 hr) (b) only MB310 signals (c) all microorganisms (DAPI) (blue)

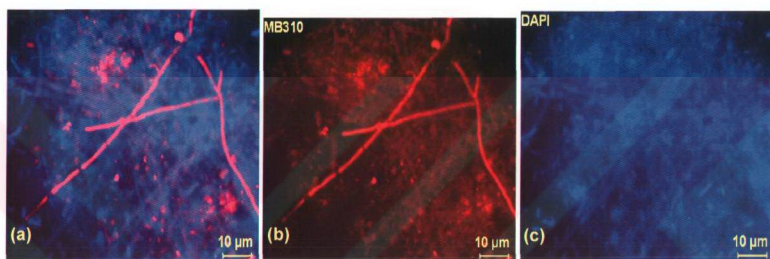


Figure 4.50: (a) DAPI staining and FISH results in mesophilic UASB reactor treating pre-settled raw sewage (HRT=9.25 hr) (b) only MB310 signals (c) all microorganisms (DAPI) (blue)

The probe specific for most Euryarchaeota (EURY498) was also used. *Methanosaeta* and *Methanosarcina*-like species were also detected by EURY498 in the granules taken from the mesophilic and psychrophilic UASB reactors treating raw sewage. Figure 4.51a-b-c also indicated short filamentous morphology of *Methanosaeta* spp. in the mesophilic UASB reactor. *Methanosarcina* like species, on the other hand, were very obvious in the psychrophilic UASB reactor treating pre-settled raw sewage (Figure 4.52a-b-c-d).

Specific probes MC1109 and MG1200 were also used for the identification of *Methanococcales* and *Methanogenium* relatives, respectively. No positive hybridization signals with either MC1109 or with MG1200 probes were found in the mesophilic and psychrophilic UASB reactors treating raw sewage.

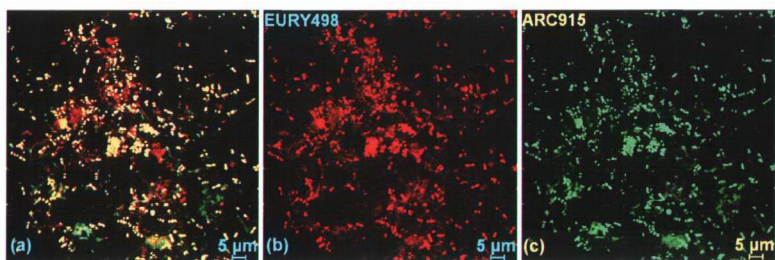


Figure 4.51: FISH results in mesophilic UASB reactor treating pre-settled raw sewage (HRT=9.25 hr) (a) dual probing with EURY498 (Cy3-red) and ARC915 (Cy5-green) (b) only EURY498 signals (c) only ARC915 signals

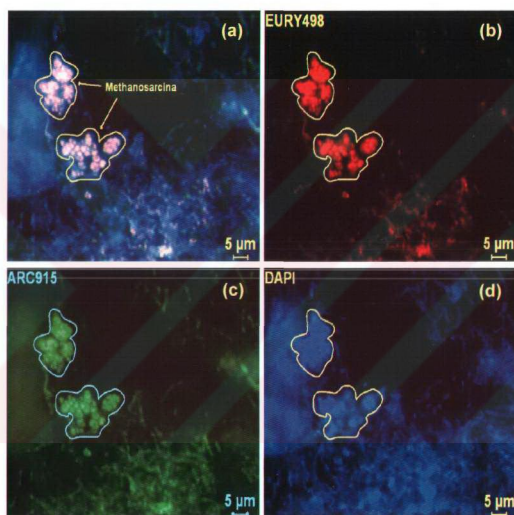


Figure 4.52: FISH results in psychrophilic UASB reactor treating pre-settled raw sewage (T=14°C, HRT=9.25 hr) (a) dual probing with EURY498 (Cy3-red) and ARC915 (Cy5-green) (b) only EURY498 signals (c) only ARC915 signals (d) all microorganisms (DAPI) (blue)

4.3.4 Granule structures in both UASB reactors

Granule samples were taken for the evaluation of the changes in the granule structures of the UASB reactors treating synthetic sewage. The diameters of the granules at both UASB reactors taken at all sampling times were varied between 1200-2200 μm . The first samples were taken at the end of the operation with an HRT value of about 11 hour. Results indicated that the granules still remained without disturbance and floc structure formations did not occur both in mesophilic (Figure 4.53a) and in sub-mesophilic (Figure 4.53b) UASB reactors. Almost no ciliated protozoa could be observed in mesophilic granules whereas *Colpidium* spp. ciliated protozoas could be detected in the sub-mesophilic granules in each image. The second samples were taken at the end of the operation at an HRT=8.65 hr. Results indicated no significant disturbance in the granules and flock structure formations did not occur both in mesophilic (Figure 4.53c) and in sub-mesophilic (Figure 4.53d) UASB reactors. Very few protozoa could be observed in both reactors in each image. Biomass formation could be observed around the granules; however this was more apparent in mesophilic granules. Algae type microorganisms were detected in both reactors which were more visible in the sub-mesophilic UASB reactor.

The sludge samples were also taken at the end of the study which was done by synthetic sewage. The HRT was around 0.23 d and the temperature of the reactor was reduced to $10\pm1^{\circ}\text{C}$ whereas the temperature of the mesophilic UASBR was $35\pm2^{\circ}\text{C}$. Some *Colpidium* spp. and *Paramecium* spp. protozoas could be observed in each image and no significant disturbance could be observed in mesophilic granules. It was also observed that organic materials were adhered on the granules in the mesophilic UASB reactor. On the other hand, too many organic material (probably biomass) and *Colpidium* spp. ciliated protozoa amounts were observed around the granules as well as formation of crystallized structure could be observed in the bulk solution of the psychrophilic UASB reactor (Figure 4.53e-f). More particulate matter was detected and the mucus or pellicle structure which was seen at the top of the psychrophilic reactor also indicated high amounts of protozoa in the reactor.

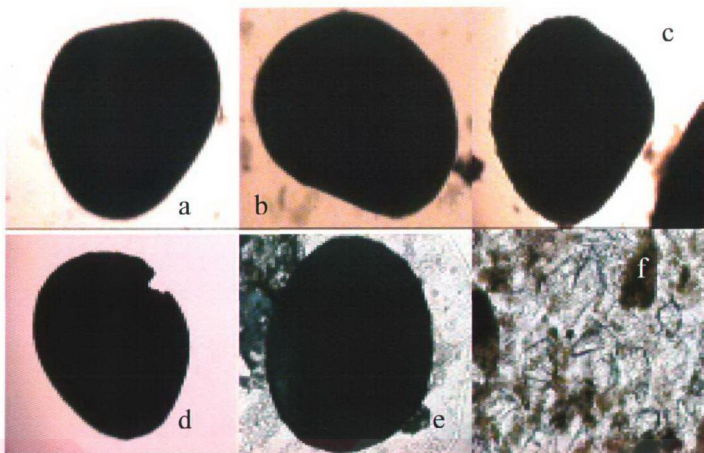


Figure 4.53: View of the granules in (a) mesophilic reactor (HRT $\cong$ 0.45 day) (b) sub-mesophilic UASB reactor (HRT $\cong$ 11 hr) (c), mesophilic reactor (HRT $\cong$ 8.65 hr) (d) sub-mesophilic reactor (HRT $\cong$ 8.65 hr) (e) psychrophilic reactor (HRT $\cong$ 5.5 hr) (f) bulk solution of psychrophilic UASB reactor (HRT $\cong$ 5.5 hr)

In Table 4.7 and 4.8, comparisons of the FISH results at different time and operating conditions treating synthetic and raw domestic sewage, respectively are presented.

4.3.5 The Z-stacking study with non-homogenized granules

Whole granulation study was done using the advanced image acquisition Z-stacking module at LSM 510 META Laser Scanning Microscopy. With this module you can generate Z-stacks. The Z-stack collects a series of XY-images along the Z axis. The software controls the Z-drive of a motorized microscope in exact steps synchronizing with the image. The focusing steps can be determined by the user or they can be computed automatically. The advantage of this module is the optimal detection of information in the third dimension. In addition, with the OrthoView function, even the entry level version of AxioVision provides a highly effective technique for the Z-stack analysis. A Z-stack is a sequence of optical sections collected at different levels from a specimen using computer controlled stepping in the Z (vertical) direction by preset distances. From the Z-stack, the various patterns of staining at different levels of the specimen can be observed.

Table 4.7: Comparison of the FISH results at different time and operating conditions treating synthetic domestic sewage (R1: Mesophilic UASB reactor, R2: Sub-mesophilic and psychrophilic UASB reactor)

Time (from start-up)	Operating Conditions							Remarks
	HRT Range (hour)	T (°C)	COD _{tot} Removal (%)				pH _{avr} (-)	
			R1	R2	R1	R2		
Synthetic Sewage	5.5-29	35±2	16-26	71-90	69-84	7.70	7.50	• significant amounts of archaeal and bacterial cells in R1 and R2 • archaeal cells representing the methanogens (ARC915) intensively dominant in R1 and R2 • archaeal and bacterial cells live together in R1 and R2 • <i>Methanosaeta</i>, <i>Methanosarcina</i>, <i>Methanobacterium</i> like species detected by general probe ARC915 in R1 and R2 • density of <i>Methanosarcina</i> species in the granules of R2 much higher compared to R1 • <i>Methanosarcina</i> cells were normally clusters and individual small and/or large clumps • <i>Methanosaeta</i> abundant in seed sludge also identified as the major methanogenic archaea in R1 and R2 • <i>Methanosaeta</i> spp. indicated short and/or long filamentous structures sometimes coming together • density of <i>Methanosaeta</i> spp. in R1 ≈ R2 • <i>Methanosaeta</i> spp. had longer filamentous forms and their filamentous structures more apparent in R2 • significant amounts of <i>Methanobacterium</i> species in R1 and R2 • no positive hybridization signals with either MC1109 (<i>Methanococcus</i> species) or with MG1200 (<i>Methanomicrobium</i> relatives) probes
Day 294 (Part I)								
OLR=0.56-2.37 kgCOD/m ³ .day								

Table 4.7: Comparison of the FISH results at different time and operating conditions treating synthetic domestic sewage (R1: Mesophilic UASB reactor, R2: Sub-mesophilic and psychrophilic UASB reactor) (continued)

Time (from start-up)	Operating Conditions							Remarks
	HRT _{avT} (hour)	T _{avT} (°C)		Average COD _{tot} Removal (%)		pH _{avT} (-)		
		R1	R2	R1	R2	R1	R2	
Synthetic Sewage	6.5-12.5	35±2	10.8	85-90	75-82	7.70	7.35	• bacterial cells decreased in R1 and R2 • more bacterial cells in R2 • <i>Methanosaeta</i> cells decreased in R1 and R2 • <i>Methanosaeta</i> cells shifted to shorter filaments which were more apparent in R1 • <i>Methanosaeta</i> cells still kept their long filamentous forms and cluster characteristics in R2 • much more <i>Methanosarcina</i> spp. in R2 • <i>Methanobacterium</i> species in very high numbers in R1 and R2 • no positive hybridization signals with either MC1109 (<i>Methanococcus</i> species) or with MG1200 (<i>Methanomicrobium</i> relatives) probes
Day 362 (Part II)								
OLR=1,05-2,05 kgCOD/m ³ .day								

The Z-stack of optical sections can be processed relatively easily into a 3D representation of the specimen. They are designed for analysis of living or fixed biological specimens in a multi-user environment. Using the “Z-Stack” software module, both microscopes can scan through a sample and obtain one or more high resolution optical sections. Users then have the option of utilizing a Z-Stack to render a 3D reconstruction of the sample. The microscopes can provide images of live or fixed cells and tissue sections of varying thickness (URL 3.2). For Z-stacking, non-homogenized granules were used. The sample preparation was explained in Section 3.2.3.3 briefly.

Anaerobic degradation is a multi-step process consisting of hydrolysis, acidogenic fermentation, acetogenesis, and methanogenesis. Slow growing *Methanosaeta* usually have a competitive advantage over *Methanosarcina* at low concentrations of acetate (Rocheleau et al., 1999). Arcand et al. (1994) observed a clear predominance of fermentative bacteria in the external layer of the anaerobic granules which was consistent with the multilayer microstructural model of cleaved granules. In contrast, acetoclastic activities were evenly distributed along the granule depth even though Scanning Electron Microscopy (SEM) had shown the core of granules of various sources to be almost exclusively composed of *Methanosaeta*-like bacteria. They also developed a detailed distributed diffusion-reaction mathematical model for predicting local substance and biomass concentrations at any depth within the granular biofilm. The model predicted pH values 1 mm inside the granule to be over one pH unit more alkaline than in the bulk liquid. Hence this significant pH gradient could be the major factor which gives a competitive advantage for *Methanosaeta*-like population to proliferate in the granule core.

It was reported that syntrophic bacterial associations would be located between an external envelope made of an heterogeneous population in the three-layered structure of the sucrose-fed granule. In the external envelope, predominately H₂-producing acidogens, and also including H₂-utilizing microorganisms such as *Methanosarcina*, *Methanococcales* and *Methanospirillum*-like species were observed. Aceticlastic core, on the other hand, was composed almost exclusively of *Methanosaeta*-like organisms (Guiot et al., 1992).

Table 4.8: Comparison of the FISH results at different time and operating conditions treating raw domestic sewage (R1: Mesophilic UASB reactor, R2: Sub-mesophilic and psychrophilic UASB reactor)

Time (from start-up)	Operating Conditions						Remarks
	HRT _{av} (hour)	T _{av} (°C)	Average COD _{tot} Removal (%)			pH _{av}	
		R1	R2	R1	R2	R1	R2
Raw Sewage	9.25	35±2	14	58	39	7.88	7.55
Day 22 (Pre-settled) OLR=0.7 kgCOD/m ³ .day							- archaeal cells representing the methanogens (ARC915) intensively dominant in R1 and R2 - bacterial cells were denser in R2 than in R1 - density of <i>Methanosarcina</i> spp. decreased in R1 and R2 - almost no <i>Methanosarcina</i> clumps could be detected in R1 <i>Methanosarcina</i> spp. were much more in R2 compared to R1 - density of <i>Methanosaeta</i> species from R1 and R2 decreased <i>Methanobacterium</i> species could be detected at high amounts in R1 and R2 - no positive hybridization signals with MC1109 and MG1200 probes
Day 58 (Mixed) OLR=0.7 kgCOD/m ³ .day	7.5	35±2	13.5	49	35	7.78	7.46
							- bacterial cells decreased at a great extent in R1 - bacterial cells were denser in R2 than in R1 - long filamentous forms of <i>Methanosaeta</i> shifted to shorter forms in R1 and R2 - decrease in the long filamentous form and density of <i>Methanosaeta</i> spp. was much more in R1 than R2 - almost no <i>Methanosarcina</i> clumps were detected in R1 <i>Methanosarcina</i> spp. were still detected at much more numbers in R2 compared to R1 <i>Methanobacterium</i> species decreased in R1 - more <i>Methanobacterium</i> species in R2 compared to R1 - no positive hybridization signals with MC1109 and MG1200 probes

On the other hand, no layered structure was detected in the UASB reactors treating propionate, ethanol, glutamate, sugar refinery wastewaters, and methanol waste. Bacteria other than *Methanosarcina* and *Methanosaeta* occupied the outer most layers, *Methanosarcina barkeri* was present in the underlying layer, followed by a layer of *M. concilii* and an inner core composed of *M. concilii* and other bacteria at anaerobic reactor treating methanol (Rocheleau et al., 1999). Diaz et al. (2003) observed different structural features at granular sludge at a UASB reactor treating different type of wastewaters. Densely packed microcolonies, loose colonies, channels and holes were observed.

In this study, dual probing with ARC915 (Cy3 labeled) and EUB338 (Cy5 labeled) was applied to the non-homogenized granules treating both synthetic and raw sewage. Different structural features of both mesophilic and psychrophilic granular sludges were observed such as densely packed microcolonies, channels and holes (Figure 4.54a and Figure 4.54b).

A primary spatial distribution of archaea and bacteria could also be observed by FISH. Bacteria always appeared in the outer layer of the granules, while archaea were always located in the interior as also reported by Diaz et al. (2003) and Sekiguchi et al. (1999). This could be attributed to the fact that facultative bacteria in the outer layer consumed oxygen (higher metabolic activity) and prevented its diffusion into the granule, therefore protecting the strict anaerobes, such as methanogenic archaea, from oxygen toxicity.

Two images were shown in Figure 4.55a-b-c and Figure 4.55d-e-f. Both images belong to the same non-homogenized psychrophilic granule treating synthetic sewage ($T=11^{\circ}\text{C}$, $\text{HRT}=6.25\text{ hr}$) at distances of $0.63\text{ }\mu\text{m}$ and $11.9\text{ }\mu\text{m}$, respectively. Figure 4.55c and Figure 4.55f indicated that the density of bacteria decreased as the z-stacking distance increased. Figure 4.56 and Figure 4.57 indicated that bacteria always appeared in the outer layer of the granules, while *Archaea* were always located as z-series images went to deeper distances. *Archaea* and bacteria were labeled with Cy3 and Cy5 and they were represented by red and green colors, respectively. In Figure 4.56 and Figure 4.57, it could be seen that green was the dominant color showing that the outer layer was mainly composed of bacteria. Since green was disappearing and red was becoming the dominant color at higher

distances, it could be concluded that *Archaea* were located in much higher numbers in the inner layers towards the core. However, the last images in Figures 4.56 and 4.57 did not indicate the cores and it was not easy to comment on the exact structures of microbiological species at the core of the granules. Nevertheless, a reliable estimation could be done with these figures.

4.3.6 Quantification of UASB granules

Quantification study was done using Image Pro-plus Version 4.0 which is the leading image analysis software. It can capture, process, measure, analyze, archive, communicate, and customize the images. Image-Pro Plus is the ultimate image analysis software package for fluorescence imaging, quality assurance, materials imaging, and various other scientific, medical, and industrial applications. The most powerful member of the Image-Pro software family, Image-Pro Plus includes extensive enhancement and measurement tools and allows you to write application-specific macros and plug-ins. The software is based on area measurements which were performed on CLSM images. The average of ten CSLM images for each sample were used for quantification in this study. The print out on computer screen when the program is running is shown in Figure 4.58. In Figure 4.58, 'image 1' represented the first image of the sample which would be used for quantification. 'Red', 'Green', and 'Blue' images represented the microorganisms stained with Cy3, Cy5, and DAPI, respectively. The application of Image Pro-plus Version 4.0 program for anaerobic cultures was not very easy due to the multispecies microconsortia in anaerobic reactors. Besides, the microbial species were found in very dense numbers which also made the application of the program difficult.

Methanosaeta spp. and *Archaea* were quantified in mesophilic and psychrophilic sludge granules. Results indicated significant amounts of *Methanosaeta* spp. at Day 294 at both reactors. *Methanosaeta* spp./*Archaea* ratios for mesophilic and psychrophilic UASB reactors were calculated as 71% and 84%, respectively. This findings agreed with the confocal scanning laser microscope examinations, in which cells morphologically resembling *Methanosaeta* were predominantly observed in granular sludges.

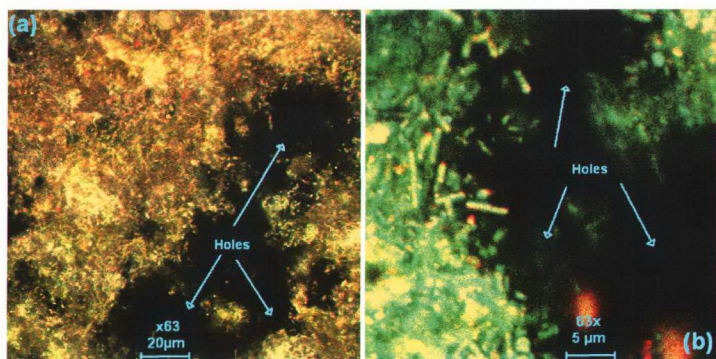


Figure 4.54: Whole granule images among the confocal Z-series images (a) mesophilic granule treating synthetic sewage (HRT=6.25 hr) at a distance of 5.5 μm (b) psychrophilic granule treating raw sewage at a distance of 0.71 μm stained with ARC915 (Cy3-red) and EUB338 (Cy5-green)

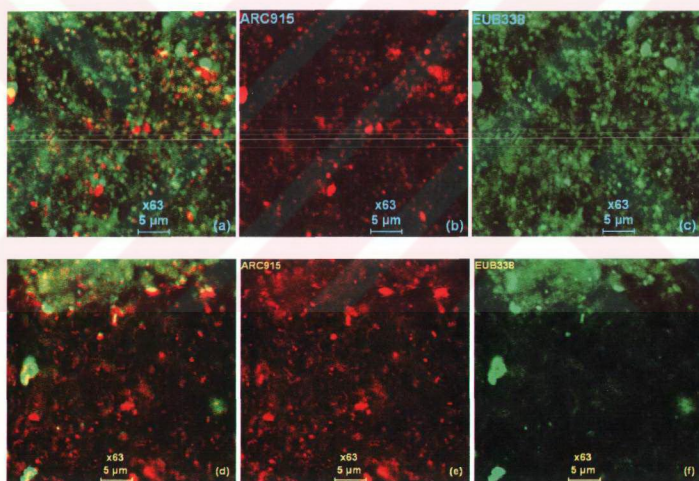


Figure 4.55: Non-homogenized psychrophilic granule treating synthetic sewage ($T=11^{\circ}\text{C}$, HRT=6.25 hr) at a distance of 0.63 μm (a) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (b) only ARC915 signals (c) only EUB338 signals; and at a distance of 11.9 μm (d) dual probing with ARC915 (Cy3-red) and EUB338 (Cy5-green) (e) only ARC915 signals (f) only EUB338 signals

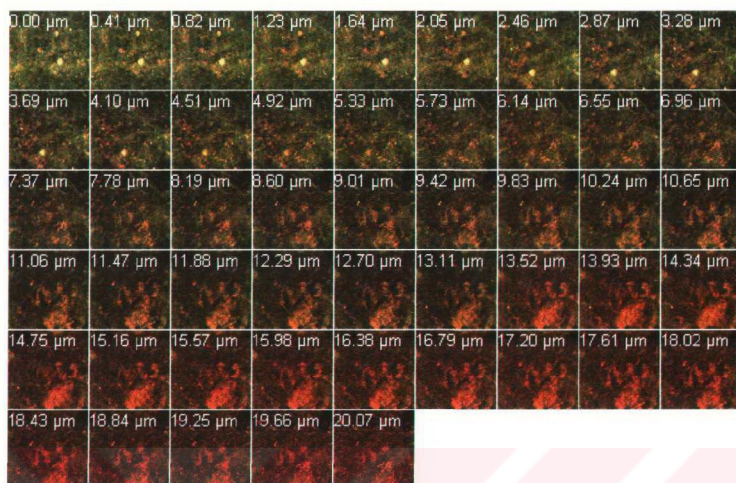


Figure 4.56: Confocal Z-series images of mesophilic granule treating synthetic sewage (HRT=6.25 hr) stained with Cy3 (red) labelled ARC915 and Cy5 (green) labelled EUB338

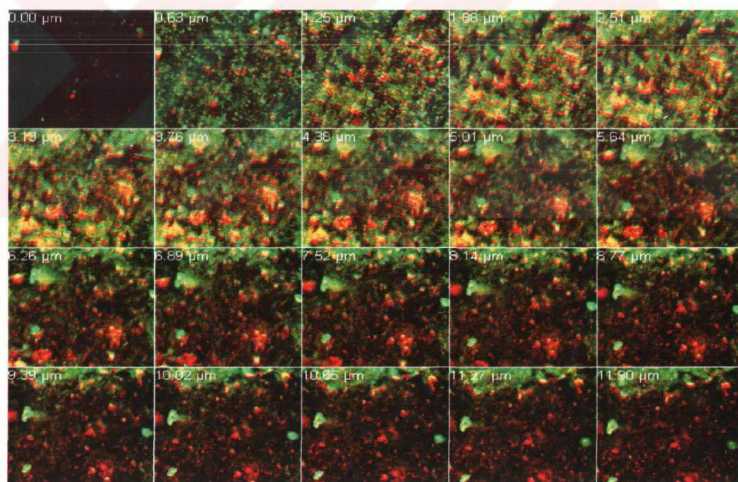


Figure 4.57: Confocal Z-series images of psychrophilic granule treating synthetic sewage (T=11°C, HRT=6.25 hr) stained with Cy3 (red) labelled ARC915 and Cy5 (green) labelled EUB338

On the other hand, archaeal cells detected with probe ARC915 were found in significant numbers, which was around 60% of the DAPI-stained cells at both reactors. Results indicated slight decreases in *Methanosaeta* spp./*Archaea* ratios at Day 362 at the end of the study with synthetic sewage.

When both reactors were fed with pre-settled and mixed raw sewage, the ratios of *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI were calculated. *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI ratios were calculated as 68% and 48% in mesophilic granule, respectively. In psychrophilic granule, *Methanosaeta* spp./*Archaea* ratio still calculated as 84%. *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI ratios did not indicate any significant decrease at psychrophilic granule, however *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI ratios decreased at a great extent at mesophilic granules during mixed sewage feeding. *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI ratios at mesophilic UASB reactor were found as 61% and 30%, respectively. On the other hand, *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI ratios at psychrophilic UASB reactor were found as 80% and 51%, respectively. In conclusion, *Methanosaeta* spp./*Archaea* and *Archaea*/DAPI ratios decreased after raw sewage was fed into both reactors, however this decrease was observed much more in mesophilic granules compared to psychrophilic granules. These findings also matched with the FISH images.

FISH results together with CSLM showed that the microbial community of anaerobic granular sludge was dominated by methanogenic *Archaea*. *Methanosaeta* abundant in seed sludge were identified as the major methanogenic *Archaea* in both reactors treating synthetic and raw sewage at all sampling days. *Methanosaeta* related species prevailing in seed sludge have noticeably lost their long filamentous forms and they deteriorated when raw sewage was fed into the reactors. When pre-settled raw sewage was fed into UASB reactors, the microbial structures were not affected very seriously which could also be seen from *Methanosaeta* spp./*Archaea* ratios in both reactors. However, when completely mixed raw sewage was fed into UASB reactors, the long filamentous forms of *Methanosaeta* shifted to shorter forms and their densities decreased significantly. This decrease was observed much more (*Methanosaeta* spp./*Archaea* was around 61%) and more apparently in the mesophilic granules compared to psychrophilic granules (*Methanosaeta* spp./*Archaea* was around 80%). FISH results revealed that the percentage of cells

morphologically similar to *Methanosaeta* decreased from 71% to 61% of the DAPI-stained cells at mesophilic granules, whereas *Methanosaeta* decreased from 84% to 80% of the DAPI-stained cells at psychrophilic granules after raw sewage feeding. Archaeal group was detected with *Archaea* specific probe (ARC915) and accounted for 60% and 30% of the DAPI-stained cells at mesophilic granules before and after feeding with raw sewage, respectively. Archaeal cells, on the other hand, accounted for 60% and 51% of the DAPI-stained cells at psychrophilic granules before and after feeding with raw sewage, respectively.

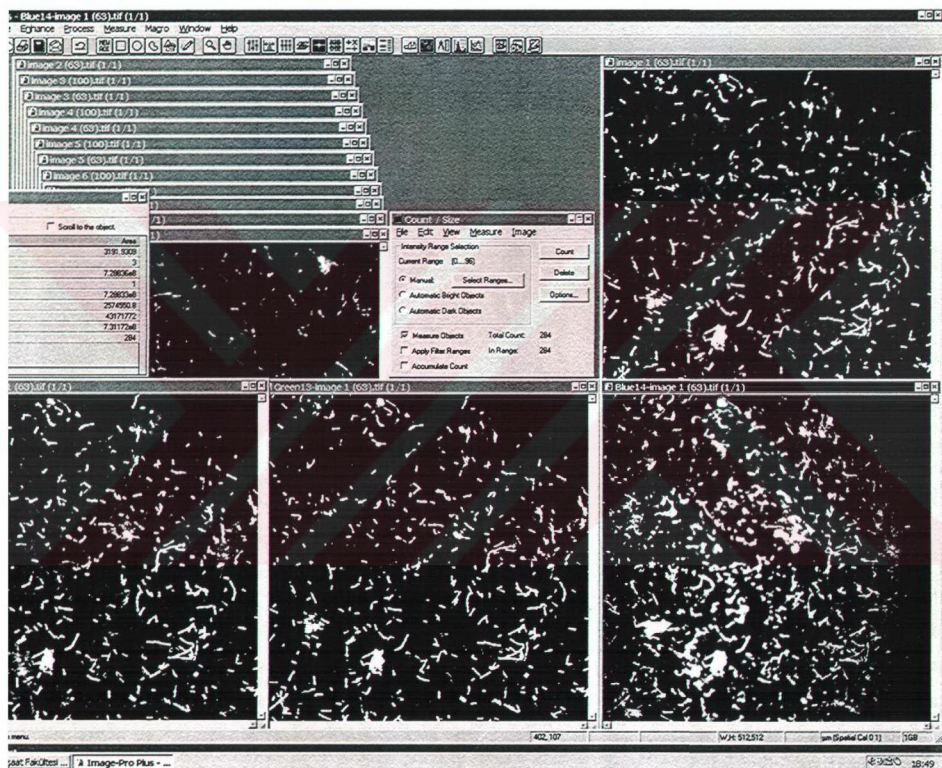


Figure 4.58: An example of the application of Image Pro-plus Version 4.0 program to one of the images

5. CONCLUSIONS AND RECOMMENDATIONS

The effect of temperature on the UASB reactor performance under relatively moderate to low temperature conditions treating low strength synthetic and raw sewage was investigated. The results were compared with a second UASB reactor performance operated under mesophilic temperature. The direct treatment of low strength wastewaters such as domestic sewage by an anaerobic process is without doubt an attractive and appropriate alternative. Low energy requirement during operation, low initial investment cost, lower sludge production, and easier maintenance make anaerobic systems more attractive than conventional aerobic processes, especially for developing countries such as Turkey. The UASB process performance was investigated at HRT values as low as 6.5 hr and 7.5 hr and at temperatures as low as $10\pm1^{\circ}\text{C}$ and 13°C for synthetic and raw domestic sewage, respectively. Results indicated a satisfactory performance in the psychrophilic UASB reactor treating synthetic sewage. Even at the lowest temperature at $10\pm1^{\circ}\text{C}$, the UASB reactor was able to achieve an average COD_{tot} removal of over 80% at an OLR value of $2.05 \text{ kgCOD/m}^3\cdot\text{day}$. On the other hand, around 92% average COD_{tot} removal could be observed in the mesophilic UASB reactor at the same OLR value. However, very low COD_{tot} removals were observed in both UASB reactors treating raw sewage. Total COD removals were obtained as 49% and 35% for the mesophilic and psychrophilic reactors, respectively. Since higher COD removals were reported in several studies, the reason for low efficiencies in this study could be very dilute influent characteristics of the raw sewage due to sea water addition during sampling times. The sewage used in this study could be characterized as a very low-strength domestic wastewater. It was observed that the COD_{tot} removal was significantly affected by the influent COD concentration rather than the operational temperature. Very low COD_{sol} removals were found in both UASB reactors treating raw sewage as well. It was reported that anaerobic treatment is of interest only for relatively concentrated municipal wastewaters having a COD value above 500 mg/l (Mergaert et al., 1992). The particulate COD in the influent was almost removed by means of

the entrapment effect of the sludge bed, and the solubilization rate of the entrapped organics was dependent on temperature. Most of the effluent COD was part of the soluble fraction indicating a low participation of biological removal processes in the overall performance. Hence most of the removal at this stage was due to physical processes like solids entrapment, adsorption, settling and filtration which was also reported by **Rodriguez et al. (2001)**.

The biogas yields indicated a similar trend in both reactors when HRT was reduced to 5.5 hr (OLR=2.37 kgCOD/m³.day). The temperature in the psychrophilic reactor was around 16°C at this OLR value. On the other hand, gas production was reduced at a great extent when the temperature of the reactor was reduced to 10±1°C. Although an improvement was observed in the biogas yield as operation continued at this temperature, the biogas yields indicated much lower values in the psychrophilic UASB reactor compared to the values in the mesophilic UASB reactor. The carbon dioxide content of the sub-mesophilic and psychrophilic UASB reactors indicated lower values compared to the mesophilic UASB reactor at the temperature values below 19°C. On the other hand, results indicated almost no biogas productions in both reactors in the case of either pre-settled or mixed raw domestic sewage. Obviously, the low influent COD resulted in low substrate levels inside the reactor and also in a low biogas production rate. This also implied low mixing intensities in the reactor and consequently a poor substrate-biomass contact (**Lettinga et al., 2001**). Besides, a considerably large fraction of methane and carbon dioxide gases might have been escaped in dissolved form in the effluent (**Uemura and Harada, 2000**).

Effects of low temperatures on UASB reactor performances treating low strength synthetic and raw sewage were also investigated in terms of the variations in methanogenic diversity using the 16S rRNA/DNA based FISH technique. Qualitative and quantitative diversity of microorganisms present in anaerobic granular sludges fed with synthetic and raw domestic sewage operated at different temperatures were done. Besides, the structure of these granules have been investigated using FISH combined with CSLM.

According to FISH results, archaeal cells representing methanogens were found intensively dominant in the bottom sampling ports of the reactors fed with synthetic

and raw domestic sewage and acetoclastic *Methanosaeta* was the abundant methanogen. Other methanogens such as *Methanosarcina* and *Methanobacterium* like species were also observed in both reactors. The abundance of originally mesophilic *Methanosaeta*-related *Archaea* under such low temperatures in all sampling days revealed the microbial adaptation to psychrophilic conditions. This might be attributed to the enzymatic alterations in *Methanosaeta* cells originated from seed sludge, which were exposed to sub-mesophilic temperatures at start-up and then to psychrophilic conditions parallel to gradual decrease in temperature. Obviously, the mesophiles in the seed sludge could still grow successfully under low temperature conditions and the psychrophilic homologs in the sludge were not able to manifest in these temperature-activity profiles because of the presence of many mesophiles (Lettinga et al., 2001). Moreover, quite high COD removals obtained in the anaerobic treatment of low-strength wastewater verified the presence of high methanogenic activity in the psychrophilic UASB reactor as an indication of adaptation. It has been reported that anaerobic bacteria can adapt quite easily to low temperatures and a high-rate anaerobic treatment can be achieved at psychrophilic conditions (Singh and Viraraghavan, 2001).

In samples taken from both psychrophilic and mesophilic reactors, the granules remained undisturbed and kept their rigidity even under psychrophilic conditions treating synthetic sewage. Diameters of the granules varied between 1200-2200 µm. Although *Methanosaeta* is known as a sensitive and slowly growing methanogen (Jetten et al., 1990), it is obvious that *Methanosaeta* species in the seed sludge of the mesophilic reactor did not loose their activities even though they were exposed to such low temperatures and hydraulic retention times during the study with the synthetic sewage. In both reactors the microbial diversity did not significantly change, while COD removal efficiencies dropped dramatically. However, long filamentous morphologies of *Methanosaeta* cells shifted to shorter filaments, consequently loosing their aggregating property, especially in granules from the mesophilic UASB reactor as the OLR increased. The tendency to shift to shorter filamentous forms was observed much less in psychrophilic granules. According to CSLM observation, even though the sludge retained in the reactor kept its granular form as a whole, the majority of the granules had a tendency to be partly broken and they lost their rigidity when raw sewage was fed into the UASB reactors. Besides,

Methanosaeta related species prevailing in seed sludge have noticeably lost their long filamentous forms and deteriorated during raw sewage feeding. Lower cell densities might have been due to long-term exposure to starvation conditions which was also reported by Uemura and Harada (2000). The *Methanosaeta* species almost disappeared and shifted from long filamentous form to short filament fragments, with a much lower density of viable cells after two months of operation with raw sewage at the mesophilic UASB reactor. On the other hand, the decrease in the viable cells and the increase in the shorter filamentous forms of *Methanosaeta* species in psychrophilic granules were not as dramatic as in mesophilic granules after raw sewage feeding.

Methanosaeta was the predominant species in both reactors, while *Methanosarcina* and *Methanobacterium* could be detected in significant amounts. Significant *Methanosarcina* spp. could be observed in both UASB reactors treating synthetic sewage. However, the density in psychrophilic granules was much higher compared to mesophilic granules. Results also indicated that the signals with the SARC1645 probe were stronger in the psychrophilic UASB reactor compared to the mesophilic reactor. The density of *Methanosarcina* spp. decreased significantly after raw sewage was fed into both UASB reactors. Almost no *Methanosarcina* clumps could be detected after pre-settled raw sewage feeding into mesophilic UASB reactor. It could be concluded that the morphology of *Methanosarcina* spp. was changed from clumps to single cells in mesophilic granules after raw sewage feeding. Since *Methanosaeta* indicates a much lower minimum threshold for acetate utilization than *Methanosarcina*, it can be concluded that *Methanosarcina* is found in environments with higher acetate concentrations and low acetate concentrations favour *Methanosaeta*. Higher amounts of *Methanosarcina* in psychrophilic granules could be attributed to higher acetate concentrations in the UASB reactor operated at low temperatures. Members of the order *Methanobacteriales* (MB310) constituted the major hydrogenotrophic methanogens present in both UASB reactors, whereas the other hydrogenotrophic methanogens-members of the order *Methanococcales* and *Methanogenium* relatives-were absent. Thus, no positive hybridization signals with either MC1109 or with MG1200 probes were found as was reported by several authors.

A multi-layered structure was observed in the granules in which channels and holes as well as densely packed micro-colonies could be detected. From the results, it was concluded that the outer shell of the granules were composed mainly of bacteria, while both *Archaea* and bacteria were observed going through the inner core. Quantification results indicated that *Methanosaeta* spp./*Archaea* ratios for the mesophilic and psychrophilic UASB reactors were calculated as 71% and 84%, respectively treating synthetic sewage which decreased after raw domestic sewage feeding.

Psychrophilic UASB reactors operated without heating is considered as an attractive and a feasible option in the treatment of dilute wastewaters, discharged at moderate to low temperatures. **Ritmann and Mc Carty (2001)** also reported that with dilute wastewaters at ambient temperature, the methane produced may be insufficient to raise the wastewater temperature, and operation at ambient wastewater temperature may be the economical option. Quite high COD removals obtained in this study during the treatment of low-strength synthetic sewage verified the presence of high methanogenic activity in the psychrophilic UASB reactor as an indication of adaptation. Besides, in addition to classical operational parameters, process performances of UASB reactors may be evaluated by tracking the variations in microbial diversity with FISH technique.

Anaerobic treatment is effective in removing biodegradable organic compounds, leaving mineralized compounds like ammonium, phosphate and sulfur in the solution. These compounds therefore have to be removed by an additional post-treatment step to meet sufficiently the criteria for a sustainable environmental protection. Besides it was reported that no pathogen removal could be achieved at low temperatures. Thus, anaerobic treatment of low strength wastewaters by UASB reactor should be considered as a pre-treatment alternative. Experiences in full scale UASB reactors in several tropical countries have indicated that treatment of domestic wastewater is feasible with reasonable organic removal. However, post-treatment is necessary to achieve disposal standards and significant bacteria removal. The sludge from UASB reactors, in general, has good dewatering capacity, but its long term effect on the environment has to be assessed. Efforts should also be made to find proper post-treatment method for UASB effluents. Aerobic polishing ponds appear to be the most commonly used method for the treatment of UASB reactors effluents

treating domestic wastewater to meet effluent quality standards (Tare et al., 1997). Arceivala (1995), on the other hand, made a cost evaluation and compared the use of UASB reactor with other methods which have been generally used for treatment of municipal wastewater such as conventional activated sludge process, extended aeration and aerated lagoon. It was reported that the UASB type plant was the cheapest while aerated lagoons stood second in terms of capitalized costs (i.e., capital and operating costs). In terms of annual operation and maintenance costs alone, the UASB was the cheapest among the four methods because of the least power requirement. Power is only needed for the slight post-aeration of UASB effluent. The benefits from power saving is so substantial that even if no gas recovery is obtained at low temperatures, the UASB becomes cheaper than other processes. Mergaert et al. (1992) also evaluated cost estimates for a plant of 100,000 inhabitant equivalents (IE) for different domestic wastewater treatment systems and reported that UASB reactor indicated nearly half expensive as the costs of capital, operation and maintenance, and sludge disposal compared to oxidation ditch, trickling filter and activated sludge system. Thus, due to operational simplicity and low cost of the system, it is believed that UASB type reactor combined with a post-treatment can become a very attractive alternative for low strength domestic sewage (having a COD concentration around 500 mg/l) treatment especially in small villages, particularly in Turkey where in rural areas wastewater treatment plants are rare. Effluents of UASB reactor after polishing may be used for irrigation. However, effluents should comply with the criteria given for the 'treated wastewaters to be used for irrigation' in the Technical Bulletin of Turkish Water Pollution Control Regulation.

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CURRICULUM VITAE



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