ELUCIDATION OF AMMONIA INHIBITION IN ANAEROBIC TREATMENT PROCESS BY USING 16S rRNA/DNA BASED MICROBIAL IDENTIFICATION TECHNIQUES

by

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Dedicated to my wife Meltem and my family ...

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ABSTRACT

A high ammonia landfill leachate was anaerobically treated for more than 1000 days in two laboratory scale anaerobic reactors configured as upflow sludge blanket and hybrid bed. By adjusting the influent pH to 4.5 to keep the free ammonia concentrations below inhibitory level, COD removal efficiencies above 90% were achieved in both of the reactors. However, when pH adjustment was terminated severe free ammonia inhibitions were observed several times. To clearly elucidate the inhibition phenomena, effects of high ammonia concentrations on reactor performances were correlated to the variations in methanogenic diversity by using 16S rRNA/DNA based microbial identification techniques such as FISH (fluorescent in-situ hybridization), DGGE (denaturing gradient gel electrophoresis), cloning and DNA sequencing. Nevertheless, sudden and unexpected fluctuations in the characteristics of leachate resulted in complications during interpretation of the results. To prevent the inconvenience of fluctuations, five laboratory scale anaerobic reactors seeded with different sludges were operated for 450 days under stable COD loads, gradually increasing total ammonia levels from 1000 to 6000 mg/l. Parallel to elevation in total ammonia, free ammonia nitrogen (FAN) was increased step by step from 50 to 750 mg/l. After gradual adaptation, moderately high COD removal efficiencies in the range of 77 to 90% were obtained in the reactors. *Methanosaeta*-related species mainly prevailing in seed sludges were replaced by *Methanosarcina* when FAN exceeded 100 mg/l. Subsequently, as FAN level elevated rather than any shifts in the methanogenic community, single coccus shaped *Methanosarcina* cells formed stringent multicellular packets. However, when the FAN exceeded 600 mg/l, disintegration of *Methanosarcina* clusters was observed. In contrast, inorganic particles originated from seed sludge provided a good support for *Methanosarcina* clusters in R4 and with this special feature it successfully resisted to FAN as high as 750 mg/l.

ÖZET

Yüksek amonyak ihtiva eden bir katı atık depo sahası sızıntı suyu 1000 günden fazla bir süreyle yukarı akışlı çamur yatağı ve hibrit olarak dizayn edilen iki laboratuar ölçekli anaerobik reaktörde arıtılmıştır. Serbest amonyak konsantrasyonunu inhibisyon seviyesinin altında tutmak için girişte pH 4.5'e ayarlanmış ve her iki reaktörde de %90'nın üzerinde KOİ giderimi gerçekleştirilmiştir. Ancak, pH ayarlamasından vazgeçilince, bir çok defa ciddi serbest amonyak inhibisyonları görülmüştür. İnhibisyon olayını daha net açıklayabilmek için, yüksek amonyak konsantrasyonunun reaktör performansı üzerindeki etkileri, 16S rRNA/DNA bazlı FISH (flüoresan in-situ hibridizasyon), DGGE (denatüre gradyan jel elektroforezi), klonlama ve DNA dizi analizi teknikleri kullanılarak tespit edilen metanojen dağılımı ile ilişkilendirilmiştir. Ancak, sızıntı suyu kompozisyonundaki ani ve beklenmedik dalgalanmalar sonuçların yorumlanmasında karmaşıklığa sebep olmuştur. Bu problemi ortadan kaldırmak için, farklı çamurlar ile aşılanan laboratuar ölçekli beş anaerobik reaktör 450 gün boyunca sabit KOİ yükü ve kademeli olarak 1000 mg/l'den 6000 mg/l'ye kadar artırılan toplam amonyak konsantrasyonu altında işletilmiştir. Yükselen toplam amonyak miktarına paralel olarak serbest amonyak azotu (SAN) 50 mg/l'den 750 mg/l'ye kadar yükselmiştir. Aşamalı adaptasyon sonrası %77 ile % 90 arasında değişen yüksek sayılabilecek KOİ giderimleri elde edilmiştir. SAN 100 mg/l' yi aşınca aşı çamurlarında baskın olarak bulanan *Methanosaeta* türleri *Methanosarcina* türleri ile yer değiştirmiştir. SAN miktarı daha da arttıkça, tür dağılımında başka belirgin bir değişim meydana gelmemiş, tekli yuvarlak yapıda bulunan *Methanosarcina* hücreleri çok hücreli dayanıklı yapılar oluşturmuştur. Fakat, SAN seviyesi 600 mg/l'yi aşınca ise, *Methanosarcina* kümelerinin dağıldığı gözlenmiştir. Bilakis R4, aşı çamurundan gelen inorganik partiküllerin *Methanosarcina* kümelerinin tutunması için uygun bir ortam sağlaması sayesinde, 750 mg/l SAN seviyesine bile dayanabilmiştir.

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FAN	Free ammonia nitrogen
TAN	Total ammonia nitrogen
TKN	Total kjeldahl nitrogen
VFAs	Volatile fatty acids
OLR	Organic loading rate
HRT	Hydraulic retention time
FISH	Fluorescent in-situ hybridization
PCR	Polymerase chain reaction
DGGE	Denaturing gradient gel electrophoresis
UASB	Upflow anaerobic sludge blanket
TSS	Total suspended solids
VSS	Volatile suspended solids
rRNA/DNA	Ribosomal ribonucleic/deoxyribonucleic acid

1. INTRODUCTION

For many decades, anaerobic treatment has been used in the digestion of wastewater treatment plant sludges, animal manures and organic solid wastes. Besides, the technology is particularly attractive for treatment of biodegradable agro-industrial wastewaters, such as those from sugar, paper, starch, fish, meat, canning industries, alcohol fermentation, breweries, tanneries and also some more complex industrial wastewaters, such as those derived from the petrochemical and pharmaceutical industries. Therefore, a lot of full-scale anaerobic reactors are in operation all over the world for the treatment of these wastewaters. Additionally, although some chemical compounds like ammonia, sulfide, volatile fatty acids and heavy metals reach to inhibitory levels, anaerobic treatment processes are also convenient for the treatment of acidogenic landfill leachate, particularly in countries in which organic fraction of municipal solid waste is mainly landfilled.

In the literature, there are numerous studies about anaerobic treatment of landfill leachate. However, owing to differences in site operations, composition of solid wastes landfilled, climatic conditions of the region and the landfill age, leachate generated in a landfill may not resemble another. Therefore, to select the appropriate process for the treatment of leachate, characterization and treatability studies should certainly be conducted. For this purpose, the anaerobic treatability study of Istanbul Kömürcüoda Landfill leachate was started in August 1998 to obtain compatible design parameters and to propose solutions for possible problems in the operation of full scale anaerobic reactors especially at the start-up, and under inhibitory levels of toxic compounds.

According to preliminary results, high total ammonia nitrogen (TAN) concentrations as high as 3000 mg/l and pH values above 8.1 were claimed to be responsible for an increase in the possibility of ammonia inhibition (Inanc *et al.*, 2000). Therefore, a long term anaerobic treatability study was conducted for more than 1000 days to investigate the effects of ammonia inhibition in detail. The results of this long term treatability study

carried out by using three different anaerobic reactors configured as upflow filter, sludge blanket, and hybrid bed reactors will be presented in Chapter 3.

Although ammonia is an important buffer in anaerobic treatment process and an essential nutrient for anaerobic microorganisms, high concentrations can be a major cause of operational failure. Although free ammonia is the more toxic form, most of the toxicity threshold concentrations were given as total ammonia. So that, there are difficulties in comparing the threshold concentrations in literature given in different forms and with lacking pH and temperature values.

On the other hand, process instability in anaerobic reactors at elevated ammonia levels is generally considered to be the result of inadequate adaptation of the methanogenic population. In an anaerobic reactor that has not previously adapted to high ammonia concentrations, or operating near the limits of its design capacity for anaerobic degradation, shock high ammonia loads generally cause rapid production of VFAs. Therefore, the buffering capacity of the system may not be able to compensate for the decrease in pH. Further depression of alkalinity and reduction of pH may result in process failure. In a reactor under steady-state conditions and adapted to high ammonia loading, relatively high concentrations of VFAs will be counteracted by the ammonia-bicarbonate buffering capacity of the system.

Adaptation of methanogens to elevated ammonia concentrations is very significant, as these microorganisms are remarkably sensitive to pH fluctuations. However, once adapted, an anaerobic reactor is capable of remaining in equilibrium under transient shock loading conditions. Today, the presence of adaptation phenomena that increase the tolerance of methanogens against ammonia-nitrogen is known but not exactly understood. Therefore, it should be realized that, to understand the phenomena exactly, actual mechanism of the inhibition and formation type of the adaptation should be entirely elucidated.

At this point, to increase our understanding on the formation of ammonia inhibition and adaptation, investigations were focused on the impacts of high free ammonia concentrations on the diversity and activity of anaerobic microorganisms especially on

methanogens in the reactors. For many decades the identification of methanogens in anaerobic reactors was limited to cultivation dependent methods and direct microscopic analyses. However, with recent developments in molecular techniques it is now possible to identify all methanogens present in anaerobic reactors – regardless of their cultivation capabilities. With these developments in identification of methanogens, formation type of adaptation may be defined as enzymatic adaptation of the same species or selection of ammonia tolerant methanogens.

Therefore, the population shifts in upflow sludge blanket and hybrid bed reactors treating high ammonia landfill leachate were investigated by using molecular techniques such as denaturing gradient gel electrophoresis (DGGE) and fluorescent in-situ hybridization (FISH), two methods that are complimentary to each other. These are powerful techniques in the identification of microbial populations in combination with cloning and DNA sequencing. The results of this study will be summarized in Chapter 4.

Nevertheless, it was actually difficult because of unexpected fluctuations in the raw leachate as sudden jumps and drops in biodegradability, pH, VFA and total ammonia concentrations. Thus, to stabilize and simplify the conditions in evaluation of free ammonia inhibition and adaptation, five upflow anaerobic reactors seeded with different sludges were fed with a synthetic wastewater and organic loading rate was kept constant under gradually increasing free ammonia levels. The details of this study will be given in Chapter 5.

Finally, changes in methanogenic populations in five laboratory scale anaerobic reactors seeded with different sludges and exposed to gradually increasing ammonia levels were monitored by using the aforementioned molecular techniques and the collected population data were linked with reactor performances. Results will be presented in Chapter 6.

2. LITERATURE REVIEW AND BACKGROUND

2.1. Anaerobic Treatment Process

Methanogenesis is an essential terminal electron accepting process in many anaerobic environments where the supply of oxygen, nitrate, oxidized forms of sulfur, iron, and manganese are limited (Ferry, 1993). Examples of such environments include freshwater and some marine sediments, flooded soils, wet wood of trees, tundra, landfills, and waste digestors. In these environments, a complex microbial community consisting of many interacting microbial species completely degrades natural polymers such as polysaccharides, proteins, nucleic acids, and lipids to methane and carbon dioxide. Because of the large amounts of organic matter that are degraded in the natural environments, methanogenesis is an important process in cycling of carbon and other elements in nature and may be responsible for up to 60% of atmospheric methane (Conrad, 1996).

The amount of energy released during methanogenesis is relatively low compared to other terminal electron accepting processes. Thus, the amount of biomass produced per unit of substrate degraded is much less than that of other terminal electron accepting processes. For this reason, methanogenesis has been used as the treatment of choice for sewage and other complex wastes since sludge yields are low and most of the energy in the original substrates is retained in the energy rich fuel, methane. Anaerobic treatment by methanogenesis is often a net energy producer, resulting in significantly lower operating costs compared to aerobic treatment (Lettinga, 1995). Although the low cell yields associated with anaerobic treatment make it attractive for wastewater treatment, it is also one of its main disadvantages because large reactor volumes and long retention times are needed to achieve the required treatment efficiency (McCarty, 1971). Therefore, only relatively high strength wastes are applicable to anaerobic treatment and additionally, it is often supposed as being an unstable process (McCarty, 1971; Anderson *et al.*, 1982; Speece, 1983). However, great advances have been achieved in the past 20 years in our

understanding of the biochemistry and energetics of anaerobic metabolism. This has allowed the description of the most sensitive steps in the process and the development of strategies to enhance the operational stability of anaerobic treatment systems. The result has been the development of novel reactors where the slow growing microorganisms are retained in the reactor even at high organic loadings. With these advances, it is now believed that almost any type of waste can be treated anaerobically (Lettinga, 1995).

2.1.1. Process Microbiology

In anaerobic treatment, organic matter is completely degraded to methane and carbon dioxide in discrete steps by the concerted action of at least four different groups of microorganisms, primary fermenting bacteria, secondary fermenting bacteria, and two types of methanogens (Fig. 2.1). The degree of mutual dependence among these different bacterial types varies considerably; whereas the later members of the food chain always depend on the earlier ones for their substrates. They may also exert a significant influence on the earlier members in the chain by removing metabolic products.

Polymers such as polysaccharides, proteins, nucleic acids, and lipids are first hydrolyzed to oligomers and monomers for instance sugars, amino acids, purines, pyrimidines, fatty acids, and glycerol, typically through the action of extracellular hydrolytic enzymes. These enzymes are produced by the fermenting bacteria, which ferment the resulting monomers further to fatty acids, succinate, lactate, alcohols, etc. (Fig. 2.1, group 1). Some of these fermentation products, especially acetate, H_2 and CO_2 can be converted directly by methanogenic archaea into methane and carbon dioxide (Fig. 2.1, groups 4a and b). For degradation of other fermentation products, especially fatty acids longer than two carbon atoms a further group of fermenting bacteria, the so-called secondary fermenters or syntrophic acidogenic fermenters (Fig. 2.1, group 2), are needed. These bacteria convert their substrates to acetate, carbon dioxide, hydrogen, and perhaps formate, which are subsequently used by the methanogens. The degradation of these compounds with H_2 production is thermodynamically unfavorable unless the concentration of H_2 (or formate) is kept low by hydrogenotrophic methanogens. Because of the diverse

number of organisms involved in these reactions and their ability to perform other types of metabolisms such as fermentation or sulfate reduction, the organisms that participate at this step are also called syntrophic metabolizers (Schink, 1997).

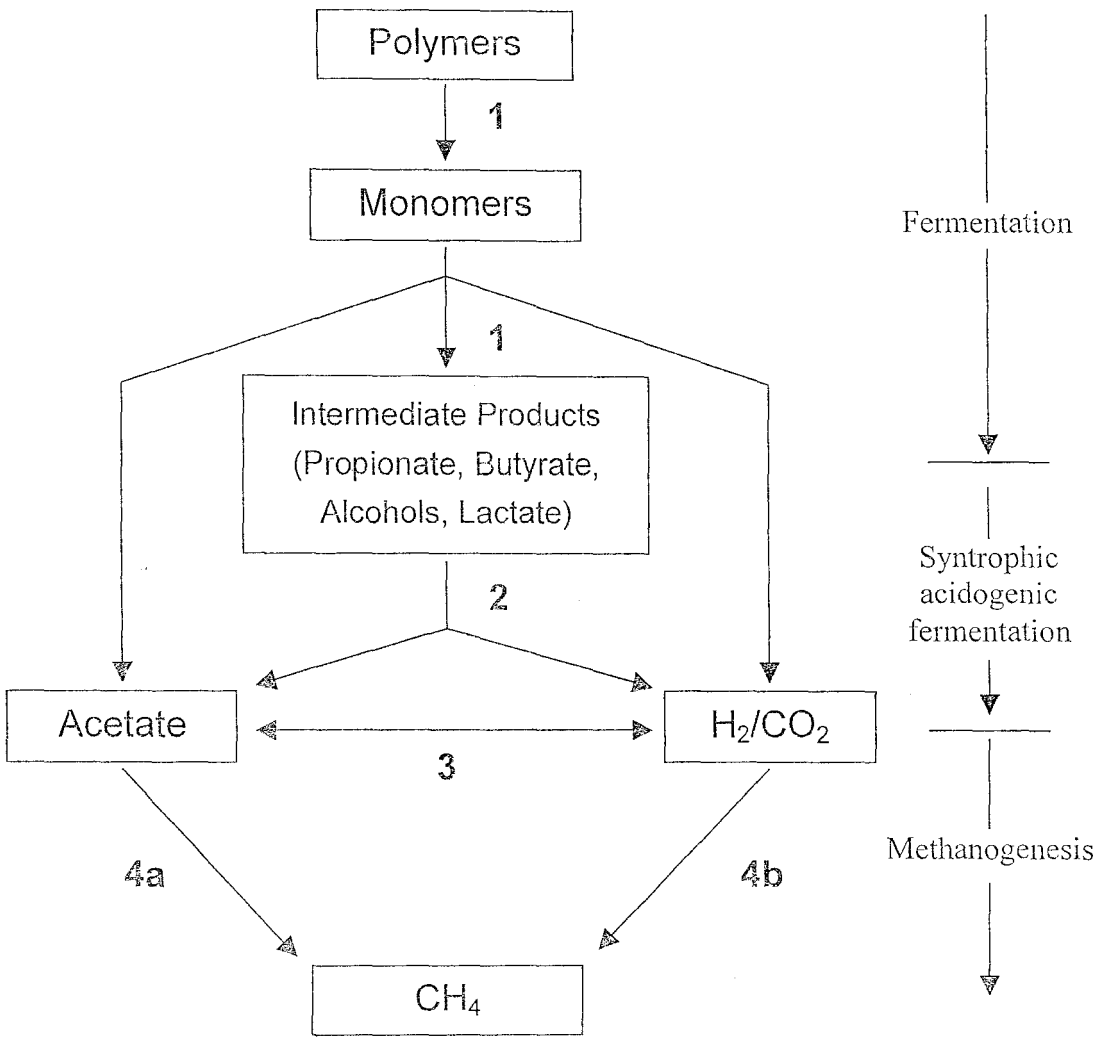

Figure 2.1. Degradation pathway through various trophic groups of microorganisms in a methanogenic reactor

In a well-balanced anaerobic reactor in which an active hydrogen-utilizing population maintains a low hydrogen partial pressure, the flux of carbon and electrons goes nearly exclusively through the outer paths of the flow schemes (Fig. 2.1), and reduced fermentation intermediates therefore play only a minor role. However, the flux through the central paths will never become zero, because fatty acids are always produced in the

fermentation of lipids and amino acids as well. The intermediate products become more important if the hydrogen concentration increases for any reason as excess supply of fermentable substrate and inhibition of hydrogenotrophic methanogens due to a drop in pH (<6.0) or to the presence of toxic compounds. Under such conditions, fatty acids accumulate and might even shift the pH further downward, thus inhibiting the methanogens even further. The consequence may be that the whole system turns over, meaning that methanogenesis ceases entirely and the fermentation stops with the accumulation of huge amounts of fatty acids, as is frequently encountered in unbalanced anaerobic reactors. Obviously, the hydrogenotrophic methanogens act as the primary regulators in the total methanogenic conversion process and the syntrophically fatty acid oxidizing bacteria are affected most severely by a failure in methanogenic hydrogen or formate removal (Schink, 1997).

The function of homoacetogenic bacteria (Fig. 2.1, group 3) in the overall process is less well understood. They connect the pool of one-carbon compounds and hydrogen to that of acetate. Due to their metabolic versatility, they can also participate in sugar fermentation and degradation of special substrates such as N-methyl compounds or methoxylated phenols (Schink, 1994). In certain environments, for instance at lower pH or low temperature, they may even successfully compete with hydrogenotrophic methanogens and take over their function to various extents.

Methanogens

A large number of microorganisms belong to Archaea domain produce methane as an internal part of their energy metabolisms. Such microorganisms are called methanogens. Methanogens are a taxonomically and phylogenetically diverse group of microorganisms. As a group, methanogens use a small number of compounds, H_2 or one-carbon atom compounds (Boone *et al.*, 1993; Zinder, 1993). This specialization makes methanogens dependent on other organisms for the supply of substrates in most anaerobic environments. Without methanogens, effective degradation of organic matter would cease due to the accumulation of nongaseous products of fermentation which have almost the same energy content as the original substrate. The ability of methanogens to use H_2 plays a key regulatory role that controls the types of products made by fermentative bacteria and sets

the thermodynamic conditions required for the degradation of fatty and aromatic acids. The favorable thermodynamics of H_2 use by methanogens allows them to metabolize H_2 to very low partial pressures. Because of the large capacity for H_2 use by methanogens, H_2 concentrations are normally very low in well operated anaerobic treatment systems, even though large amounts of H_2 are produced. The ability of methanogens to maintain low levels of H_2 affects the types of products formed by fermentative bacteria and is essential for the degradation of fatty and aromatic acids by syntrophic associations. Formate is a common fermentation product, especially by bacteria that use pyruvate–formate lyase in their metabolism, and may be an essential intermediate for syntrophic metabolism (Thiele and Zeikus, 1988). Many methanogens are able to use formate and it serves as a source of electrons for methane formation equivalent to H_2. Acetate is a major product of fermentative metabolism and is quantitatively the most important substrate for methane production. About 60–90% of the methane produced in anaerobic reactors is derived from the methyl group of acetate (Mountfort and Asher, 1978; Mackie and Bryant, 1981; Boone, 1982). At thermophilic temperatures or at high ammonium levels, the oxidation of the methyl group of acetate to H_2 may be the predominant route for acetate metabolism. Acetate using methanogens include members of the genera *Methanosarcina* and *Methanosaeta* (Boone *et al.*, 1993). *Methanosarcina* sp. have faster growth rates, higher apparent K_S (half saturation constant) values for acetate use, and higher threshold acetate values than *Methanosaeta* sp. (Zinder, 1993). The differences in the apparent K_S values for acetate use have been attributed to differences in respective enzymes used to activate acetate (Jetten *et al.*, 1990). Since *Methanosarcina* sp. and *Methanosaeta* sp. have different threshold values for acetate use but use the same reaction for acetate metabolism, the threshold values cannot represent a thermodynamic limitation. Acetate threshold values may result when a critical or inhibitory concentration of unionized acetic acid is reached which, for *Methanosarcina* sp., is between 4 and 7 mM (Fukuzaki *et al.*, 1990). Consistent with the known growth characteristics of the acetoclastic methanogens, a drop in acetate concentrations below 1 mM was correlated with a displacement of *Methanosarcina* sp. by *Methanosaeta* sp. in a thermophilic digestor (Zinder *et al.*, 1984). Generally, high-rate anaerobic sludge blanket reactors usually have granules composed of *Methanosaeta* sp. rather than *Methanosarcina* sp. (Grotenhuis *et al.*, 1991; Wu *et al.*, 1993). Interestingly, Raskin *et al.* (1996) found that *Methanosaeta* sp. and *Methanosarcina* sp. were present at approximately equal levels in glucose degrading anaerobic biofilm reactors even though

acetate levels were below the threshold values for *Methanosarcina* sp. The maintenance of *Methanosarcina* in these biofilms could not be attributed to the utilization of other substrates such as methanol, suggesting that there may be *Methanosarcina* sp. that have a lower threshold value than has been reported in the literature. Strayer and Tiedje (1978) found that acetate was converted to methane at or near the maximal rate in eutrophic lake sediments since the addition of acetate to these sediments did not result in a corresponding increase in the rate of acetate utilization or methane production. This suggests that there is little additional capacity to metabolize acetate if the acetate production rates increase. A similar conclusion was reached by Hickey and Switzenbaum (1991) for anaerobic sewage digestors. This is probably the reason why acetate concentrations in digestors increase when organic or volume loading rates increase. Methanol, methylamine and trimethylamine also serve as substrates for *Methanosarcina* sp. and other methylotrophic methanogens (Boone *et al.*, 1993). These compounds may be important substrates for methanogenesis in marine systems (Oremland and Polcin, 1982). Some hydrogenotrophic methanogens can oxidize secondary alcohols to ketones and primary alcohols to carboxylic acids (Widdel, 1986).

2.1.2. Start-up and Operation

The bacteria involved in the fermentative step grow relatively rapidly, because the fermentation reactions give a greater energy yield than the reactions that lead to methane formation. For this reason, the methanogens are more slowly growing and tend to be rate-limiting in the process. This generalization is true with sewage, sewage sludges, and most industrial wastewaters. However, with certain organic materials, for example the anaerobic decomposition of lignocellulosic materials such as grasses, agricultural crop residues, or newsprint, the hydrolysis step may be very slow and rate-limiting.

The successful start-up and operation of an anaerobic system requires that a proper balance be maintained between the fermentative and methanogenic organisms (Fig. 2.1). This balance is accomplished through proper seeding, as well as through control of organic-acid production and pH during the start-up, when the microbial populations are

establishing themselves. Ideally, an anaerobic reactor is seeded with digested sludge or biomass from an active anaerobic treatment system. This kind of balanced, active seeding is necessary because of the slow doubling time (ca. 4 d at 35 °C) of methanogens. If the seed contains only a small number of methanogens, the start-up time may be long. For example, about 10^8 to 10^9 of methanogens are required per ml of reactor volume to ensure successful operation of an anaerobic treatment system. If a seed with only 10^3 per ml of methanogens is available, the population would need to be increased by a factor of about 10^6. This requires about 20 doubling times, or about 80 d at 35 °C. At lower temperatures, the doubling time increases by a factor of about two for each 10 °C drop in temperature (Rittmann and McCarty, 2001).

During reactor start-up, the operator must maintain a sufficiently small loading on the reactor so that organic acids produced by the much faster growing fermentative bacteria do not exceed the buffering capacity of the system. If this occurs, the pH will drop, and the methanogenic population can be killed. The crucial steps during start-up are: (1) begin with as much good anaerobic seed as possible, (2) fill the digester with this seed and water, (3) bring the system to temperature, (4) add buffering material in the form of a chemical such as sodium bicarbonate to protect against pH drop, and (5) add a small amount of organic waste sufficient to let the organic acid content from fermentation reach no more than about 2,000 to 4,000 mg/l, while keeping pH between 6.8 and 7.6. These organic acids are the food source required for the methanogenic population to grow. The time when sufficient doublings have occurred will be evidenced through a drop in the organic acid concentration. Feeding with additional waste can then be initiated, slowly at first, until a balance is reached between fermentation and methanogenesis in the system. At such balance, the organic acid concentration will generally remain below 100 to 200 mg/l, depending upon the loading on the system.

Organic acid concentration and reactor pH should be determined on a daily basis can be evidenced through a sudden increase in the organic acid concentration. If the buffering capacity is becoming depleted, chemical base (bicarbonate) must then be added quickly to prevent a drop in pH from occurring, which would kill the critical methanogenic population. Thus, monitoring the organic acids and the buffer capacity provides the first

line of defense for control of anaerobic systems so that the acid producers and the acid consumers achieve and maintain a proper balance (Rittmann and McCarty, 2001).

2.1.3. Process Monitoring

As with any biological treatment process, anaerobic treatment systems should be monitored to ensure successful operation. Upsets of the systems may be caused by both hydraulic and organic overloads, and the presence of inorganic and organic toxic substances. Some of the more commonly used indicators include; pH, alkalinity, volatile fatty acids concentrations, biogas production rates and biogas composition (methane and carbon dioxide) and volatile solids or COD reduction. Usually several of these are monitored together, as they supply complementary information. The best operation is achieved by daily monitoring and the charting of trends of these parameters and applying corrective actions before the process gets out of control (Switzenbaum *et al.*, 1990).

pH is an important monitoring parameter in the control of anaerobic treatment systems, because of the inhibitory effects of low pH on the activity of reactor microorganisms especially on methanogens. It controls the fraction of unionized fatty acids that freely permeate the cellular membrane of microorganisms. After permeating the membrane, fatty acids internally ionized, thus lowering the cytoplasmic pH and affecting microbial metabolism (Zoetemayer *et al.*, 1982). Nevertheless, the occurrence of low pH is the result of a well-developed unbalance and as such is not useful as an early warning indicator.

Determination of volatile fatty acid (VFA) concentration in liquid phase is the most popular parameter for monitoring of system performance. The question is what organic acids should be measured on a routine basis, and how can this be accomplished? The key organic acids are the series of short chain fatty acids and which vary in chain length from formic acid with one carbon per mole to octanoic acid with eight carbon atoms per mole. These acids have been termed *volatile acids* because, in their unionized form, they can be distilled from boiling water. This meaning of the term *volatile* is different from its meaning

in *volatile organic compounds,* a term generally used to describe organic compounds that are readily removed from water by simple air stripping. The short-chain fatty acids cannot be removed from water by air stripping.

The volatile acids that are generally found present in highest concentrations as intermediates during start-up of an anaerobic system or during organic overload are acetic, propionic, butyric, and isobutyric acids. These comprise the bulk of the organic acids found in anaerobic systems. Other nonvolatile organic acids also are formed as intermediates of waste organic degradation such as lactic, pyruvic, and succinic acids but their concentrations generally are much below those of the volatile acids and thus are of less general concern for control. The volatile acids are all quite soluble in their ionized and unionized forms and are present as dissolved species. At normal pH of operating systems, they all are present for the most part in the ionized form. Typical values at 35 ^{o}C for the negative logarithm of the acidity constant, (the pH at which the acids are 50 percent in the acid form and 50 percent in the ionized form), vary from a low of 3.8 for formic acid to more typical values of 4.8 for acetic and n-butyric acids and 4.9 for propionic acid.

The routine measurement of volatile acid concentration is of the greatest importance in the operation and control of anaerobic systems. Various analytical procedures can be used to measure volatile acids. Methods vary from those that require expensive instrumentation, such as gas chromatography and high performance liquid chromatography, to relatively inexpensive wet chemical procedures involving distillation, column chromatography, or acid/base titration. The instrumental approaches allow one to differentiate among the various organic acids present, which can at times be used to help diagnose the cause of digester failure. The wet chemical procedures generally provide information only on the total organic acid or total volatile and concentration present, but this is often sufficient for routine control (Rittmann and McCarty, 2001).

Alkalinity is another monitoring parameter. A direct relationship exists between alkalinity concentration in the system and VFA accumulation. However, as a control parameter effectiveness of alkalinity is not clear under toxicity circumstances (Jenkins *et al.*, 1983).

Biogas production rates and more specifically the methane yield can potentially be a good indicator of the metabolic status of the anaerobic treatment system. Reduction in methane production rates, when compared to the organic loading, gives warning of the accumulation of soluble acid products in the liquid phase. Unfortunately, this is again the result of an unbalance rather than a warning of it.

There is a potential to characterize the metabolic status of anaerobic treatment systems by monitoring the concentration of certain intermediate gases such as hydrogen (H_2) and carbon monoxide (CO) which are present at trace levels in the biogas. Besides, in contrast to liquid phase sampling, where sample pretreatment, extraction and etc are not adapted for on-line monitoring, gas analysis are amenable to real time data acquisition. CO and H_2 are two gases that appear to lend useful information concerning the two principal terminal reactions in methanogenic ecosystems. H_2 monitoring allows some measure of the status of the carbon dioxide reduction to be obtained, while CO may give insight into the acetate cleavage reaction. Both trace gases have been found respond quickly to changes in loading and accumulation of volatile fatty acids and can be monitored on-line (Switzenbaum *et al.*, 1990). However, as the microbial composition of the system is not completely determined, these parameters should also be used in concert with conventional indicators of process performance.

A desirable monitoring analysis would be that for the active population of microorganisms present in the system, especially of the populations responsible for the critical steps, including the methanogens. At this point, microbial identification tools for such analyses are not available for routine use. However, through promising 16S rRNA/DNA based molecular methods, it is now possible to identify microorganisms present in anaerobic reactors - regardless of their cultivation capabilities (Raskin *et al.*, 1994; Sekiguchi *et al.*, 1999; Chan *et al.*, 2001). Therefore, such monitoring analyses are becoming progressively more essential in control of anaerobic treatment systems (Fernandez *et al.*, 1999; McMahon *et al.*, 2001). Insights into the diversity, structure and function of mixed microbial communities in anaerobic reactors are also necessary to improve the stability against inhibitory compounds. So far, oligonucleotide probes have been used successfully to determine the relative abundances of methanogenic and suldidogenic populations as well as to differentiate among different methanogenic and

sulfidogenic populations (Raskin *et al.*, 1996). Analyses for more complex fatty acids, that comprise bacterial cell wall has also been used to characterize bacterial populations in complex systems (Nishihara *et al.*, 1995).

2.2. Identification of Microorganisms in Anaerobic Treatment Systems

To start-up and operate an anaerobic treatment systems properly the microbial diversity in the bioreactor should be continuously investigated. These investigations should include the characteristics of microorganisms that lead to the formation and stability of biomass, and the physiological and ecological properties of the microorganisms in the anaerobic bioreactor. Insights into the diversity, structure and function of mixed microbial communities in anaerobic reactors are essential for advancing treatment efficiency and stability against inhibitory compounds. Knowledge about microbial diversity and activity of the seed biomass are needed for a successful start-up, however, as a general application, seed biomass is taken from another anaerobic reactor unadapted to the new wastewater.

2.2.1. Classical Identification Techniques

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

Cultivation dependent methods: For many decades, the identification of microorganisms was limited to cultivation dependent methods. The major limitations of these traditional techniques are that only a relatively small fraction of all microorganisms making up a natural community can generally be cultured, and those that can be cultured are often difficult to identify. Environmental studies indicate that only 0.1-10 % of all microorganisms can be cultivated (Amann *et al.*, 1995; Hugenholtz *et al.*, 1998; Muyzer *et al.*1993; Muyzer, 1999; Torsvik *et al.*, 1998). Moreover, several studies proved the

existence of cultivation shifts, which means that some microbial groups are favored under cultivation conditions whereas other groups have no chance to compete.

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

Microscopic Analyses: The methanogens are structurally diverse and display no unique features by which all species can be characterized. All basic morphological types found among bacteria, including cocci and packets of cocci, rods of different shape and size, spirillum, and filamentous forms are represented in methanogens. Although, except for *Methanosaeta*, methanogens can be recognized by epifluorescence microscopy by detecting coenzyme F_{420}-dependent auto-fluorescence, this technique can be used only for counting not for identification. Moreover, fading of the autofluorescence and the presence of weakly or non-fluorescent methanogens (*Methanosaeta*) create inaccuracy (Morgan *et al.*, 1991). The conclusion was that, despite developments in microscopy, direct microscopic analyses alone still posses many limitations in identification of methanogens. However, accurate identifications may be obtained by combining epifluorescence or confocal scanning laser microscopy with rRNA-based molecular hybridization techniques (Raskin *et al.*, 1994; Harmsen *et al.*, 1996; Sorensen *et al.*, 1997; Rocheleau *et al.*, 1999; Sekiguchi *et al.*, 1999).

2.2.2. Molecular Identification Techniques

Recent studies on anaerobic bacteria and methanogens have been greatly augmented by the use of molecular methods as immuno detection, membrane lipid fatty acid analysis and rRNA/rDNA based identification methods. These molecular methods provide tools for analyzing the entire microbial community in anaerobic bioreactors, covering also those that have not been cultured on artificial growth medium in the laboratory. Therefore, such

methods are becoming increasingly important in the optimization of anaerobic treatment processes.

Immuno detection: Immuno detection techniques are based on the fact that microbial cell wall biopolymers have strong antigenic properties that can be used to raise antibodies. When labelled with fluorescent dyes or with gold particles, they can be used for the detection of specific microorganisms. Immunological methods for the detection of methanogens in anaerobic bioreactors have been presented in a number of papers in the literature (Bryniok and Trösch, 1989; Koornneef *et al.*, 1990; Macario and Conway de Macario, 1988; Macario *et al.*, 1991). In the latest study, Sorensen and Ahring (1997) were developed an enzyme-linked immunosorbent assay (ELISA) for the detection of whole cells of methanogens in samples from continuously stirred anaerobic bioreactors treating slurries of solid waste. The assay was found well suited for quantitative detection of the main groups of methanogens in complex samples from anaerobic digesters. They claimed that, the limited preparatory work required for the assay and the simplicity makes the test amenable for routine analysis of large numbers of samples. Nevertheless, to increase the applicability of this method pure methanogenic cultures are needed to raise specific antibodies. Moreover, sensitivity reduces with the cross-reaction of antibodies with other non-related strains.

Membrane lipid fatty acid analysis: Although, membrane lipid fatty acid analysis has been extensively used to identify unknown microbial isolates, it is not always suitable for the characterization and quantification of microorganisms in complex biomass (Böttger, 1996). Bacteria and archaea that lack signature lipid biomarkers are especially difficult to distinguish in the mixed culture profile. In addition, lipid analyses are not very sensitive and therefore microorganisms that are present in low numbers are beyond detection. Moreover, changes in the environmental conditions affect the membrane lipid fatty acid patterns. Nevertheless, there are some applications of lipid analysis in the literature to detect and quantify the methanogens (Ohtsubo *et al.*, 1993; Nishihara *et al.*, 1995), sulfate reducers and syntrophic propionate degraders (Oude Elferink *et al.*, 1998a).

Ribosomal RNA/DNA (rRNA/rDNA) based methods: Without doubt, rRNA/DNA-based molecular methods have become the most important detection and identification

methods in the determination of genetic diversity of complex microbial populations. Sequence analysis of the rRNA or the rRNA gene (rDNA) has revealed that 16S and 23S rRNA can be used as evolutionary biomarkers (Stams and Oude Elferink, 1997; Oude Elferink *et al.*, 1998b). The 16S and 23S rRNA contain both highly conserved and highly variable regions. At present, a large database of 16S rRNA sequences is available, which is expanding daily, and 16S rRNA analysis has become a significant tool for the identification of new isolates. The results of the studies indicate that molecular techniques provide an excellent method for the rapid and quantitative monitoring of microorganisms in their communities. Therefore, these techniques are bridging the 'gap' between engineer (reactor operation) and microbiologist (culture-based study) and lead to an interactive communication between them for improving the performances of reactors used in wastewater treatment (Sekiguchi *et al.*, 2001).

Several rRNA based methods have been developed to identify and quantify microorganisms in anaerobic bioreactors. These methods even can be used without cultivation of the microorganisms (Fig. 2.2) (Oude Elferink *et al.*, 1998b).

One of the methods used for the identification of microorganisms in anaerobic bioreactors is hybridization with rRNA-based oligonucleotide probes (Raskin *et al.*, 1994; Rocheleau *et al.*, 1999; Sekiguchi *et al.*, 1999) which are short single-stranded oligomers of 15-40 nucleotides that can be synthesized chemically. The oligonucleotide probes are complementary to either variable or conserved parts of the rRNA. For the detection and quantification of microorganisms, the oligonucleotide probes either are made radioactive by ^{32}P-labeling or are chemically linked to fluorescent dyes. The best probe hybridization will be obtained when all nucleotides bind to the target; one mismatch may weaken the binding. The probes can be applied after extraction of the rRNA from the sludge (dot-blot hybridization), but can also be used in situ (fluorescent in-situ hybridization; FISH) in combination with fluorescent microscopy or confocal scanning laser microscope (CSLM).

The major advantage of oligonucleotide hybridization methods over other hybridization methods (eg. immuno labeling) is the fact that the probe specificity can be controlled. Species-specific probes can be designed by targeting the most variable regions of the rRNA, whereas more general probes (group or genus) are complementary to the

more conserved regions. Various groups of microorganisms have been detected and quantified with rRNA hybridization probes in anaerobic bioreactors (Raskin *et al.*, 1994; Harmsen *et al.*, 1996; Sekiguchi *et al.*, 1999; Tagawa *et al.*, 2000). The combined use of different fluorescent dyes offers very interesting possibilities to study the ecology of certain strains within a community of related organisms.

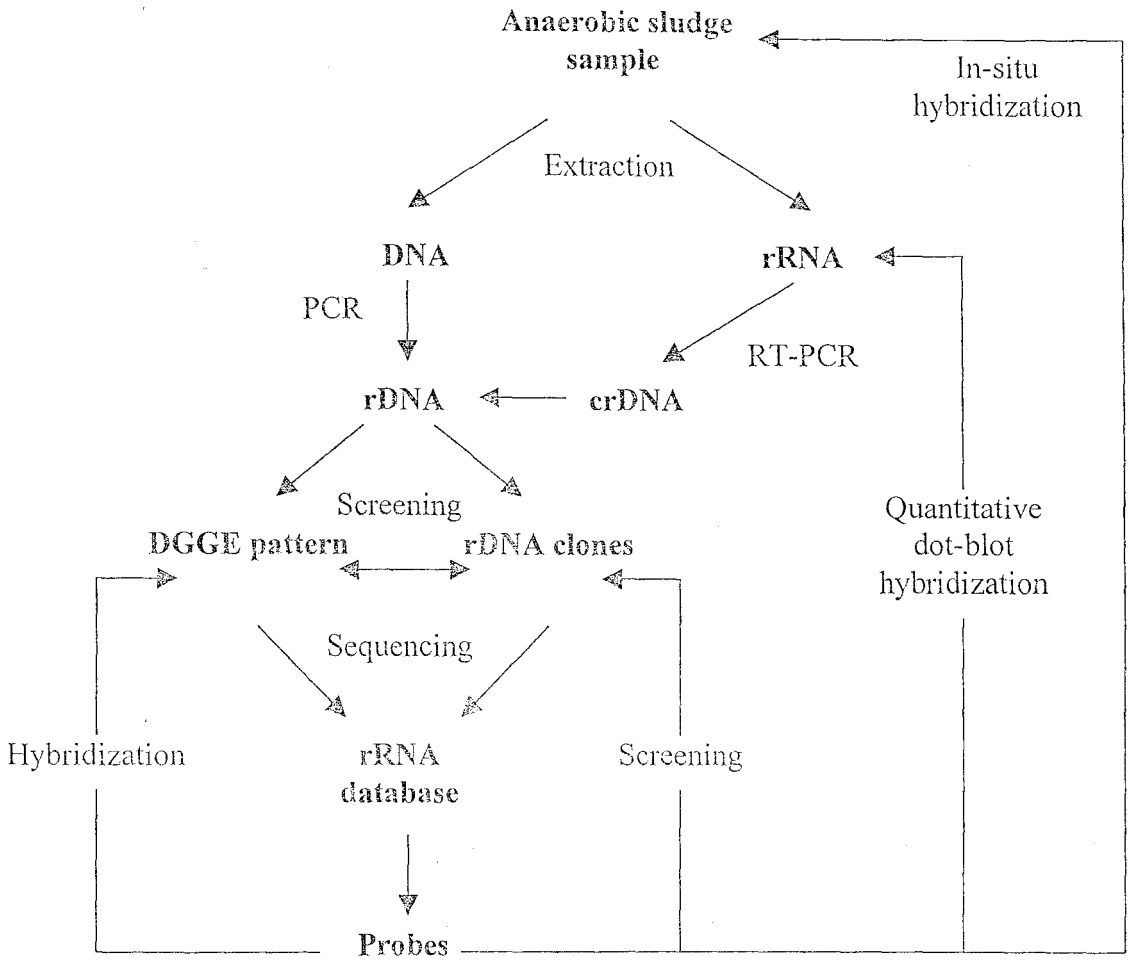

Figure 2.2. The 16S rRNA/DNA based approach for identification and quantification of microorganisms in anaerobic bioreactors

At present, the most time-consuming part of the rRNA hybridization method is the development of probes and the evaluation of their specificity. This explains why detailed sludge characterization studies are still hampered by the lack of suitable probes. Fortunately, the number of probes suitable for sludge studies is still increasing. There are also some limitations in rRNA hybridization methods including questions of RNA

extraction or probe insertion efficiency (Sekiguchi *et al.*, 1999) and molecular preservation (Raskin *et al.*, 1994).

The PCR amplification of rRNA genes (rDNA) is another rDNA-based detection method. A few target rRNA genes can be amplified with polymerase chain reactions to make them detectable and quantifiable. The selection of PCR primers determines which rRNA gene and which part of the rRNA gene will be amplified. If PCR amplification with universal primers combined with cloning and sequencing analysis, it is possible to get information about the microbial composition of anaerobic sludges (Sekiguchi *et al.*, 1998; Godon *et al.*, 1997; Delbes *et al.*, 1998; Fernandez *et al.*, 1999; 2000; Zumstein *et al.*, 2000). Besides, if selective primers are used, it is possible to amplify rRNA genes from specific groups of microorganisms present in the biomass. PCR amplification with archaea and eubacteria -specific primers in combination with cloning and restriction fragment length polymorphism (RFLP) analysis or amplified rDNA restriction analysis (ARDRA) was used to identify the acidogenic and methanogenic populations in anaerobic bioreactors (Hiraishi *et al.*, 1995; Fernandez *et al.*, 1999; 2000; Ficker *et al.*, 1999).

Instead of difficult and expensive cloning technique, it is also possible to separate the PCR products with denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). Separation of DNA fragments in DGGE or in TGGE is based on the decreased electrophoretic mobility of partially melted double-stranded DNA molecules in polyacrylamide gels containing a linear gradient of DNA denaturants (a mixture of urea and formamide) or a linear temperature gradient. Molecules with different sequences may have a different melting behaviour, and therefore will stop migrating at different positions in the gel and similar fragments denature at the same position. After staining, a banding pattern is obtained with each band theoretically representing a microbial species. By using selective PCR-DGGE in combination with partial DNA sequencing or hybridization by using specific DNA probes, complex microbial populations in anaerobic bioreactors can be studied (Santegoeds *et al.*, 1998; Chan *et al.*, 2001; Perreira *et al.*, 2002).

Whereas DGGE analysis of PCR products is a powerful tool to analyze diversity and dynamics of microbial communities, it has some severe limitations.

The method involves extraction of nucleic acids and subsequent PCR, which may both cause some bias: Not all cells lyses under the same conditions and preferential amplification of certain templates can occur. Therefore, different intensities of DGGE bands must not be interpreted as quantitative measures of the abundance of species relative to each other. For qualitative statements on the development of a certain band (population) over time, i.e., to monitor its appearance or disappearance, identical treatment of all samples has to be ensured.

Separation of DNA fragments with high resolution is restricted to a maximum size of about 500 bp. Consequently, the phylogenetic information that can be retrieved by sequencing is relatively little. In case of full identity with an rRNA sequence in a database it might be sufficient for identification, but in cases in which only distantly related sequences are available classification becomes difficult if not impossible. Also characterization of bands by hybridization analysis with rRNA-targeted oligonucleotide probes is only possible if the probe target region is within the amplified fragment, which is only the case for a small fraction of the full set of available probes.

The main difficulty, however, is the "one band – one species" hypothesis. Especially in complex communities, bands might originate from two or more fragments that co-migrate on the denaturing gradient gel. Furthermore, single species might result in two or more DGGE bands due to inter operon microheterogeneity (Nübel *et al.*, 1996).

2.3 Toxic Substances in Anaerobic Treatment

To maintain a stable process in anaerobic treatment, there should be a balance between volatile fatty acid production and consumption rates. Since the susceptibility of methanogens to toxic compounds is much greater than that of acidogens, the presence of toxic compounds in the reactor can easily disrupt the balance between acidogenesis and methanogenesis. Therefore, this unbalance may be the start of a fatal upset of the anaerobic treatment process. If some precautions are not taken as reducing the organic loading rate

and pre-treating the toxicant in the influent, the ultimate consequence will be a significant pH-drop below the tolerance level of methanogens, because of volatile fatty acid accumulation in the system (Stronach *et al.*, 1986). In that case, recovery of the reactor is only possible by means of completely starting-up again after addition of viable methanogenic seed sludge.

Inhibition of the anaerobic treatment process can be mediated to varying degrees by toxic compounds present in the system. These substances may be components of the influent waste stream, or by-products of the metabolic activities of the microorganisms in the reactor. Inhibitory compounds, for instance, may interfere with metabolic enzymes of one or more members of a trophic group, or with energy metabolism by uncoupling of growth and ATP production, or with cell membranes, creating intracellular changes of the pH or the salt concentration (Kadam and Boone, 1996; Gallert *et al.*, 1998).

Common toxic substances in anaerobic digestion causing severe operational failures are volatile fatty acids especially propionate, sulfide, ammonia, heavy metals, cyanide, organic solvents and etc. Ammonia inhibition will be discussed in details in the following section.

2.4. Ammonia Nitrogen Inhibition

A very common metabolic end product in the anaerobic digestion of protein-containing wastes is ammonium-nitrogen. Although ammonium is an important buffer in anaerobic digestion processes and an essential nutrient for methanogens, high concentrations can be a major cause of operational failure. Organic fraction of municipal solid waste (Kayhanian, 1994; Gallert and Winter, 1997; Varaldo *et al.*, 1997; Gallert *et al.*, 1998), waste activated sludge (Lay *et al.*, 1998), wastewaters originating from potato starch, baker's yeast, dairy and seafood processing industries (Koster and Lettinga, 1984 and 1988; Koster, 1986; Omil *et al.*, 1995; Guerrero *et al.*, 1997; Vidal *et al.*, 2000), young landfill leachates (Borzacconi *et al.*, 1999; Inanc *et al.*, 2000a) and animal manures

(Angelidaki and Ahring, 1993 and 1994; Borja *et al.*, 1996; Krylova *et al.*, 1997; Hansen *et al.*, 1998; Sterling *et al.*, 2001) contain ammonium concentrations at inhibitory levels.

The inhibitory effects of ammonia, as far as is known, influence mainly the phase of methanogenesis in anaerobic reactors (van Velsen, 1979; Koster & Lettinga, 1984; Koster, 1986; Koster & Lettinga, 1988; Bhattacharya & Parkin, 1989; Blomgren *et al.*, 1990; Heinrichs *et al.*, 1990; Poggi-Varaldo *et al.*, 1991; Angelidaki & Ahring, 1993; Kayhanian, 1994; Gallert & Winter, 1997; Poggi-Varaldo *et al.*, 1997; Lay *et al.*, 1998). Other sensitive reactions, such as those performed by the acetogenic bacteria, may also be either directly or indirectly affected by accumulation of volatile fatty acids or hydrogen in the system (van Velsen, 1979; Koster, 1986; Koster & Lettinga, 1988; Heinrichs *et al.*, 1990)

Two species of ammonia nitrogen, the gaseous form free ammonia and the aqueous form ammonium exists together in equilibrium as indicated in the following equation. The relative abundance of the phases depends upon both pH and temperature of liquid phase of the anaerobic reactor

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \qquad (2.1)$$

As pH increases, equilibrium shifts to the left and ammonium is converted to more inhibitory free ammonia form. Increasing pH from 7 to 8 actually leads to an eight-fold increase in free ammonia fraction of total ammonia nitrogen.

Free ammonia concentration can be calculated by using the following formula (Koster, 1986; Heinrichs *et al.*, 1990; Varaldo *et al.*, 1991; Angelidaki and Ahring, 1993; Borja *et al.*, 1996):

$$FAN = \frac{TAN}{1+10^{(pK_a - pH)}} \qquad (2.2)$$

$$pK_a = 0.09018 + \frac{2729.92}{T + 273.15} \qquad (2.3)$$

TAN : Total ammonia nitrogen, mg/l

FAN : Free (unionized) ammonia nitrogen, mg/l

pK_a : Dissociation constant for ammonium ion, 8.95 at 35°C

T : Temperature, °C

Despite the toxic form of total ammonia is the temperature and pH depending free (unionized) ammonia, most of the toxicity threshold concentrations in the literature are given as ammonium nitrogen. Therefore, there are difficulties in comparing the threshold concentrations given in different forms with lacking pH and temperature values.

McCarty (1964) reported ammonia inhibition to occur at TAN concentrations in the range of 1500 to 3000 mg/l at pH levels above 7.4, whereas at concentrations in excess of 3000mg/l the ammonium ion was claimed to be toxic irrespective of pH. Likewise, Koster and Lettinga (1984) reported ammonia inhibition to occur at 1700 mg/l TAN at pH 7.5. These studies have mostly been carried out using anaerobic digester sludge, in which TAN concentrations were typically below 1000 mg/l, as a seed material.

Van Velsen (1979) has determined that the time needed to achieve an acceptable anaerobic degradation was increased with increasing ammonium concentrations, when anaerobic digester sludge unadapted to high ammonia concentrations was used as seed. However, when sludge from a swine manure digester adapted to 2400 mg/l TAN was used; the lag period was shorter or non-existent at ammonium concentrations as high as 5000 mg/l TAN

Similar results have been given for granular sludge, where methane production was fully inhibited at 1900 mg/l TAN for a potato juice fed reactor (Koster, 1986). After adaptation of the same sludge, methanogenesis occurred at 11.8 g/l TAN with 90% inhibition and at 16 g/l TAN methanogenic activity became nil (Koster and Lettinga, 1988). After the adaptation process in which the sludge gained the ability to produce methane at ammonium concentrations exceeding the initial toxicity threshold level, the maximum tolerable ammonium concentration was 6.2 times higher than the initial toxicity threshold level.

Kayhanian (1994) reported that by adapting the methanogens to ammonia it was possible to operate the high-solids digester at TAN concentrations up to 2300 mg/l, whereas 1100 mg/l was originally considered to be the upper limit.

Free ammonia has been pointed out as the active form of total ammonia causing inhibition. 50-100 mg/l FAN has been proposed as the minimum inhibitory level (Koster and Lettinga, 1984; DeBaere *et al.*, 1984; Soubes *et al.*, 1994). McCarty and McKinney (1961) found 150 mg/l FAN to be the inhibitory concentration. Anaerobic digesters operating with animal manures often have high pH values around 8 and especially at thermophilic temperatures (Angelidaki and Ahring, 1993; 1994; Hansen *et al.*, 1998) inhibitory FAN concentrations were reported as high as 700 - 1100 mg/l.

Borzacconi *et al.* (1999) reported an ammonia inhibition at pH 8.5-9.0 and 1470 mg/l TAN in a lab-scale UASB reactor treating young landfill leachate. By adjusting the inlet pH, it is given that ammonia inhibition was reverted and 80% COD removal efficiency at 20 kg COD/m^3.day organic loading was obtained. Similar results were reported by Inanc *et al.* (2000a) in anaerobic filter, UASB and hybrid bed reactors treating high ammonia landfill leachate. High TAN concentrations of about 2700 mg/l with pH values around 8.0 in anaerobic reactors were claimed to increase the possibility of ammonia inhibition.

In another study conducted with a wastewater originating from a sea-food processing industry Omil *et al.* (1995) reported that, however, the control of the influent protein content is necessary, FAN concentrations higher than 200 mg/l cause severe inhibitions in anaerobic treatment of this wastewater. In a similar study, Guerrero *et al.* (1997) successfully operated an upflow anaerobic filter in the treatment of the wastewater of a fishmeal factory. By using an acclimated biomass, no ammonia inhibition has been observed at 350 mg/l FAN even at the start-up.

It has been suggested that the interaction between free ammonia, volatile fatty acids and pH lead to an "inhibited steady state", which is a condition where the process is running stable but with a lower methane yield. This has been shown to happen when the substrate has a high ammonia concentration (Angelidaki and Ahring, 1993; Angelidaki *et al.*, 1993)

Acetoclastic methanogens seem to be more sensitive to ammonium, at concentrations above the threshold level of 1700mg/l TAN, than the hydrogentrophic methanogens (Koster and Lettinga, 1984). This corresponds with the importance of the acetoclastic methanogens for the overall performance of the anaerobic reactors.

Calli *et al.* (2000) found 320 mg/l to be the inhibitory FAN concentration (3500 mg/l TAN, pH 8.0, 35°C) in granular anaerobic sludge fed with acetate. In flocculant sludge, this inhibitory concentration was reported as 130 mg/l (3500 mg/l TAN, pH 7.5, 35°C). At higher ammonium concentrations with lower pH and free ammonia levels, reduction in biogas production that is the indication of ammonium inhibition was determined. Likewise, as to free ammonia inhibition, granular anaerobic sludge was found more tolerant than flocculent sludge to ammonium inhibition.

For hydrogenotrophic methanogens Angelidaki and Ahring (1993) and Hansen *et al.* (1998) reported higher ammonia inhibition concentrations (>1200 mg/l FAN) than given for a whole anaerobic swine manure digestion process. Since about two thirds of the methane produced in an anaerobic reactor is derived from acetate, a decrease in the activity of acetoclastic methanogens severely affects the anaerobic degradation process (Blomgren *et al.*, 1990; Schnürer *et al.*, 1994; 1999; Hansen *et al.*, 1998).

A sigmoidal pattern of ammonia inhibition of the aceticlastic methanogens was observed by Varaldo *et al.* (1991) and Angelidaki and Ahring (1993), who found that the growth rate and the specific acetate uptake rate were affected by the free ammonia concentration in a three stage pattern: initial inhibition, plateau and final inhibition. This inhibition pattern could indicate that two inhibition mechanisms are involved, acting at different concentration levels. The hydrogenotrophic methanogens exhibited, however, a more linear pattern of inhibition.

In the experiments conducted by Sprott and Patel (1986) with pure cultures, referred to above, the aceticlastic methanogens *Methanosarcina barkeri* and *Methanothrix concilii* (*Methanosaeta concilii*) also seemed to be more sensitive than the hydrogenotrophic methanogens to increasing ammonium concentrations. *Methanospirillum hungatei*, which

needs acetate as a carbon source even though it is a hydrogenotroph, was very sensitive to ammonium.

Methanogens used or identified in very limited ammonia inhibition studies in the literature are presented in Table 2.1 according to their sensitivity and tolerance to ammonia. For each methanogen, morphological properties, substrates and optimum growth conditions are also given.

Table 2.1. Classification of methanogens according to sensitivity and tolerance to ammonia

			Optimum growth conditions		
Ammonia sensitive	Morphology	Substrates	pH	Temp.	Ref.
Methanospirillum hungate	sheathed spiral	H, F	6.6-7.4	30-37	[1]
Methanosarcina barkeri	irreg. coccus	H, Ac, Me	6.5-7.5	30-40	[1]
Methanosaeata concilii	sheathed rod	Ac	7.1-7.5	35-40	[1]
Methanobacterium bryantii	rod	H, 2P, 2B	6.9-7.2	37-39	[1]
Methanobacterium formicicum	rod	H, F, 2P, 2B	6.6-7.8	37-45	[1]
Methanolobus taylorii	irreg. coccus	Me, Ma, DS	8.0	37	[2]
Ammonia tolerant					
Methanobrevibacter aboriphilus	coccobacillus	H, F	7.8-8.0	30-37	[1]
Methanobrevibacter smithii	coccobacillus	H, F	6.9-7.4	37-39	[1]
Methanobacterium strain	rod	H, F	6.2-8.4	30-40	[1]
Methanoculleus bourgense	irreg coccus	H, F	7.4	37	[3]
Methanohalophilus zhilinaeae	irreg coccus	Me, Ma	9.2	45	[2]

H= hydrogen/carbon dioxide; F= formate; Ac= acetate; Me= methanol; Ma= methylamines; 2P= 2-propanol; 2B= 2-butanol; DS= dimethylsulfide

[1] Sprott and Patel, 1986

[2] Kadam and Boone, 1996

[3] Schnürer *et al.*, 1999

It was reported that *Methanobacterium formicicum* was partially inhibited in the presence of TAN concentration of about 3000 mg/l and a pH of 7.1, with some loss of growth and CH_4-forming capacity, whereas 4000 mg/l completely inhibited the

microorganism (Hobson and Shaw, 1976). In another study, Hajarnis and Ranade (1993) reported a 50% inhibition for *Methanobacterium* at ammonium concentration of 10g/l, which resulted in a FAN concentration of 113 mg/l at pH 7.0 and 35°C. However, the efficient functioning of non-methanogenic anaerobic bacteria has been noted at concentrations of TAN in excess of 6000 mg/l and a pH value of 8.0.

Sprott and Patel (1986) had shown that some of the methanogens do not require adaptation period to resist high concentrations of ammonia. While an adaptation through genetic changes of an initially sensitive strain could occur, they claimed that the recovery period of a reactor is more likely to represent the growth period required to select for those resistant strains already present in the sludge.

Similarly, Blomgren *et al.* (1990) and Schnürer *et al.* (1994) suggested that acclimation to high levels of ammonium in enrichment cultures occurred through selection for methane production by synthrophic acetate oxidation. In the presence of high ammonium concentrations the aceticlastic methanogens were inhibited and acetate was, instead, converted to hydrogen and carbondioxide by a homoacetogenic bacterium. Then a hydrogenotrophic methanogen, presumably less sensitive to ammonium than aceticlastic methanogens, used the hydrogen formed to reduce carbon dioxide to methane. In the absence of ammonium, acetate was directly split to methane and carbon dioxide by aceticlastic methanogens. In their subsequent study, Schnürer *et al.* (1999) identified the hydrogenotrophic methanogen as *Methanoculleus bourgense* with an identical partial 16S rDNA sequence. The conversion of acetate to hydrogen and carbon dioxide can only occur at low partial pressures of hydrogen in the presence of a hydrogen consuming microorganism. In standard conditions the oxidation of acetate is a thermodynamically unfavorable reaction. So far, methane formation by syntrophic acetate oxidation has only been reported by Zinder and Koch (1984), Petersen and Ahring (1991) and Schnürer *et al.* (1994).

2.4.1. Engineering Solutions for Ammonia Inhibition in Anaerobic Treatment

According to engineering significance, it is reported that for ammonia toxicity, sludge or biomass retention time is a very important parameter in the design and control of anaerobic rectors (Bhattacharya and Parkin, 1989; Varaldo *et al.*, 1997; Angelidaki and Ahring, 1993). With continuous addition of ammonia, a high sludge retention time (SRT) system shows less adverse effects than a low SRT system. With slug additions, however, low SRT systems have the advantage of lower toxicity because of a higher dilution rate of ammonia (Bhattacharya and Parkin, 1989).

Toxic ammonia concentrations might be mitigated by the addition of ion exchangers or adsorbers, such as zeolite (Borja *et al.*, 1996; Kayhanian, 1994) and phosphorites (Krylova *et al.*, 1997). Alternatively antagonistic cations like Mg^{+2}, Ca^{+2} or Na^{+} may be added to stabilize anaerobic degradation (McCarty and McKinney, 1961; Sprott *et al.*, 1984; 1985; Sprott and Patel, 1986).

Van Velsen (1979) emphasized that to ensure the rapid and safe start-up of anaerobic reactors, which ammonium concentrations are expected to become high, it is essential that a proper inoculum be chosen. Koster and Lettinga (1984) reported that application of anaerobic treatment needs a careful management of the process, particularly during the start-up, even if granular seed sludge is used.

Control of the pH in the influent of anaerobic reactor is very important for reducing the ammonia inhibition possibility by maintaining lower free ammonia levels (Borzacconi *et al.*, 1999; Inanc *et al.*, 2000a). In case of insufficient buffering capacity of the reactor content, such a volatile fatty acid build-up because of ammonia inhibition may result in a beneficial pH-drop, naturally reducing the inhibitory effect of ammonium (Koster, 1986; Koster and Lettinga, 1988). Moreover, investigations of Angelidaki and Ahring (1994) clearly showed that decreasing the process temperature could be a good option for overcoming ammonia inhibition in anaerobic reactors. Besides, changes in growth rate and the effect on ammonia inhibition with temperature should be balanced.

Inanc *et al* (2000b) has reported that attached growth systems; anaerobic filter and hybrid bed reactors are more resistant to ammonia inhibition than UASB reactor. This might be explained by the diffusion limitation of the ammonia into the depths of the biofilms. Domination of ammonia resistant methanogens in the anaerobic filter and hybrid bed reactors is also possible (Inanc *et al.*, 2000b). The types and percent distribution of methanogenic populations should be determined in the reactors for bringing an explanation to the phenomenon.

In a previous study (Calli *et al.*, 2000) it was found that granular anaerobic sludge was more tolerant than flocculent sludge against free ammonia inhibition. A comparable suggestion was stated for biofilms by Inanc *et al.* (2000b) that surface diffusion of free ammonia is limited to the deeper parts of biofilm as to the inner parts of granules. In addition, similar situations are also valid for some methanogens. For instance, *Methanosarcina* packets are more resistant since the cells inside the packets are protected from inhibitory compounds by comparison with single cells (Macario *et al.*, 1999).

2.4.2 Postulated Ammonia Inhibition Mechanisms on Methanogens

At least two possible formation mechanisms for ammonia inhibition have been postulated by pure culture studies (Sprot *et al.*, 1984; 1985; Sprott and Patel, 1986; Kadam and Boone, 1996):

1. The activity of methane synthesizing enzymes may be directly inhibited by free ammonia,
2. Hydrophobic free ammonia molecule probably diffuses passively into the cell. Inside the cell ammonia is rapidly converted to ammonium owing to the intracellular pH conditions. Ammonium accumulated inside the cell may be toxic by altering the intracellular pH or the concentration of other cations such as K^+ (Figure 2.3).

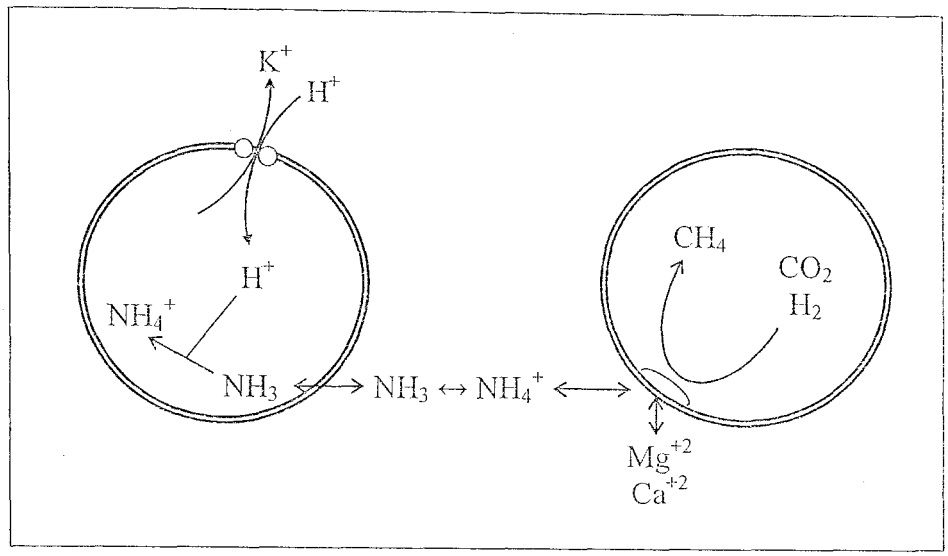

Figure 2.3. Model hypothesized by Sprott and Patel (1986) to illustrate two separate interactions of ammonia with *Methanospirillum hungatei*

Either way, high pH and total ammonia concentration could exert their toxicities synergistically. At higher pH values, a larger fraction of ammonia is unionized; about 1.1% at pH 7, but almost 78% at pH 9.5 at 35°C. Passive diffusion of free ammonia into the cytoplasm disrupts the internal cell pH by taking up protons to establish the NH_3/NH_4^+ equilibrium. In order to try to maintain the internal pH, the cells will activate systems, which actively import protons. Many methanogens can achieve this by exchanging potassium from the cytoplasm with protons from the environment, a so-called K^+/H^+-antiporter. Also if methanogens growing at a higher pH establish a more negative pH difference between inside and outside to maintain a near-neutral cytosol, then the potential toxicity due to NH_4^+ accumulation would also be greater (Sprott and Patel, 1986; Kadam and Boone, 1996).

3. ANAEROBIC TREATMENT OF HIGH AMMONIA LANDFILL LEACHATE WITH AND WITHOUT pH CONTROL

3.1. Anaerobic Treatment of Landfill Leachate

In Istanbul, municipal solid wastes are disposed into Odayeri and Kömürcüoda Sanitary Landfills located on the European and Asian sides of the city, respectively. Kömürcüoda Sanitary Landfill is under operation since 1995 on the Asian Side. It has been constructed on an old lignite and clay mining site with a total area of about 100 Ha. Its operating capacity is approximately 20 years and currently, about 4000 tons of solid waste is landfilled everyday.

Leachate generated in Kömürcüoda Landfill is collected in a concrete collection tank located at the lowest elevation of the site with perforated pipes laid on the impervious clay layer and geomembrane liner. The concrete collection tank is connected to the adjacent large earthen basin geomembrane covered holding pond. Recently, the excess leachate of about 1500-2000 m^3/day is transferred by tankers to the nearest sewer ending at Tuzla Municipal Wastewater Treatment Plant. Despite high leachate transportation costs, Istanbul Metropolitan Municipality, the owner and the operator of the landfills, would like to construct leachate treatment plants consisting of anaerobic and aerobic biological units for current and future landfills.

In literature, there are numerous studies about anaerobic treatability of leachate generating from different landfills in different countries. Some data from these studies are presented in Table 3.1. However, because of the differences in the composition of solid wastes landfilled, climatic conditions of the region, field operations and the landfill age, the leachate generated in other landfills may not resemble Kömürcüoda Landfill leachate in characteristics and treatability. Therefore, to select the appropriate processes for the treatment of leachate, characterization and treatability studies should certainly be conducted. For this purpose, the anaerobic treatability and characterization study

mentioned in this and subsequent chapters was started in August 1998 to obtain compatible design parameters and to propose solutions for possible problems in the operation of anaerobic reactors especially at start-up. Moreover, the data obtained from this study may also be used in the design and operation of other leachate treatment plants with similar leachate characteristics.

Table 3.1. Data of some previous studies about anaerobic treatability of leachate

Leachate source	Reactor type	Operational parameters			COD rem. eff., %	Ref.
		Temp., °C	OLR, kgCOD/m^3.day	HRT, days		
Colchester, England	Hybrid bed	30+/-2	3.75	5.0-2.5	81-97	[1]
Espoo, Finland	UASB	13-23	1.4-4.0		50-75	[2]
A Coruna, Spain	Anaerobic filter	37	0.2-1.2	10.7-1.8	55-60	[3]
Montevideo, Uruguay	UASB	30	9-15	2.0	75-95	[4]
Izmir, Turkey	Anaerobic SBR	35+/-2	6.5	10-1.5	74	[5]
	Hybrid Bed	35+/-2	0.8-16.5	5.1-0.9	81.4	

[1] Nedwell & Reynolds, 1996
[2] Kettunen & Rintala, 1998
[3] Mendez *et al.*, 1989
[4] Borzaconni *et al.*, 1999
[5] Timur & Ozturk, 1997

According to the first 250 days of the anaerobic treatability study including the start-up of the reactors, high ammonia concentrations of about 2700 mg/l with pH values as high as 8.2 were claimed to be responsible for an increase in the possibility of ammonia inhibition (Inanc *et al.*, 2000a). In a similar study, inhibition is reported at pH around 8.5 and ammonium nitrogen concentration of 1470 mg/l in a laboratory scale UASB reactor treating young landfill leachate in Montevideo, Uruguay (Borzacconi *et al.*, 1999).

In this chapter, results of the long-term anaerobic treatability study under low and moderately high free ammonia concentrations concerning with pH control in the reactor influents are presented.

3.2. Characterization of Leachate

For characterization of Kömürcüoda Landfill leachate, samples were taken from the leachate holding tank located at the lowest elevation of the landfill and brought to laboratory once every week between August 1998 and April 2001. The parameters analyzed were COD, BOD_5, total and volatile suspended solids, pH, alkalinity, total Kjeldahl and ammonium nitrogen, total phosphorus, chloride, metals as iron, manganese, copper, zinc, lead, cadmium, chromium and nickel.

In this study, Kömürcüoda Landfill leachate was characterized by high COD and ammonia concentrations ranging from 5850 to 47800 mg/l, and from 980 to 3260 mg/l, respectively. General characteristics are listed in Table 3.2 as minimum, maximum and average values with the numbers of analyses and duration.

Although pH values have varied between 7.0 and 8.2 in dry seasons, the organic content of the leachate was entirely composed of volatile fatty acids. Relatively elevated pH values were actually due to high alkalinity levels, which were affected strongly by extraordinarily high ammonia concentrations. BOD_5/COD ratio above 0.6 for most samples was an indication of high biodegradability and acidic phase of decomposition in the young landfill. NH_4-N/TKN ratio around 0.9 indicates that; most of the nitrogen was in the form of ammonia nitrogen.

3.3. Operation of Anaerobic Reactors

Three different laboratory scale anaerobic reactors namely upflow anaerobic sludge blanket (UASB), anaerobic upflow filter (AF) and hybrid bed reactors were operated for

1015 days in the treatment of high ammonia Kömürcüoda landfill leachate. Configurations and dimensions of the reactors are shown in Figure 3.1.

Table 3.2. Characteristics of Kömürcüoda Landfill leachate

Parameters	Numbers of analyses	Max.	Min.	Average	Duration
pH	73	8.5	6.2	7.6	Aug 98-Apr 01
Alkalinity, mg/L	73	13050	3800	9960	Aug 98-Apr 01
COD, mg/L	73	47800	5850	20650	Aug 98-Apr 01
BOD_5, mg/L	67	38500	3790	13600	Aug 98-Apr 01
NH_4-N, mg/L	73	3260	980	2135	Aug 98-Apr 01
TKN, mg/L	66	3590	1050	2392	Aug 98-Apr 01
SS, mg/L	62	4720	670	2171	Aug 98-Feb 01
TP, mg/L	63	6.4	0.14	3.0	Aug 98-Jan 01
Color, PtCo	70	47200	7400	18400	Aug 98-Mar 01
Chloride, mg/L	60	8500	725	3670	Aug 98-Feb 01
Fe, mg/L	68	365	4.9	62	Aug 98-Feb 01
Mn, mg/L	68	25.5	0.09	3.8	Aug 98-Feb 01
Cu, mg/L	68	1.1	0.01	0.23	Aug 98-Feb 01
Zn, mg/L	68	3.6	0.17	0.98	Aug 98-Feb 01
Pb, mg/L	66	3.6	0.10	0.78	Aug 98-Feb 01
Cd, mg/L	68	30.4	0.02	1.00	Aug 98-Feb 01
Cr, mg/L	68	10.2	0.01	0.53	Aug 98-Feb 01
Ni, mg/L	68	4.6	0.12	0.67	Aug 98-Feb 01
Sulfide, mg/L	12	37.5	0.50	10.4	Nov 00-May 01

The reactors were seeded with sludge taken from the anaerobic treatment plant of a baker's yeast factory. Mesophilic temperature range was maintained in the reactors by placing them in a constant temperature room at 35 ± 2°C. In the start-up and in some periods that COD in leachate suddenly jumps, dechlorinated tap water was used for dilution of the leachate to adjust the organic loading. Phosphoric acid was added to the

feed to provide a COD/Nitrogen/Phosphorus ratio of 500/7/1 due to phosphorus deficiency of the leachate.

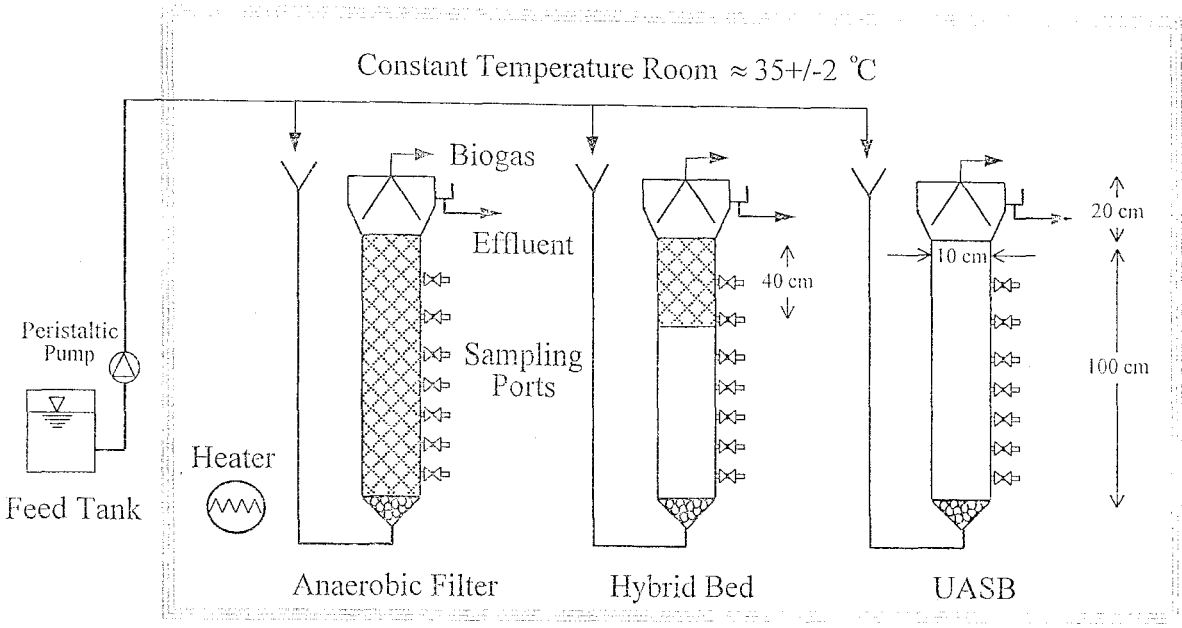

Figure 3.1. Experimental set-up and reactor configurations

In the reactor influents and effluents, pH and alkalinity were monitored daily, while COD and ammonium nitrogen were monitored three times in a week. Volatile fatty acid analyses were also conducted in periods when COD removal efficiencies have dropped. Meanwhile, sludge concentrations along the height were measured several times throughout the operation of the reactors. The time schedule of the experimental study is given in Figure 3.2.

The characterized leachate was fed to the reactors without any pH adjustment until Day 175. Subsequently, pH was adjusted to 4.5 in the influents to keep the free ammonia concentration below inhibitory level in the reactors between Day 175 to Day 860. In this period organic loading was increased gradually from 1.3 to 23.5 kg COD/m^3.day during start-up and then fluctuated between 2.9 and 23.5 kgCOD/m^3.day according to fluctuations in COD concentrations in raw leachate. Hydraulic retention time was reduced from 2.4 to 2.0 days after Day 180 and kept constant until the end of the study. Meanwhile, all the reactors showed similar performances with COD removal efficiencies between 75% and 95% as free ammonia concentration kept below inhibitory levels.

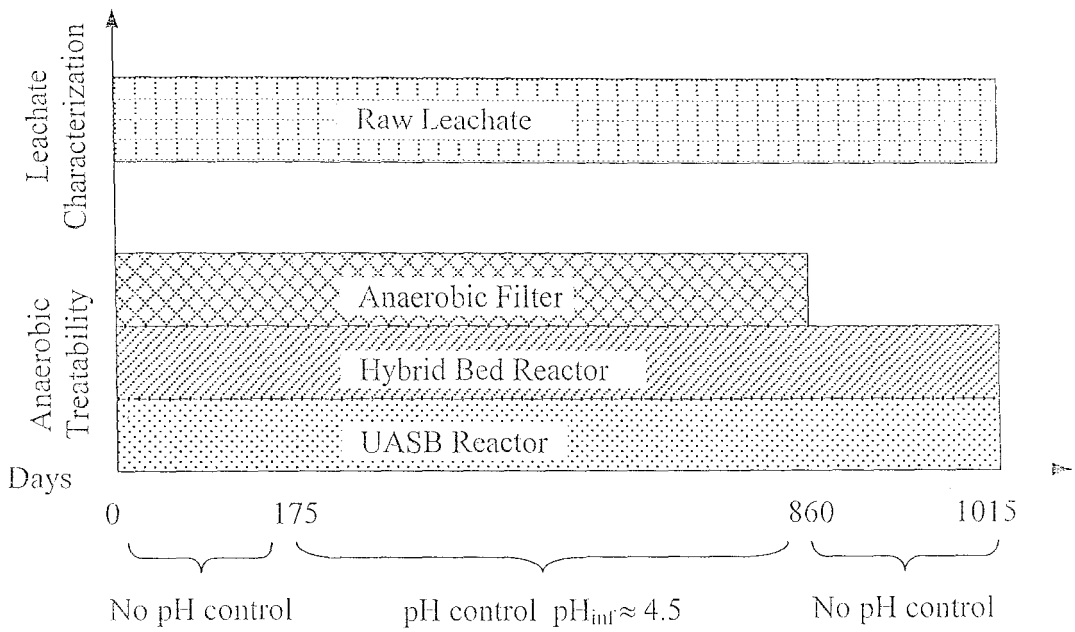

Figure 3.2. Time schedule of the experimental study

After Day 860, pH adjustment was terminated to monitor the effects of high free ammonia and pH levels on COD removal efficiency and microbial diversity in the reactors. Meantime, feeding to the Anaerobic Filter was stopped to investigate the biofilm populations on filter materials inside the reactor. After Day 860, organic loadings to UASB and Hybrid bed reactors were varied between 2.4 and 18.7 kg COD/m^3.day.

3.4. Results of Long Term Anaerobic Treatability Study

All three reactors were started with an organic loading rate of 1.3 kg COD/m^3.day. The raw leachate was diluted with de-chlorinated tap water for adjusting the appropriate organic loading. Therefore, the parameters other than COD were also diluted during this start-up period. After Day 150, the leachate was fed to the reactors without any dilution, so that influent COD concentrations were increased from about 3000 mg/L to about 45000 mg/L with stepwise increase in organic loading up to 23 kg COD/m^3.day at Day 421 (Fig. 3.3 and 3.4). Constant organic loading rates could not be applied continually as a result of low COD concentrations due to precipitation dilutions in winter months.

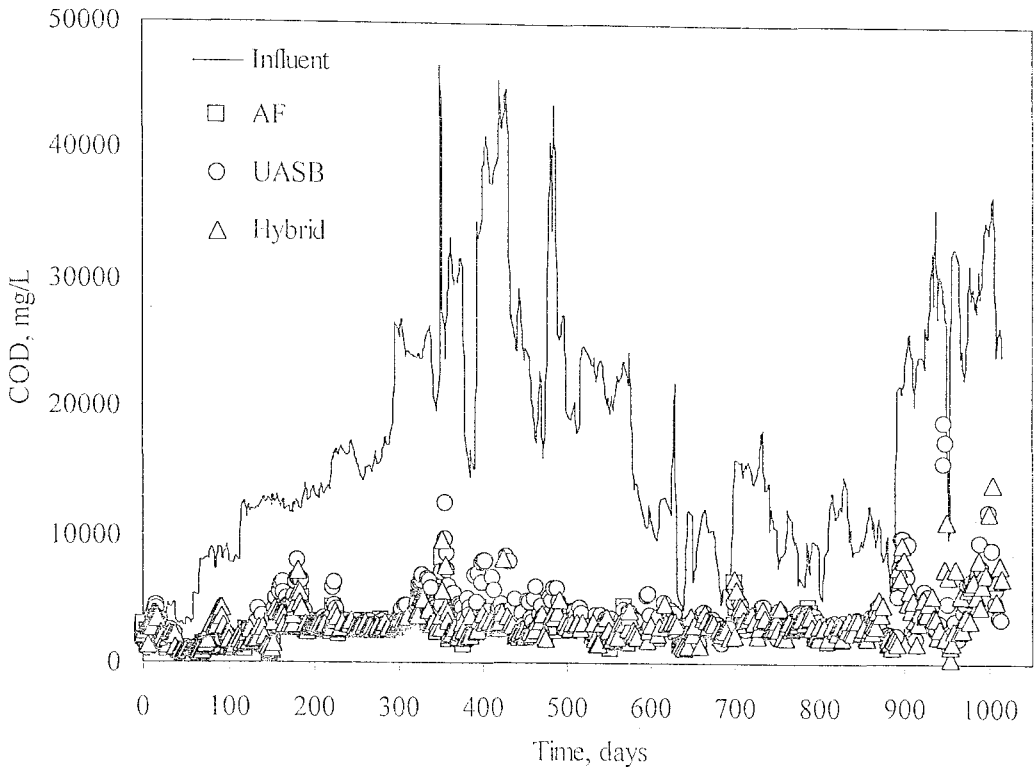

Figure 3.3. COD concentrations in the effluents of the reactors

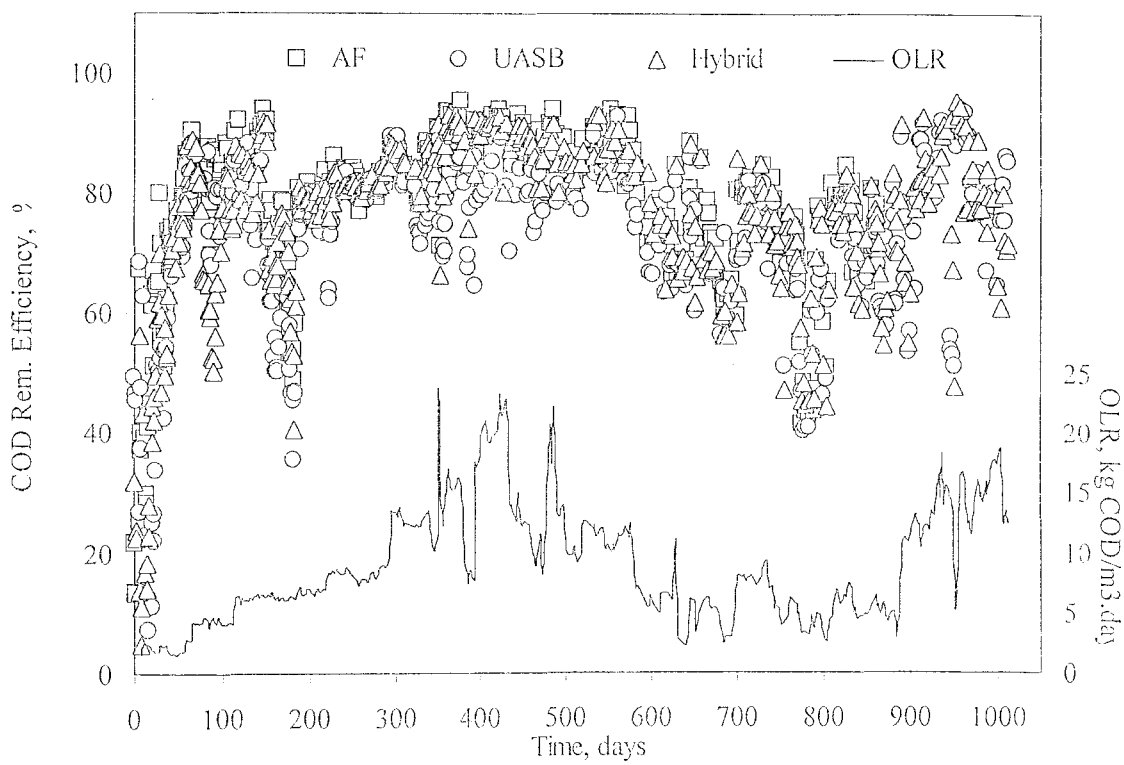

Figure 3.4. Organic loading rate and COD removal efficiencies

Until Day 175, all reactors showed similar performances against organic loadings with efficiencies between 78 and 94%. However the reactors have experienced high ammonia concentrations several times throughout the experimental period and showed different inhibition levels. The ammonia inhibition has occurred around between Day 75 and Day 100. Other severe ammonia inhibitions were between Day 130 and Day 145, Day 150 and Day 170, Day 175 and Day 190. No pH control was done until Day 175 where ammonia inhibition became more severe. Since free ammonia is more toxic to methanogens, total ammonia concentration is not a good parameter for understanding ammonia inhibition. Therefore, free ammonia concentrations in the reactors have been calculated and plotted with COD removal efficiencies as seen in Figure 3.5. pH values in the reactors were always above 8.0 and exceeded 8.5 for some duration in this period (Fig. 3.6 and 3.7). In the meantime, total alkalinity concentrations above 11000 mg/l were experienced in the reactor effluents.

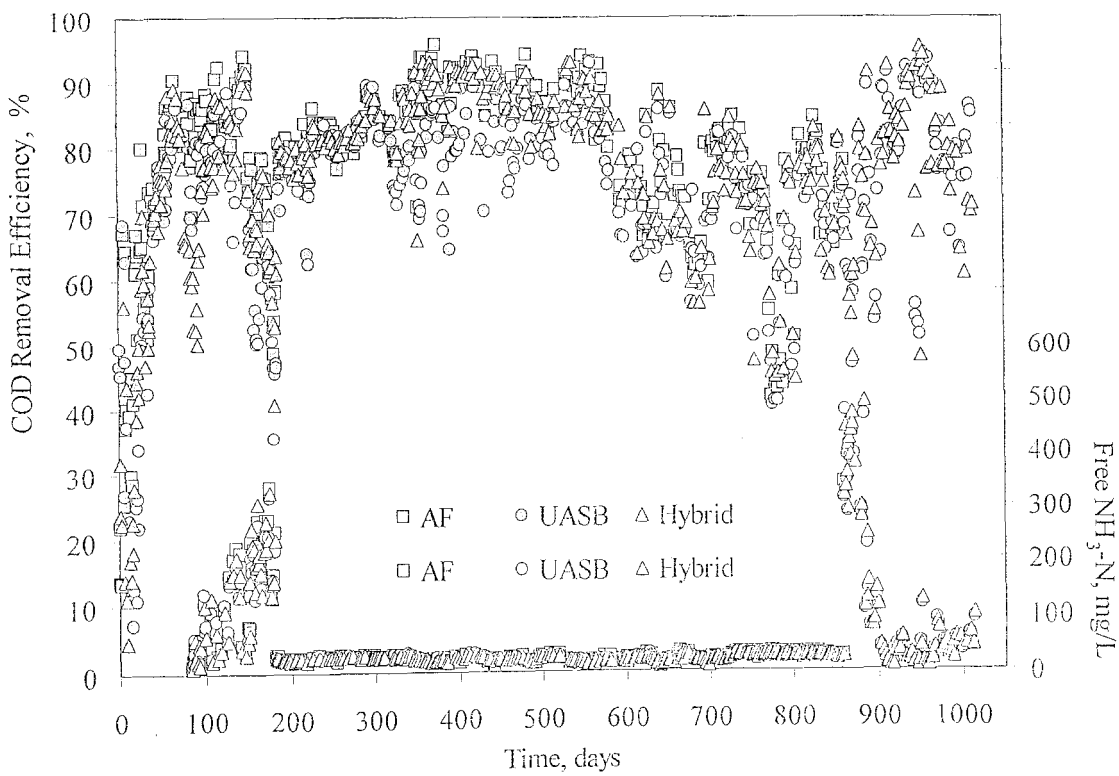

Figure 3.5. Effects of free ammonia concentrations on COD removal efficiencies

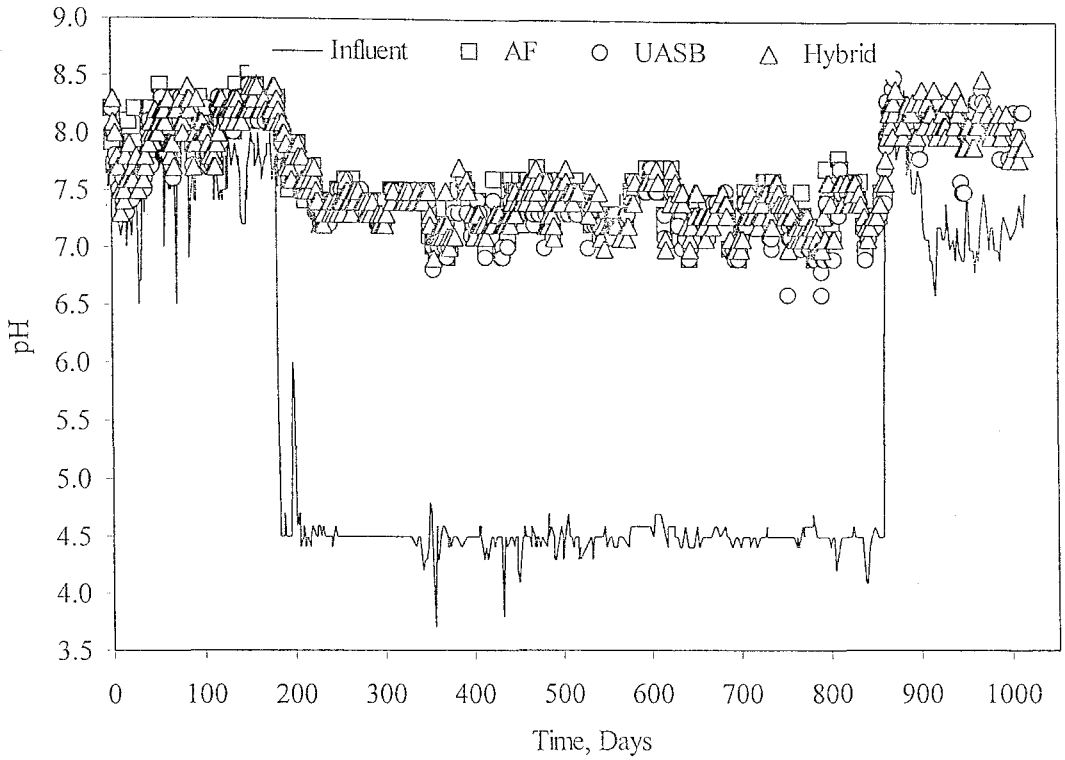

Figure 3.6. Influent and effluent pH values

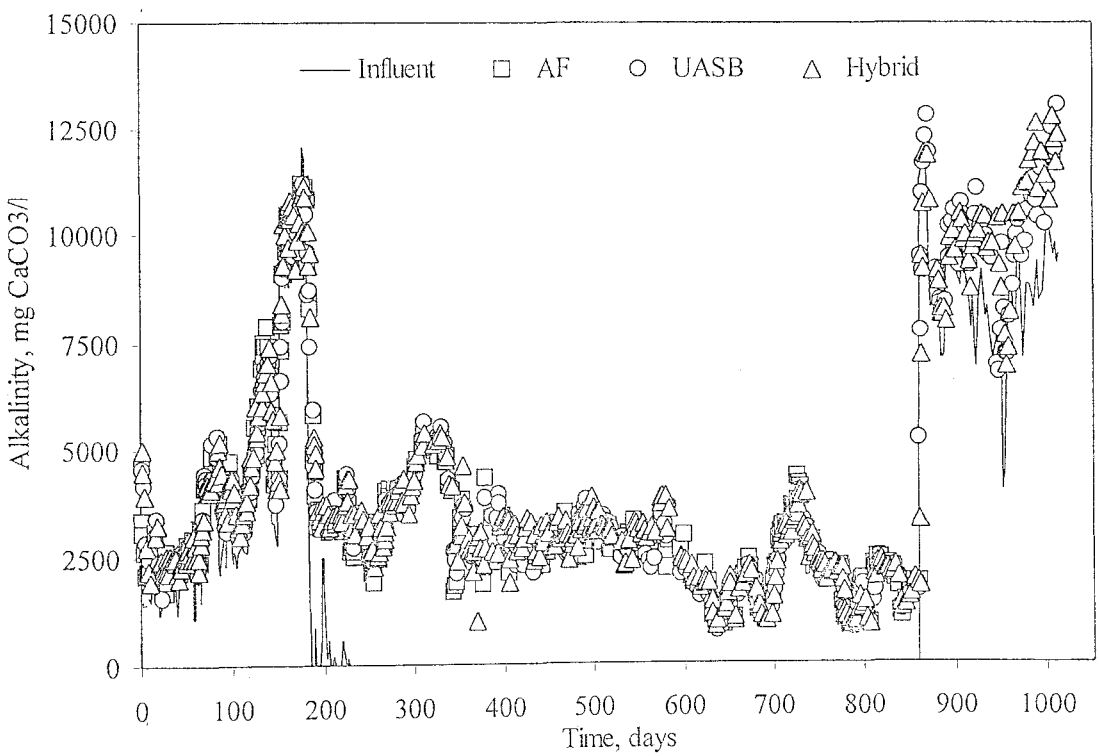

Figure 3.7. Influent and effluent alkalinity concentrations

Ammonia inhibition was reversible, as it can be seen from rapid increases in COD removal efficiencies of reactors, immediately after drop of free ammonia concentrations after Day 175 (Fig. 3.5). Attached growth systems namely, anaerobic filter and hybrid bed reactors were more resistant to ammonia inhibition than UASB reactor. Until Day 175, COD removal efficiencies as high as 92% and 94% were observed in the hybrid bed reactor and anaerobic filter, respectively, while the lowest efficiencies were always associated with the UASB reactor (Fig. 3.4). This was clearly seen in all cases of ammonia inhibition. The resistance of anaerobic filter and hybrid bed reactors to ammonia might be due to limited diffusion of free ammonia into the biofilm or conversion of free ammonia to ammonium ion at higher pH values in the biofilm. Another reason can be the dominance of ammonia resistant methanogens in these reactors which will be discussed in the next chapter.

To control the ammonia inhibition, pH of the influent was decreased to 4.5 with concentrated hydrochloric acid after Day 175. Therefore, pH values of reactors decreased gradually below 8.0 following the pH control in the influent (Fig. 3.6). Besides, even though ammonium nitrogen concentrations were almost stable in the range between 2200 and 2400 mg/l, free ammonia concentrations also decreased from about 330 mg/L to 30 mg/L parallel to drop in pH (Fig. 3.8 and 3.9). COD removal efficiency in all reactors were restored back to 80% levels soon after the drop in free ammonia concentrations and levels as high as 95% were obtained until Day 580 (Fig. 3.4).

Between Day 580 and Day 860, COD removal efficiencies in the reactors were usually in the range of 60 to 85% corresponding to low organic loading rates due to low COD concentrations in the leachate (Fig. 3.4). In this period, contradictory to free ammonia concentrations below inhibitory levels, high non-biodegradable portion in the leachate resulted in lower COD removal efficiencies in the reactors under lower organic loading rates. This situation was severely experienced between Day 776 and Day 805 and COD removal efficiencies dropped below 45% parallel to decrease in the organic loading rate to 2.6 kg COD/m^3.day with high non-biodegradable portion (Fig. 3.4).

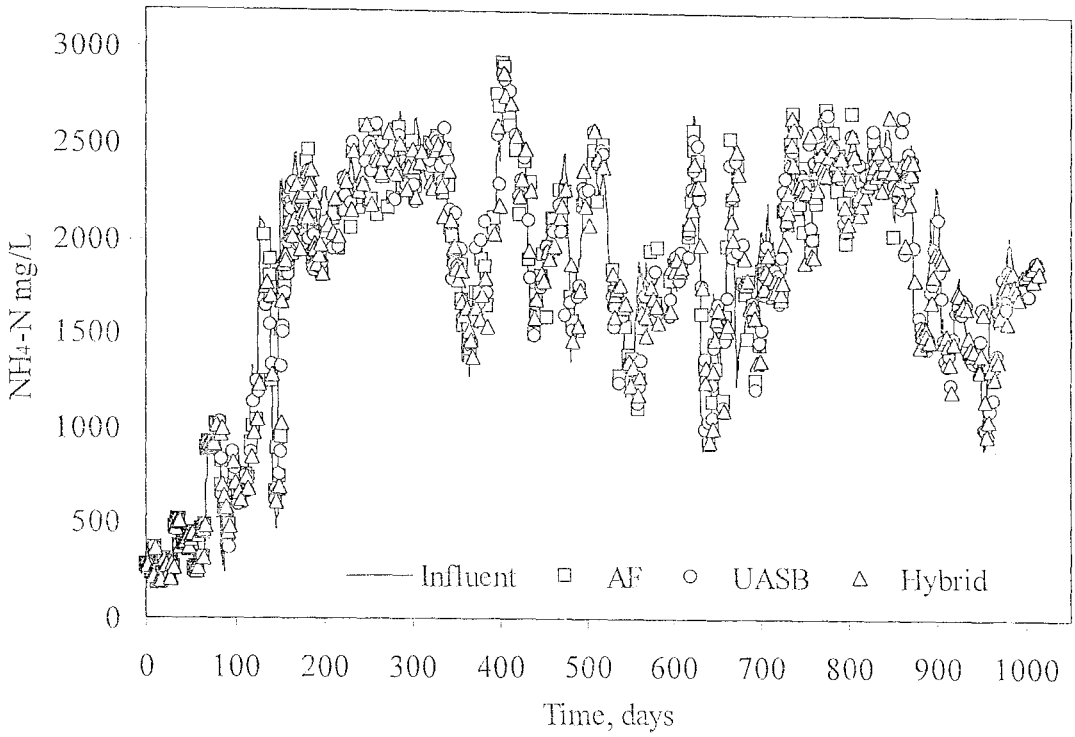

Figure 3.8. Influent and effluent total ammonia nitrogen (TAN) concentrations

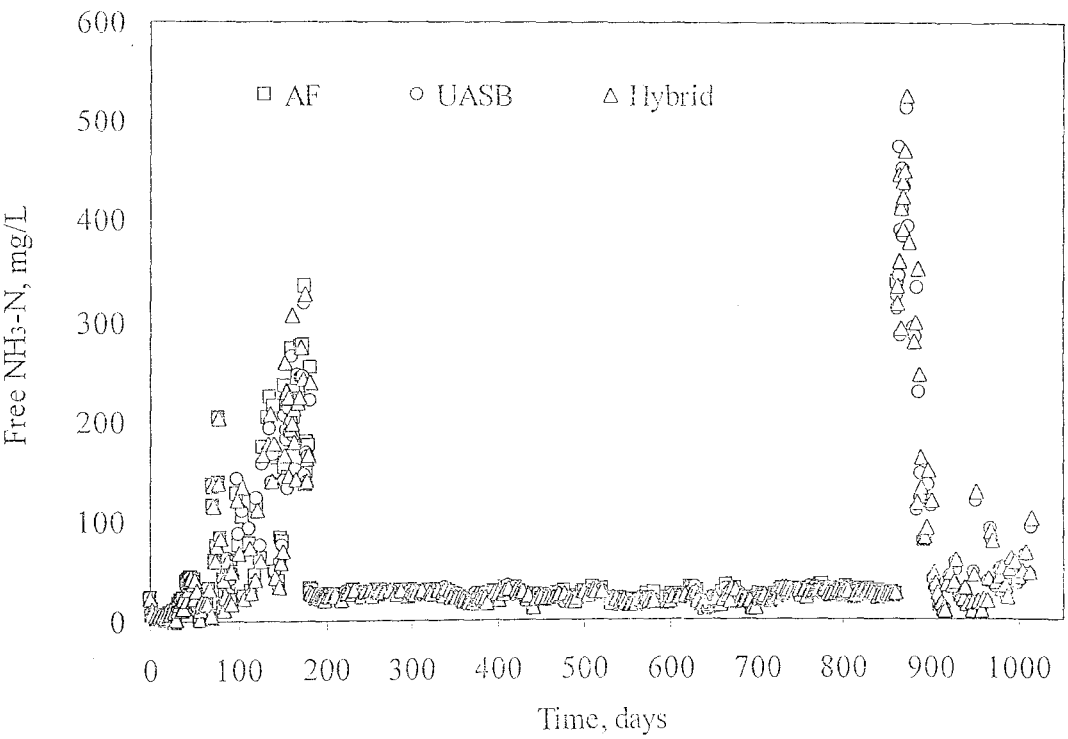

Figure 3.9. Free ammonia nitrogen (FAN) concentrations in the reactors

The sudden increase in influent COD concentration from 5800 to 16000 mg/l at Day 699 (Fig. 3.3) resulted in VFA accumulation in both of the reactors and hence increments in effluent COD concentrations. However, the increase in the effluent COD concentrations was not clearly reflected to COD removal efficiencies since the easily biodegradable portion of COD has also increased. In this period, the highest organic loading rates were 8.1 and 9.3 kg COD/m^3.day at Day 703 and Day 734, respectively. In the meantime, ammonium concentrations fluctuated between 1110 and 2690 mg/l according to variations in the characteristics of leachate (Fig. 3.8).

Before Day 860, by adjusting the influent pH to 4.5, free ammonia concentrations were kept below 30 mg/l in the reactors, despite fluctuations in pH values and ammonium concentrations of leachate (Fig. 3.6 and 3.9). To adjust the influent pH to 4.5, high amounts of hydrochloric acid were consumed in this period because of high alkalinity concentrations of about 10000 mg/l in raw leachate (Fig. 3.7). Thus to minimize the acid consumption that will be a significant cost in the operation of the full scale leachate treatment plant and to investigate the effects of increasing free ammonia concentrations and pH values on the performances of the reactors, pH adjustment in the influent of Hybrid bed and UASB reactors was terminated after Day 860. Meanwhile, feeding of Anaerobic Filter was stopped for further microbial studies on biofilm.

Just after the termination of pH adjustment, between Day 860 and Day 890, organic loading rate was nearly stable between 4.9 to 6.4 kg COD/m^3.day (Fig. 3.4). However, COD removal efficiencies dropped significantly from 82 to 42% within two weeks parallel to a sudden jump in the free ammonia concentrations up to 530 mg/l (Fig. 3.5). Nevertheless, after the unexpected decrease of the ammonium concentration in raw leachate to 1400 mg/l levels (Fig. 3.8) and drop in pH values in the reactors due to acetate accumulation, at Day 881 COD removal efficiencies were restored back to 81% and 83% for UASB and Hybrid bed reactors, respectively (Fig. 3.4). The rapid improvement in the reactor performances has corroborated the reversibility of ammonia inhibition, which was also experienced several times at the start-up until Day 175.

At Day 891, the COD concentration of raw leachate brought from Kömürcüoda Landfill was unexpectedly increased to 20800 from 9500 mg/l (Fig. 3.3). Parallel to the

increase in influent COD concentration, the organic loading rate jumped to 11.3 kgCOD/m^3.day. After this shock organic load, the VFA accumulation did not severely affect the performances of reactors because of high alkalinity in the influent (Fig. 3.7). In the meantime, ammonium concentration of the leachate decreased step by step to 1000 mg/l contradictory to the increase in organic loading (Fig. 3.8). The reduction in ammonium concentration has sustained the rapid degradation of accumulated VFAs in the reactors by reducing the free ammonia concentration. Consequently, after three weeks COD removal efficiencies have restored back to 89% in UASB and to 82% in hybrid bed reactors (Fig 3.4). The difference in removal efficiencies can be explained with the presence of acetate and propionate in the effluent of the hybrid bed reactor as 525 mg/l and 470 mg/l, respectively.

Disregarding the period between Day 947 and Day 952, where there were problems with heating, both of the reactors showed similar performances until Day 970 with efficiencies above 90%. In this period, low ammonium concentrations and pH values in the influent have kept the free ammonia concentration below inhibitory levels within the first 30 cm of the reactors where COD removal was essentially carried out.

In the last part of the study after Day 970, the organic loading rate has reached to its highest value since Day 486 as 18.7 kg COD/m^3.day (Fig 3.4). In consequence of high organic loading rate with free ammonia concentrations in the range of 23 to 102 mg/l, COD removal efficiency slightly dropped and fluctuated between 66 and 88% (Fig 3.5).

3.5. Discussions

The leachate characteristics have indicated that, Kömürcüoda Landfill possesses the properties of a young landfill. High VFA concentrations and BOD_5/COD ratio above 0.6 are the indications of high acidogenic activity in the landfill. Relatively elevated pH values were mainly owing to ammonium nitrogen concentrations extraordinarily as high as 3260 mg/l.

By adjusting the influent pH to 4.5 to keep the free ammonia concentrations below inhibitory level, COD removal efficiencies above 90% were achieved with laboratory scale, anaerobic filter, upflow sludge blanket and hybrid bed reactors in anaerobic treatment high ammonia Kömürcüoda landfill leachate.

It is understood that temporary pH adjustment in the influent can be used without discernible adverse effects on anaerobic microorganisms, for preventing free ammonia inhibition when high pH values and ammonium concentrations are experienced.

Sudden and unexpected fluctuations in the characteristics of leachate made it complicated to investigate the free ammonia inhibition in the reactors and understand the effects on microbial populations and activities. Therefore, similar reactors were operated under more stable COD loading and at higher free ammonia concentrations by feeding with a synthetic wastewater simulating the characteristics of Kömürcüoda Landfill leachate which will be discussed in Chapter 5.

4. INVESTIGATION OF VARIATIONS IN MICROBIAL DIVERSITY IN ANAEROBIC REACTORS TREATING LANDFILL LEACHATE

4.1. Introduction

Anaerobic treatment is widely used in digestion of animal manures, primary and waste activated sludges and in the stabilization of organic solid wastes. Besides, the technology is particularly attractive for the treatment of biodegradable agro-industrial wastewaters and some other complex wastes such as those derived from petrochemical and pharmaceutical industries. Anaerobic treatment is also convenient as a pretreatment for young landfill leachate although some compounds such as ammonia, volatile fatty acids and heavy metals may reach to inhibitory concentrations in the reactor (Borzacconi *et al.*, 1999; Inanc *et al.*, 2000a; Calli *et al.*, 2002).

To operate an anaerobic reactor properly, in addition to some efficiency control parameters, such as pH, alkalinity, COD, volatile fatty acids and biogas composition, microbial activity and diversity should also be monitored by identifying the microorganisms that lead to biomass stability. By this means, some other essential unknowns in anaerobic degradation such as the selection of fermentation pathways, physical manipulation of intermediate products, acclimation to inhibitory compounds and formation of granules may be elucidated. Moreover, the reactor operations may be optimized in accordance with the correlation between performances of the reactors and variations in microbial diversity. Knowledge about microbial activity and diversity is also needed in the selection of appropriate seed sludge for a successful start-up. However, for many decades the identification of microorganisms in anaerobic reactors was limited to cultivation dependent methods because, syntrophic interactions, low growth rates, unknown growth requirements and obligate anaerobiosis make these microorganisms difficult to isolate (Oude Elferink *et al.*, 1998a). Particularly, methanogens having a key role in anaerobic biodegradation are among the microorganisms that are most difficult to study with these techniques (Stams and Oude Elferink 1997). Through the promising 16S

rDNA based molecular methods, it is now possible to identify methanogenic archaea and fermentative bacteria present in anaerobic reactors - regardless of their cultivation capabilities (Raskin *et al.*, 1994; Sekiguchi *et al.*, 1999; Chan *et al.*, 2001). Therefore, such methods are becoming progressively more essential for a proper start-up and in the optimization of operational processes to advance the treatment efficiency (Fernandez *et al.*, 1999; McMahon *et al.*, 2001). Insights into the diversity, structure and function of mixed microbial communities in anaerobic reactors are also necessary to improve the stability against inhibitory compounds.

In this study, variations in microbial diversities in UASB and hybrid bed reactors treating Istanbul Kömürcüoda Landfill leachate were monitored by using denaturing gradient gel electrophoresis (DGGE) and fluorescent in-situ hybridization (FISH) techniques that are complimentary to each other. These are powerful techniques in the identification of microbial populations in combination with cloning and DNA sequencing. The microbial distribution was monitored along the height of the reactors in addition to pH, COD, free ammonia, SS and VSS analyses. Afterwards, correlation between the variations in microbial diversity and performances of reactors were investigated.

4.2. Experimental Methods

To investigate the variations in microbial diversity in hybrid bed and UASB reactors, sludge samples were taken from sampling ports located along the height of the reactors on Days 794, 824, 860, 895, 919, 953, 982 and 1012. These sludge samples were stored in a freezer at -20°C before DNA isolation. Experimental procedure followed in the identification of microorganisms in sludge samples is given in Figure 4.1.

4.2.1. DNA Isolation and PCR Amplification

To start with DNA isolation, initially sludge samples were mechanically bead beaten. After the mechanical disruption, released DNA was isolated and purified according to the

procedure outlined in Oude Elferink *et al.*, 1997. 1 ml of sludge sample was transferred to a 3 ml polypropylene tube. 500 µl TE buffer (10 mmol/l Tris/HCl, 1mmol/l EDTA, pH 8.0), 500 µl Tris/HCl buffered phenol (pH 8.0) and approximately 0.6 g zirconia/silica beads (diameter 0.1 mm Biospec Products Inc., Bartlesville, OK) were added on the sludge to the same tube. The cells were disrupted by treatment for 15 to 20 minutes in a vortex mixer. The aqueous phase of the supernatant fluid, obtained after 10 min of centrifugation (14000 rpm), was extracted with phenol/chloroform/isoamyl alcohol 25:24:1 (v:v:v), followed by chloroform/isoamyl alcohol 24:1 (v:v). Subsequently, the volume of the sample was adjusted to 0.5 ml, and DNAs were precipitated with 0.5 ml of isopropanol and 250 µl of ammonium acetate (7.5 M) at -20°C overnight. After 10 min centrifugation (14000 rpm), DNA pellet was washed with 70% ethanol, dried at room temperature and resuspended in 100 µl of TE buffer. The isolated DNA was used for PCR after judging the quality of the extract by agarose gel electrophoresis and ethidium bromide staining.

The V6 to V8 region of the 16S-rDNA genes, from the isolated DNA, was amplified using polymerase chain reaction (PCR) with two sets of PCR primers for the DGGE analysis. The primer set specific to the *Bacteria* domain was GC-968 for and UNI-1401 rev. The other primer set specific to the *Archaea* domain was A-109 for and GC-515 rev. Sequences of the primers used in PCR are given in Table 4.1. For the amplification of the 16S-rDNA gene for cloning, as the primer set specific to the *Archaea* domain A-109 for and 1510 rev, to *Bacteria* domain 7 for and 1510 rev were used. The PCR programs were performed in a Progene thermocycler (Techne). In the PCR for total archaeal 16S-rDNA and for DGGE the following program was used: pre-denaturation (94°C, 5 min), 34 cycles of annealing (52°C, 40 sec), elongation (68°C, 1 min), and denaturation (94°C, 30 sec), a final annealing (52°C, 40 sec) and post elongation (68°C, 5 min). In the PCR for bacterial 16S-rDNA the following program was used: pre-denaturation (94°C, 5 min); 25 cycles of denaturation (94°C, 30 s), annealing (52°C, 20 s), and elongation (68°C, 40 s); finally post-elongation (68°C, 7 min). The reactions were subsequently cooled to 4°C. For DGGE, a specific region of eubacterial 16S rDNA (V6-V8 region) was amplified using the same thermocycle program but with 35 cycles and an annealing temperature of 56°C. PCR products were examined on ethidium bromide-stained agarose gels, and were used for DGGE analysis and cloning.

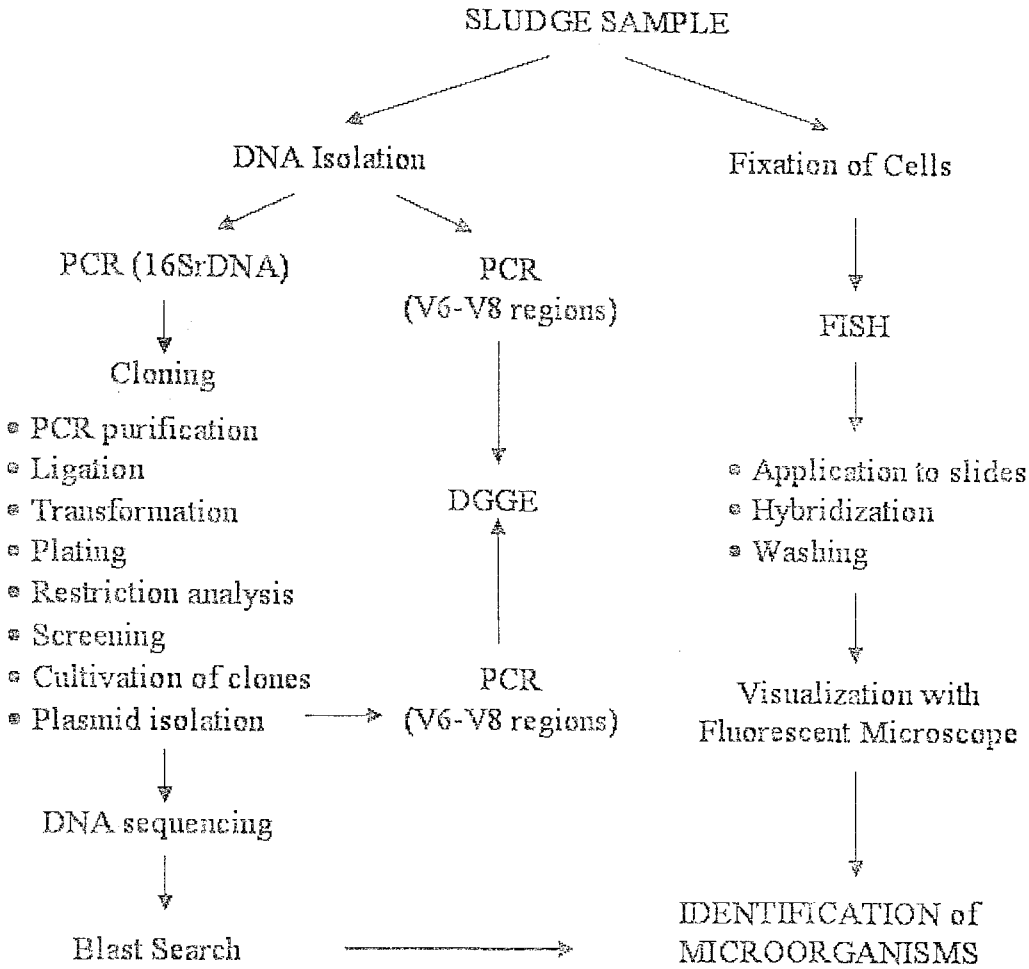

Figure 4.1. Experimental procedure followed in identification of microorganisms in sludge samples

4.2.2. Denaturing Gradient Gel Electrophoresis (DGGE) and Cloning

DGGE of the PCR amplified bacterial and archaeal partial 16S rDNA was performed with the BioRad D-Code Universal Mutation Detection System (BioRad) in accordance with Nübel *et al.*, 1996. The polyacrylamide concentrations used in the analysis of DGGE were 8% and the denaturing gradients were 35–50% for archaeal and 35-55% for bacterial 16S-rDNA. The denaturing gradient gel was electrophoresed at 85 V and 60°C for 16 hours. Previously a voltage of 200 V was applied for 5 min. Silver-staining and development of the gels were performed as described in Sanguinetti *et al.*, 1994.

The staining procedure consisted of an initial pre-stain fixation for 3 min in 10% ethanol, 0.5% acetic acid, staining for 10 min in fixing solution plus 0.2% silver nitrate, washing of gel in water for 2 min and development for approximately 45-60 min in 1.5% NaOH and 0.3% formaldehyde and 80 µg/l sodium borohydrate in deionized water. Following the staining, gels were fixed for a further 5 min and washed in deionized water to provide a permanent record of the experiment. Subsequent to this second fixation, gels were racked for 7 min in 25% ethanol and 10% glycerol preservation solution and covered with porous hydrophilic cellophane. Finally, they are dried overnight at 37°C.

Table 4.1. Primers used in polymerase chain reaction (PCR)

Primer	Target	Product	Sequence (5' - 3')	Ref.
7 for	Bacterial forward	Total 16S rDNA	GAC GGA TCC AGA GTT TGA T(C/T)(A/T) TGG CTC AG	1
1510 rev	Bacterial reverse	Total 16S rDNA	GTG AAG CTT ACG G(C/T)T ACC TTG TTA CGA CTT	1
GC968 for	Bacterial forward	DGGE	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	2
UNI1401 rev	Bacterial reverse	DGGE	GCG TGT GTA CAA GAC CC	2
A-109 for	Archaeal forward	Total 16S rDNA & DGGE	AC(G/T) GCT CAG TAA CAC GT	3
1510 rev	Archaeal reverse	Total 16S rDNA	GTG AAG CTT ACG G(C/T)T ACC TTG TTA CGA CTT	1
GC-515 rev	Archaeal reverse	DGGE	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC	1
T7 for	pGEM-T plasmid	Sequencing	AAT ACG ACT CAC TAT AGG	4
Sp6 rev	pGEM-T plasmid	Sequencing	ATT TAG GTG ACA CTA TAG	4

[1] Lane *et al.*, 1991
[2] Nübel *et al.*, 1996
[3] Grosskopf *et al.*, 1998
[4] Zoetendal *et al.*, 1998

The amplified archaeal 16S rDNA products were purified with a QIAquick PCR purification kit (Qiagen) and cloned in competent *E. coli* JM109 cells by using the pGEM-T Easy vector system (Promega) with ampicillin selection and blue/white screening, according to the manufacture's manual. The inserts were screened by Restriction Fragment Length Polymorphism (RFLP) analysis with the enzyme *MspI* (Fermentas) and by mobility comparison on DGGE. Plasmids of selected transformants were purified using the Wizard Plus SV miniprep DNA purification kit (Promega). Sequencing analysis was carried out in a private sequence laboratory. A similarity search, in the GenBank database, with the derived partial (app. 600-800 bp) 16S rDNA sequences from the clones, was performed by using the NCBI sequence search service, available on internet.

4.2.3. Fluorescent In-situ Hybridization (FISH)

The sludge samples were fixed overnight in 4% paraformaldehyde-phosphate-buffered saline at 4°C. Fixed cells were spotted on gelatin-coated [0.1% gelatin and 0.01% $KCr(SO_4)_2$] multiwell glass slides (10 wells/slide; 4 µl of sample/well) and allowed to dry at room temperature (Raskin *et al.*, 1994).

Hybridizations were performed at 46°C for 2 h with a hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 8.0, 0.01% SDS) containing each labeled probe (30 ng/well for Cy3 and TRITC 50 ng/well for FLUOS) (MWG Biotech, Ebersberg). Formamide was added to the final concentrations listed in Table 4.2 to ensure the optimal hybridization stringency. After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer containing the same components of the hybridization buffer except the probes.

For detection of all DNA, 4,6-diamidino-2-phenylindole (DAPI) was added to the wash buffer (100 µl of 0.1% DAPI). Slides were subsequently incubated at 48 °C for 10 min with washing buffer, rinsed briefly with ddH_2O and immediately air-dried. Vectashield (Vector Laboratories) was used to prevent photo bleaching. The slides were

examined with an Olympus BX50 microscope. Digital images of the slides were taken with a digital camera.

Table 4.2. Oligonucleotide probes used in fluorescent in-situ hybridization

Probe name	Label	Formamide (%)	Sequence (5' - 3')	Target group	Ref.
EUB 338	FLUOS	20	CTGCTGCCTCCCGT AGGAGT	Bacteria domain	1
ARC 915	Cy3	20	GTGCTCCCCCGCCA ATTCCT	Archaea domain	2
MX 825	Cy3	35	TCGCACCGTGGCCG ACACCTAGC	*Methanosaeta*	3
MS 821	FLUOS	35	CGCCATGCCTGACA CCTAGCGAGC	*Methanosarcina*	3
MB 1174	TRITC	35	TACCGTCGTCCACT CCTTCCTC	*Methanobacteriales*	3
MC 1109	FLUOS	35	GCAACATAGGGCA CGGGTCT	*Methanococcales*	3
MG 1200	TRITC	35	CGGATAATTCGGG GCATGCTG	*Methanogenium*-relatives	3

[1] Amann *et al.*, 1990
[2] Stahl *et al.*, 1991
[3] Raskin *et al.*, 1994

4.3. Results of Experiments

As mentioned in Chapter 3 Kömürcüoda Landfill leachate containing high ammonia concentrations in the range of 980 and 3260 mg/l was treated anaerobically for 1015 days in three different laboratory scale anaerobic reactors configured as upflow sludge blanket, anaerobic filter and hybrid bed. In this chapter, last 265 days of the study was presented by investigating the effects of high free ammonia concentrations on microbial diversity and performances of UASB and hybrid bed reactors. COD removal efficiency and organic loading rate, pH and ammonia graphs reflecting the performances of reactors in this period are presented in Figure 4.2. Sludge sampling days were also pointed out on time scale of the same graphs.

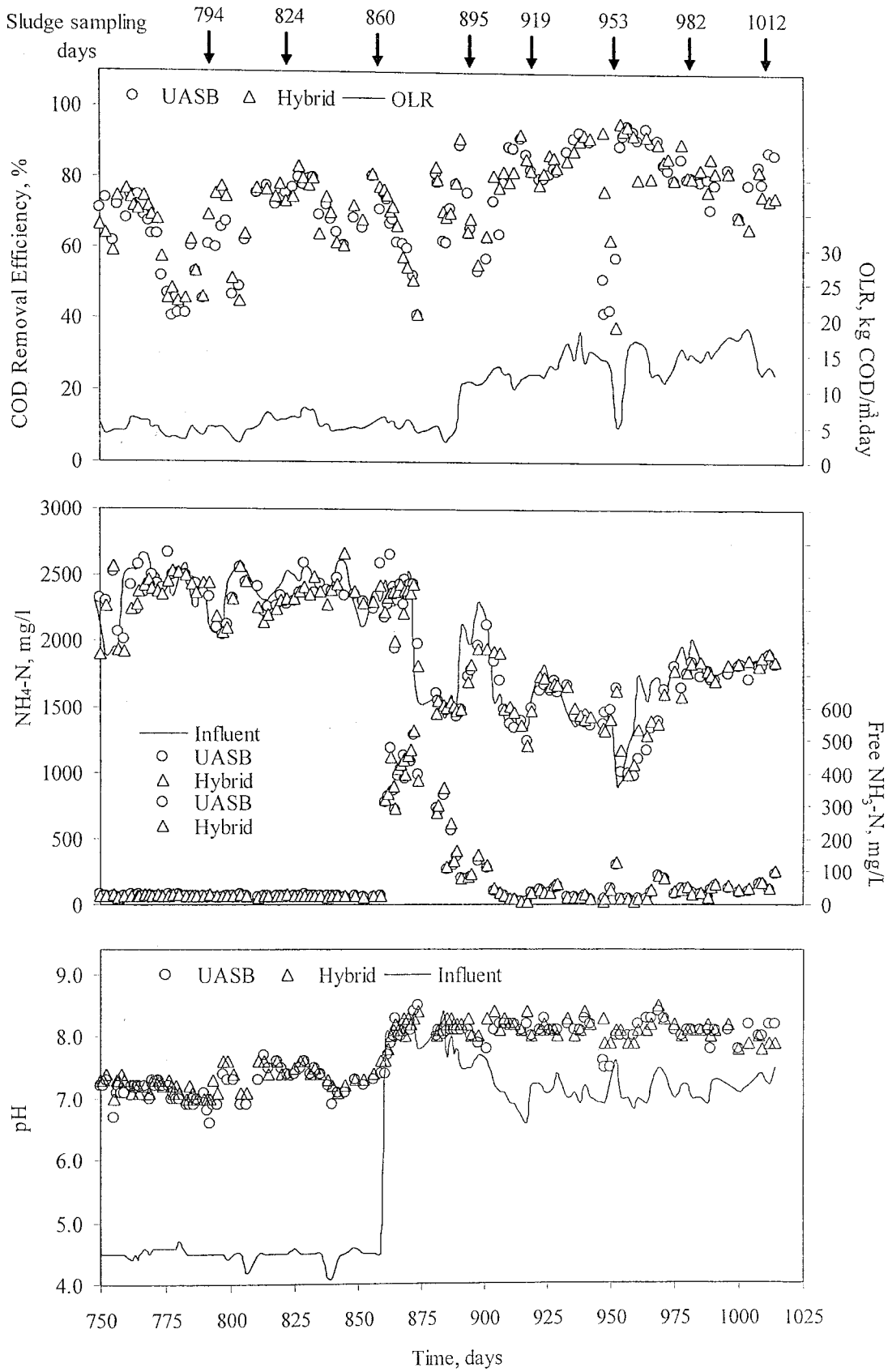

Figure 4.2. Graphs of some control parameters for UASB and hybrid bed reactors

Until Day 860, the influent pH was adjusted to 4.5 to keep the free ammonia concentration in the reactors below inhibitory level experienced several times in the start-up. Between Day 750 and 860, quite low organic loading rates in the range of 2.6-7.5 kg COD/m^3.day were applied to the reactors because of low COD concentrations in raw leachate in the rainy season. As a result of low organic loading with high non-biodegradable organic constituent, COD removal dropped below 45% similarly in both reactors (Fig. 4.2).

A pH profile increasing from 4.5 to about 7 within the first 20 cm in hybrid bed reactor was determined in the port analysis at Day 794 and Day 860, while this was not observed in the UASB reactor until 50 cm. A similar pattern was also observed in COD profiles (Fig. 4.3).

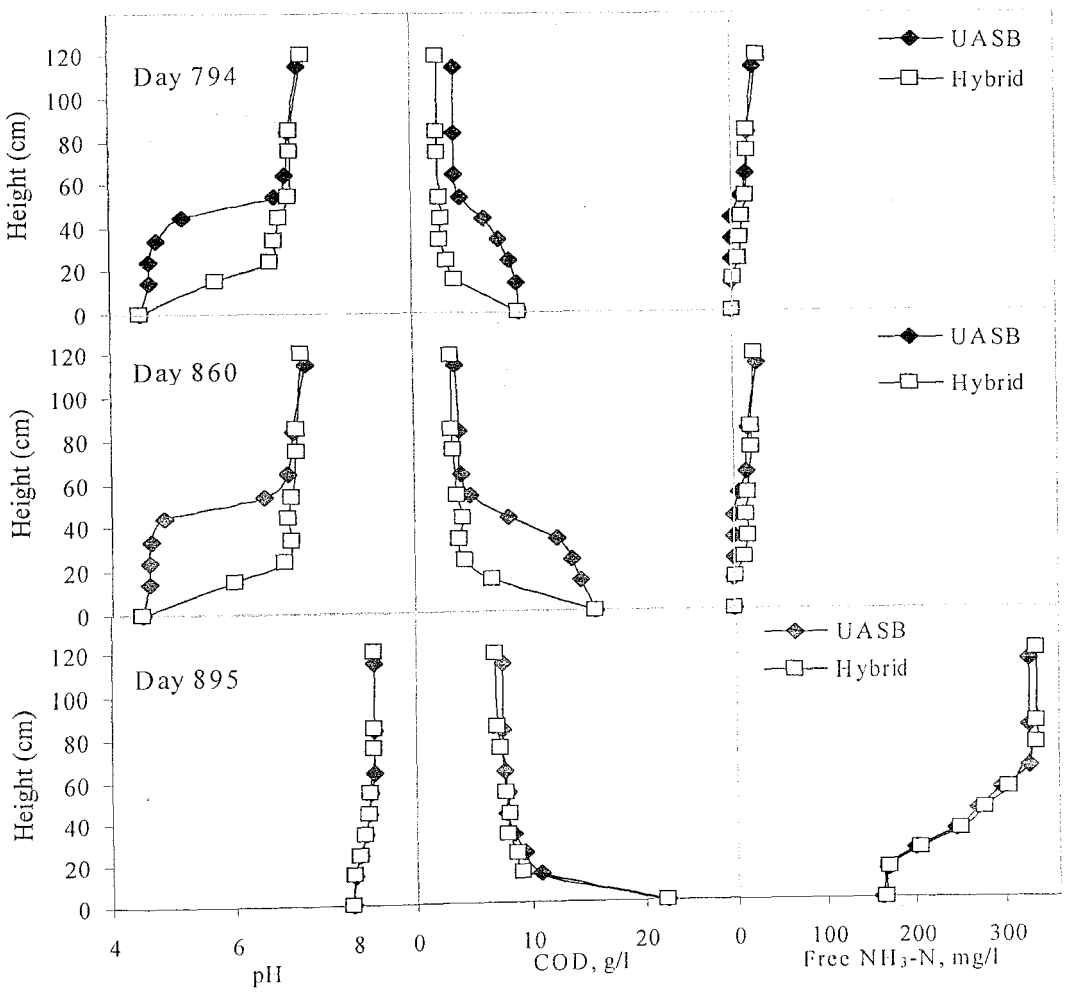

Figure 4.3. Changes of pH, COD and free ammonia parameters with reactor heights

In-situ hybridization results have indicated that archaeal cells representing the methanogens (ARC 915) were intensively dominant in the bottom sampling ports of UASB and hybrid bed reactors between Day 794 and Day 860 (Fig. 4.4 and 4.5). Although the methanogens were dominant in the low-pH bottom part of the UASB reactor because of settling due to low upflow velocity, the COD profile and activity tests indicated that they were almost inactive in that pH level. In contrast, main reason of the dominance of methanogenic archaea over acidogenic bacteria is that acetic acid was the prevailing VFA present in the raw leachate in this period. Comparing the results of FISH experiments carried out with oligonucleotide probes (Table 4.1) specific for the different groups of methanogens, it is revealed that *Methanosaeta* (MX825) species was the major methanogenic archaea present in both of the reactors (Fig. 4.6 and 4.7). Some methanogenic populations of *Methanobacteriaceae* were also detected at Day 794 in both of the reactors during the low COD removal efficiency period between Day 774 and 806 while other species were virtually absent until Day 860 in this period (Fig. 4.8).

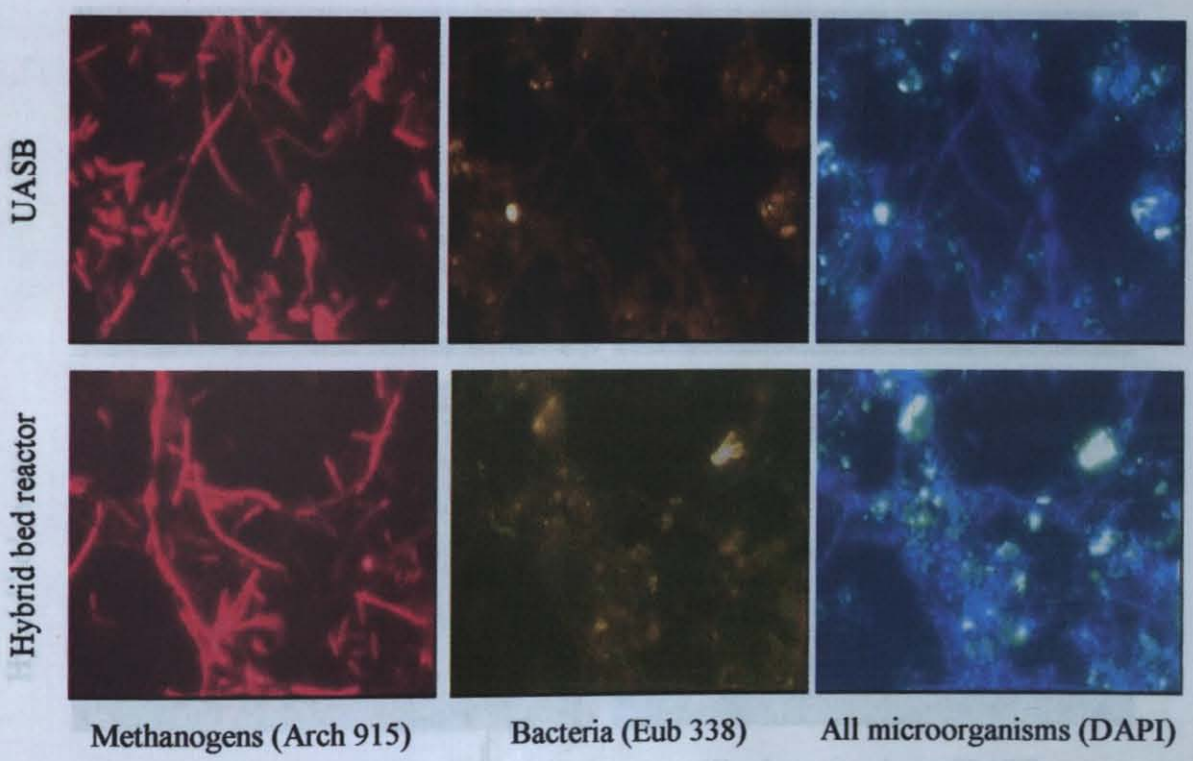

Figure 4.4. DAPI staining and FISH (Arch 915-Eub 338) photomicrographs at Day 824

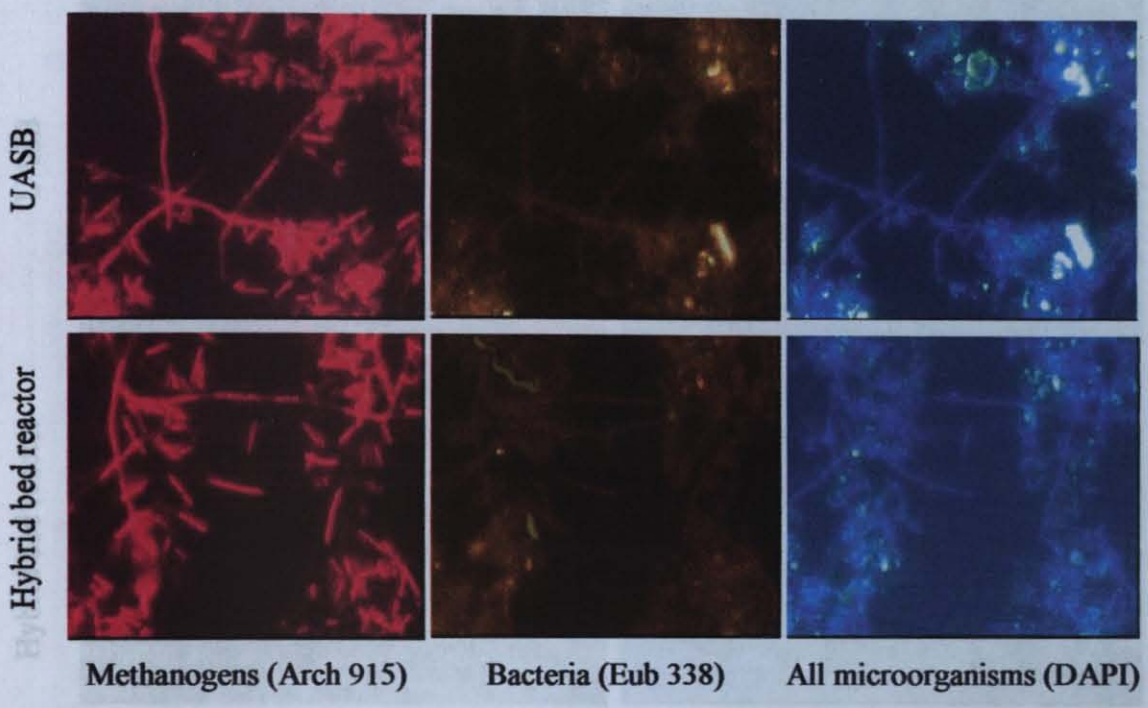

Figure 4.5. DAPI staining and FISH (Arch 915-Eub 338) photomicrographs at Day 860

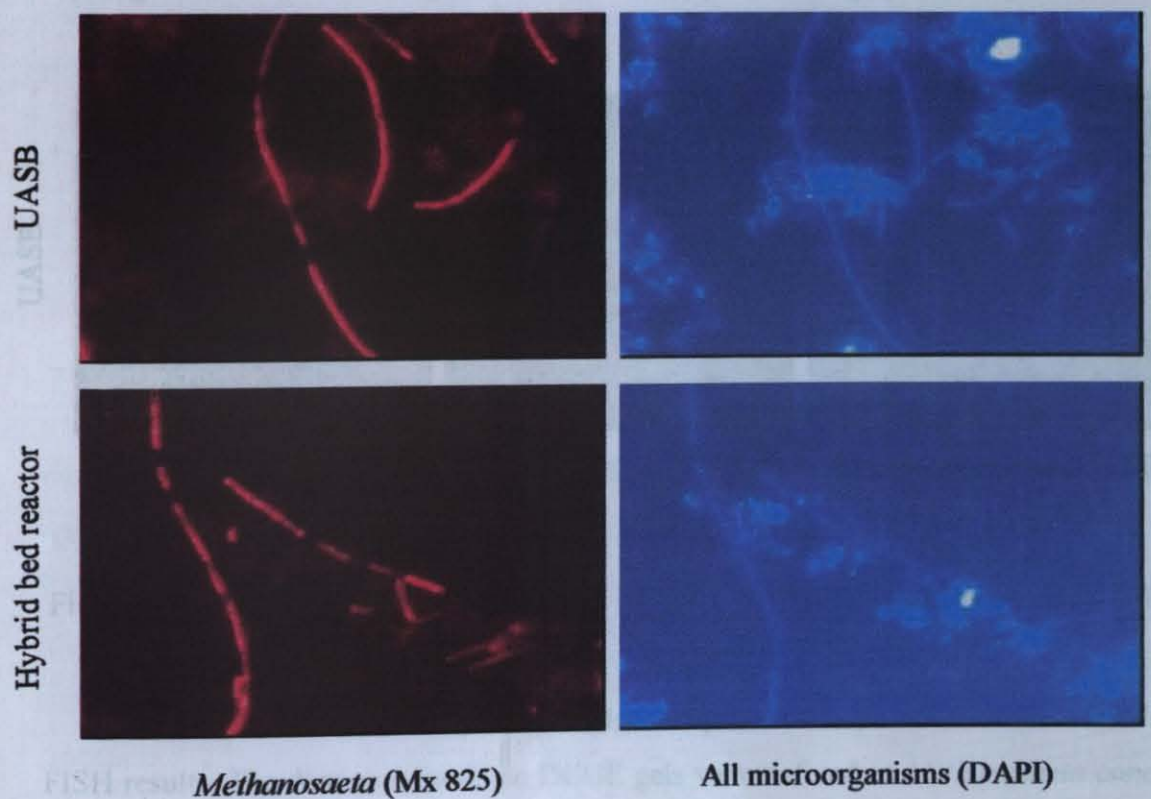

Figure 4.6. DAPI staining and FISH (Mx 825) photomicrographs at Day 824

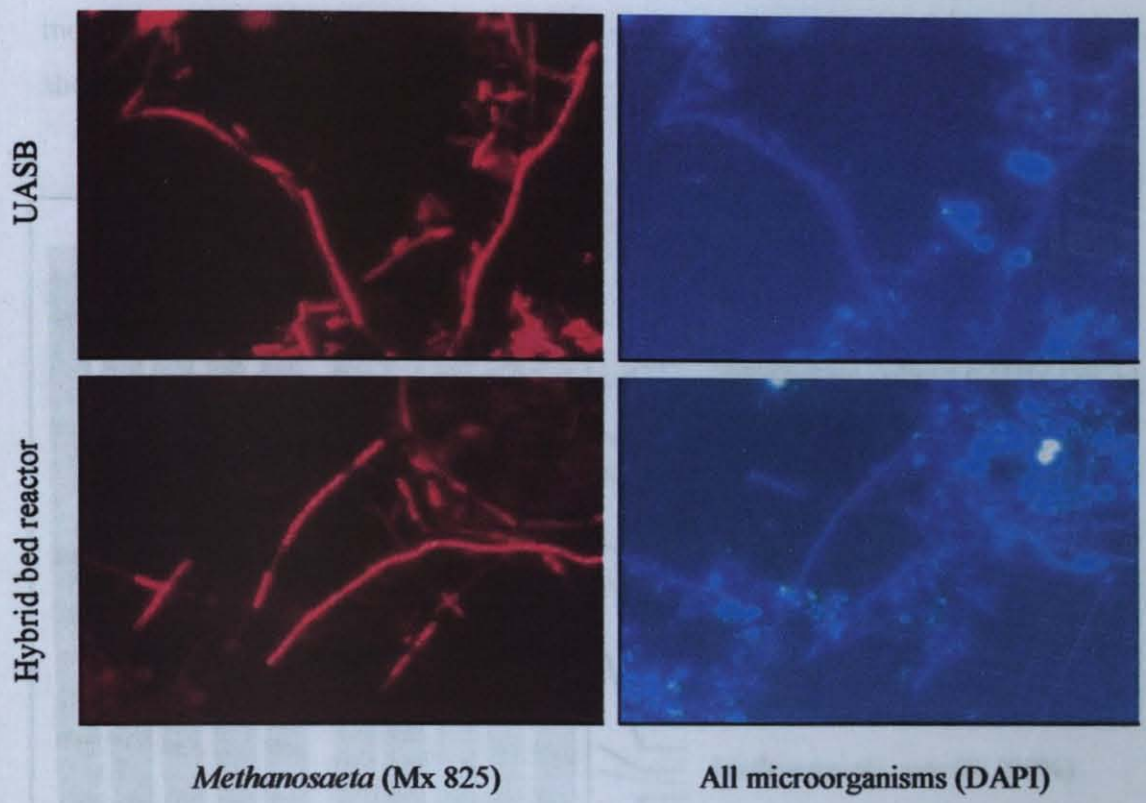

Figure 4.7. DAPI staining and FISH (Mx 825) photomicrographs at Day 860

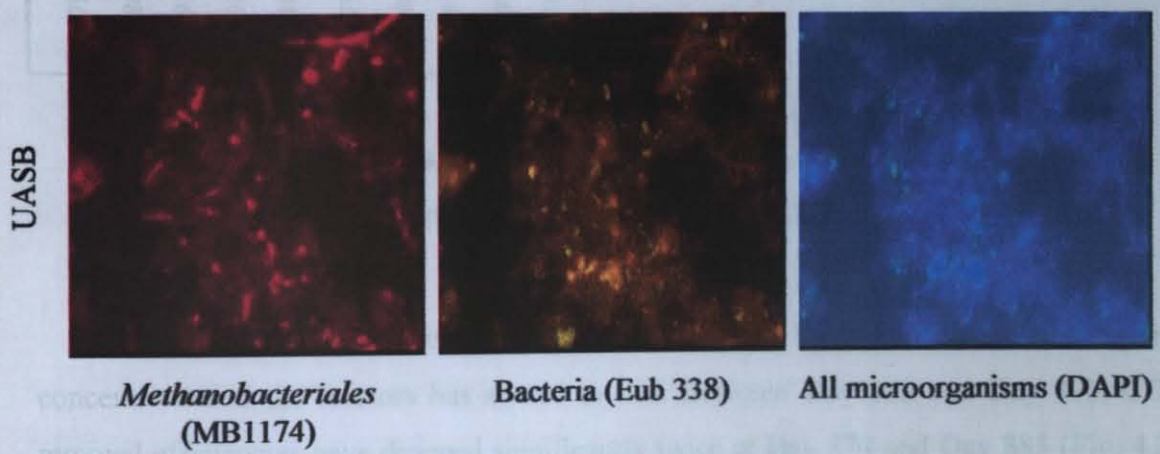

Figure 4.8. DAPI staining and FISH (Mb 1174-Eub 338) photomicrographs at Day 794

The results of DGGE analysis on sludge samples of this period have confirmed the FISH results. The dominant bands on DGGE gels were defined as *Methanosaeta concilii*, while the other few faint ones were related to *Methanosarcina* and hydrogentrophic *Methanoculleus*-related species (Figure 4.9). The results were also supported by the

morphological analysis, in which dominant cells were characterized as long filaments with sheathed structure.

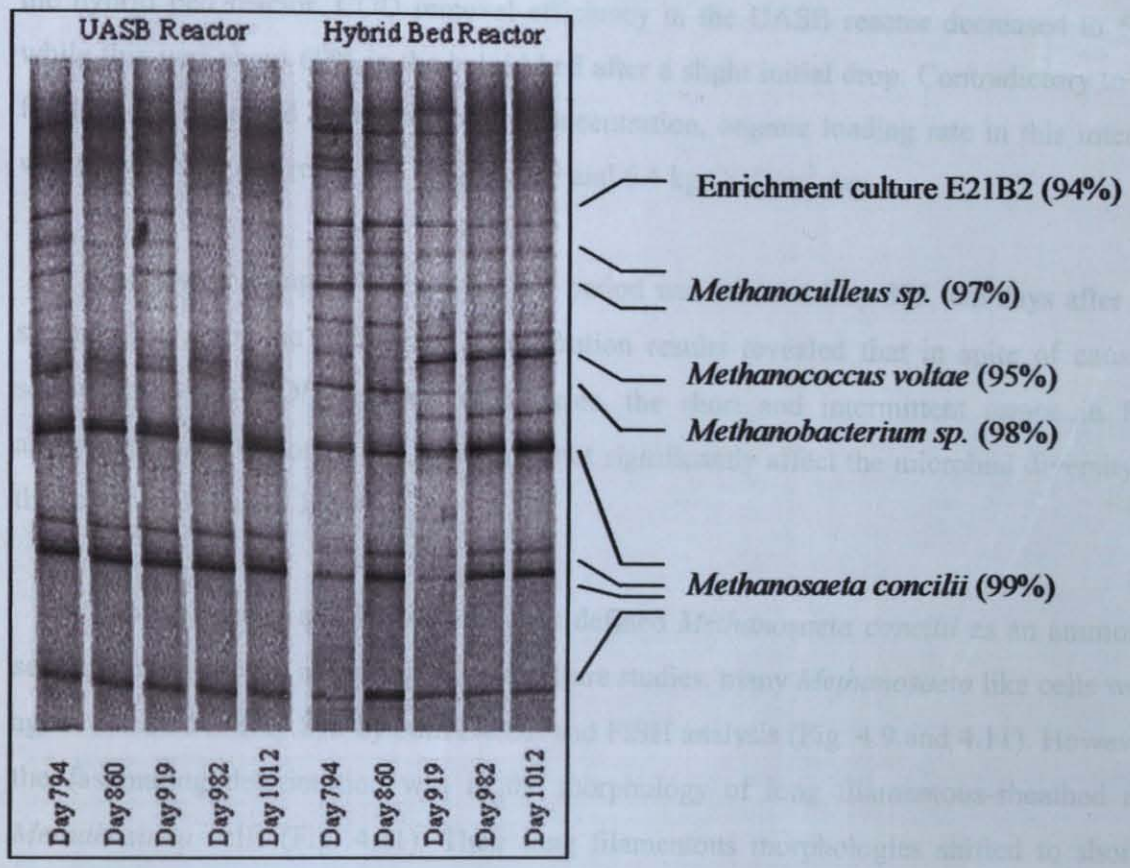

Figure 4.9. DGGE profiles and correspondent position of matching clones for PCR amplified partial archaeal 16S rDNA

After Day 860, pH adjustment in the influent was terminated and free ammonia concentration in the reactors has started to rise. Between Day 860 and Day 890, COD removal efficiencies have dropped significantly twice at Day 874 and Day 885 (Fig. 4.2). Parallel to the increase in free ammonia concentration up to 430 mg/l within two weeks after the termination of pH adjustment, COD removal efficiency has dropped significantly to 40% and 42% in hybrid bed and UASB reactors respectively at Day 874. After the unexpected decrease in ammonium nitrogen concentration from 2500 to 1400 mg/l and pH from 8.3 to 7.8 in raw leachate, at Day 881 COD removal efficiencies were restored back to 81 and 83% for UASB and hybrid bed reactors, respectively (Fig. 4.2). The rapid improvement in the reactor performances has corroborated the reversibility of ammonia

inhibition, which was also experienced several times in the start-up period. At Day 885, the reactors experienced a second but a less severe inhibition when the free ammonia concentration reached to 350 mg/l. This time, the UASB reactor was affected more than the hybrid bed reactor. COD removal efficiency in the UASB reactor decreased to 61% while this was about 69% in the hybrid bed after a slight initial drop. Contradictory to the fluctuations observed in free ammonia concentration, organic loading rate in this interval was more stable and remained between 4.9 and 6.4 kg COD/m^3.day

The sludge sample representing this period was taken at Day 895, ten days after the second free ammonia inhibition. Hybridization results revealed that in spite of causing serious drops in COD removal efficiencies, the short and intermittent jumps in free ammonia concentration in this period did not significantly affect the microbial diversity in the reactors (Figure 4.10).

Though, Sprott and Patel (1986) has defined *Methanosaeta concilii* as an ammonia sensitive methanogen according to pure culture studies, many *Methanosaeta* like cells were again detected at Day 895 by both DGGE and FISH analysis (Fig. 4.9 and 4.11). However, the fascinating deterioration was in the morphology of long filamentous-sheathed rod *Methanosaeta* cells (Fig. 4.11). Their long filamentous morphologies shifted to shorter filaments and they lost their aggregating property. This alteration might also be a substitution between two different strains as it was concluded that two types of *Methanosaeta* existed in anaerobic reactors (Kamagata and Mikami, 1990).

At Day 893, the organic loading to the reactors unexpectedly jumped to 11.3 kgCOD/m^3.day in parallel to the increase in COD concentration of raw leachate from 9,500 to 20,800 mg/l as the dry season had suddenly started. Meanwhile, in addition to acetic acid, significant concentrations of higher fatty acids were fed to the reactors, an unusual occurrence in previous periods. Because of high alkalinity in the leachate, shock organic load did not result in any serious trouble on the performances of reactors. Meanwhile, total ammonia concentration in the leachate decreased down to 1000 mg/l contradictory to the further increases in organic loading. Thus, this diminished the ammonia inhibition possibility and furthermore rapidly sustained the degradation of accumulated VFAs in the reactors. Consequently, after three weeks COD removal

efficiencies were restored back to 87 and 85% in the UASB and hybrid bed reactors, respectively (Fig. 4.2).

Disregarding the short interval between Day 947 and Day 952, in which the heating problem caused sharp drops in COD removal efficiencies, both UASB and hybrid bed reactors showed similar performances until Day 970 with efficiencies above 90%. In the last part after Day 970, the organic loading rate increased up to 18.7 kg COD/m^3.day. As a result of high organic loading with some increase in non-biodegradable portion, COD removal efficiencies slightly dropped and fluctuated between 75 and 85%.

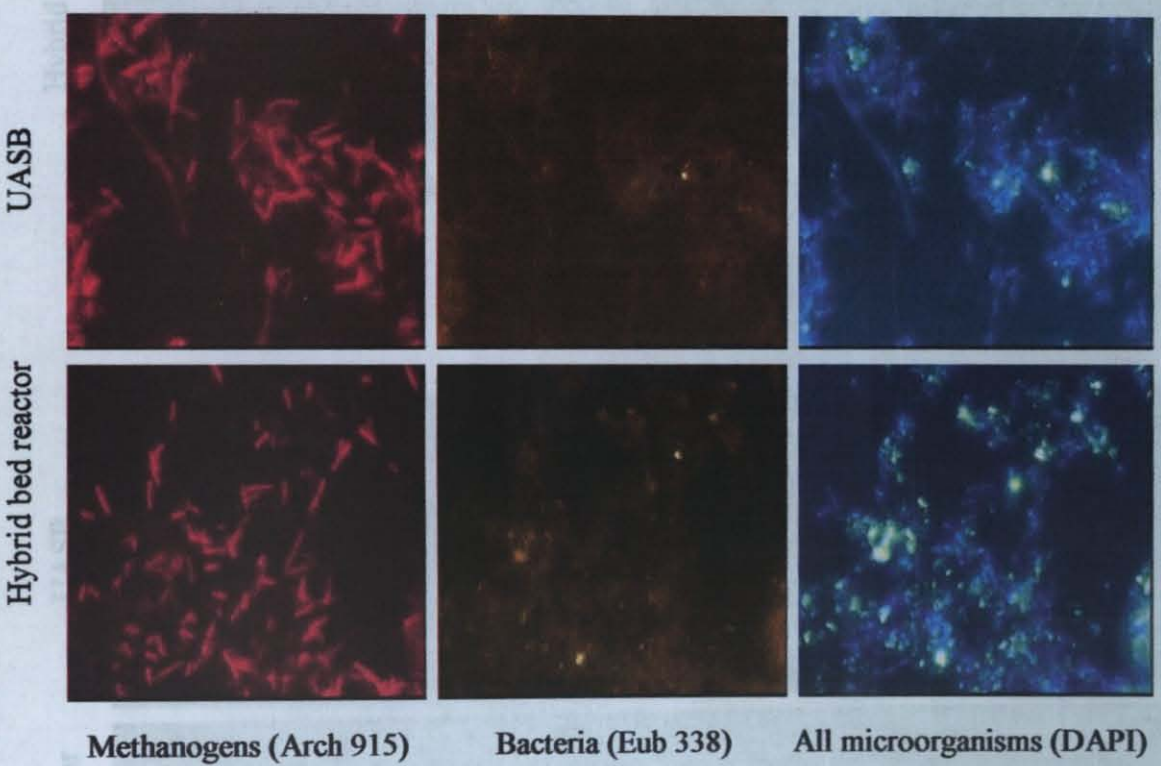

Figure 4.10. DAPI staining and FISH (Arch 915-Eub 338) photomicrographs at Day 895

The alteration in VFA composition of influent leachate immediately affected the microbial diversity in the reactors. The results of FISH experiments have indicated that in both of the reactors, bacterial populations (EUB 338) have dominated over previously prevailing archaeal cells after Day 895 (Fig. 4.12).

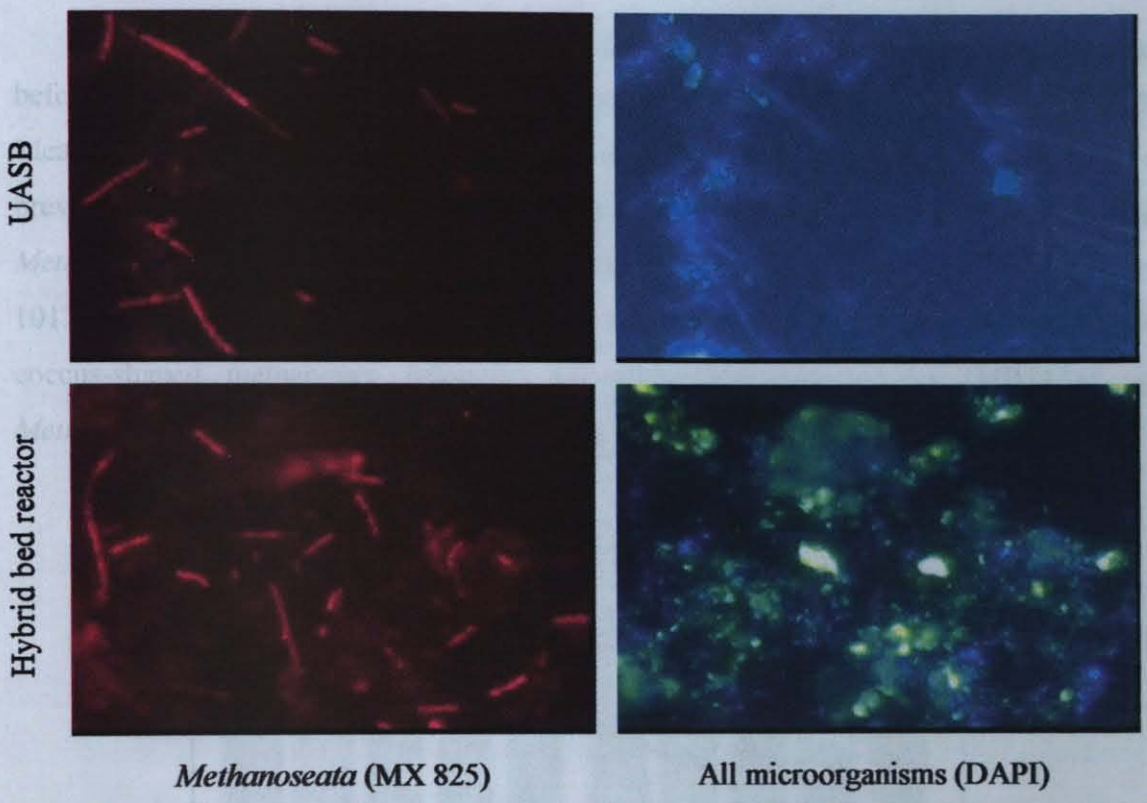

Figure 4.11. DAPI staining and FISH (Mx 825-Eub 338) photomicrographs at Day 895

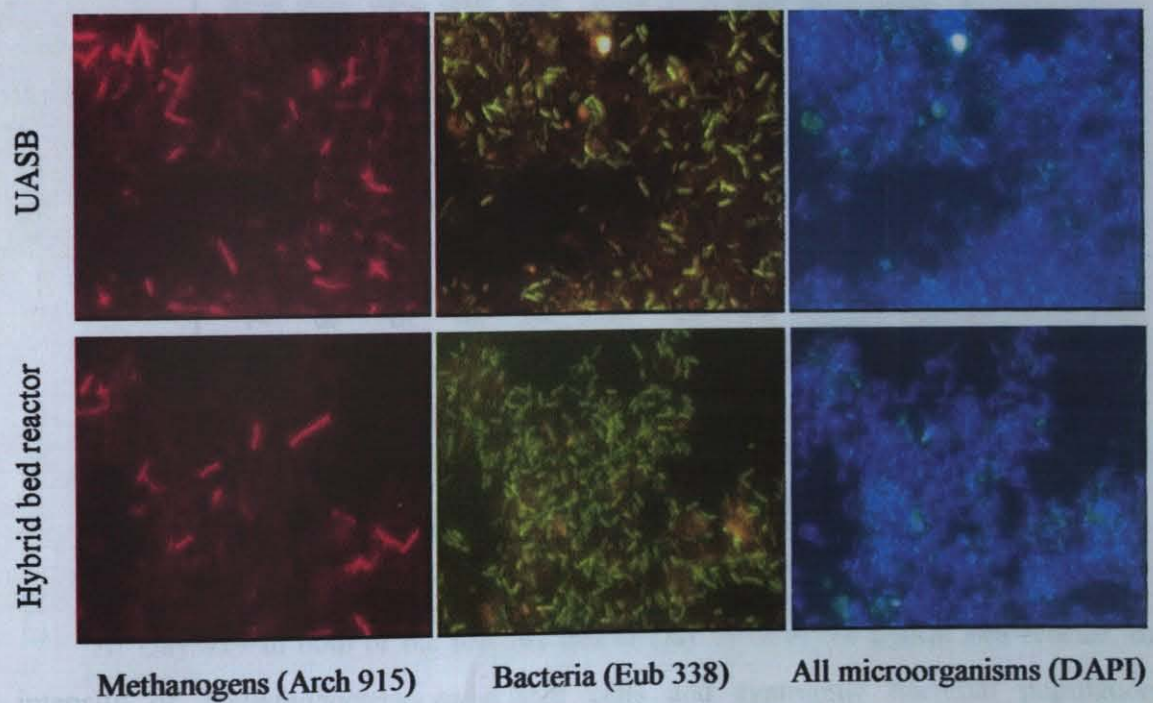

Figure 4.12. DAPI staining and FISH (Arch 915-Eub 338) photomicrographs at Day 919

Intensive bacterial 16S-rDNA bands on DGGE gels that were not distinctly obtained before overloading have also supported the abundance of bacterial populations (Fig. 4.13). Meanwhile, the methanogenic diversity has also considerably changed. Instead of previously dominant long sheathed rod *Methanosaeta* population, very few, shorter *Methanosaeta*-like cells were identified with in-situ hybridization between days 919 and 1012. Thus, the total archaeal population was almost represented by a rod and an irregular coccus-shaped methanogen belonging to order *Methanobacteriales* (MB1174) and *Methanococcales* (MC1109), respectively (Fig. 4.14).

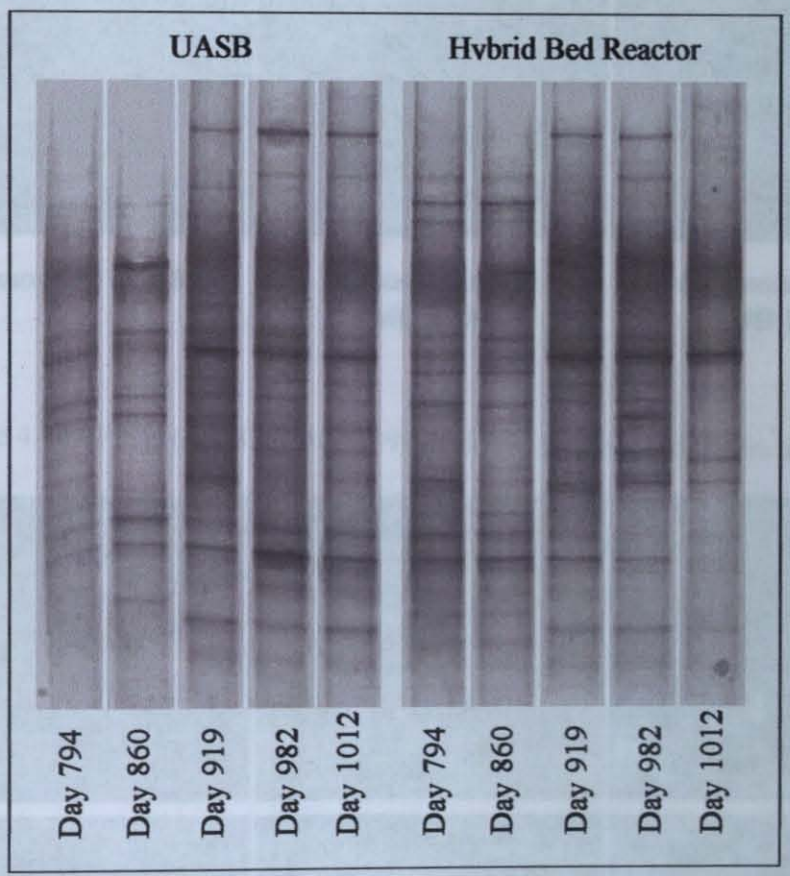

Figure 4.13. DGGE profiles of PCR amplified partial bacterial 16S rDNA

At Day 919 in both of the reactors and at Day 1012 in the hybrid bed reactor, the intensity of *Methanobacteriaceae*-related cells and syntrophic bacterial populations increased as the reactors became unbalanced. In a similar study, a sharp decrease in archaeal abundance parallel to the increase in *Methanobacteriaceae*-related cells in archaeal population was also observed under unstable reactor operations (McMahon *et al.*,

2001). However, the numbers of *Methanosaeta*-like cells have gradually increased from Day 919 to Day 982 parallel to the improvements in the stability of reactor performances. According to FISH experiments more *Methanosaeta*-like cells were identified in the hybrid bed reactor than in the UASB reactor between days 919 and 982 (Fig.4.15). Consequently, it is concluded that, the upper filter media of the hybrid bed may maintain this difference by keeping more flocs in the reactor.

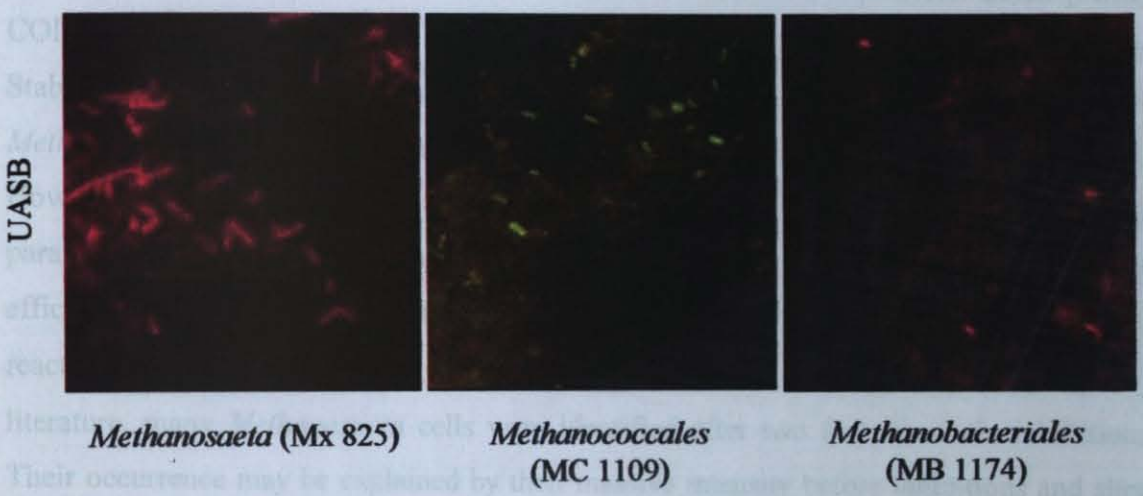

Figure 4.14. FISH (Mx 825-Mc 1109-Mb 1174) photomicrographs at Day 919

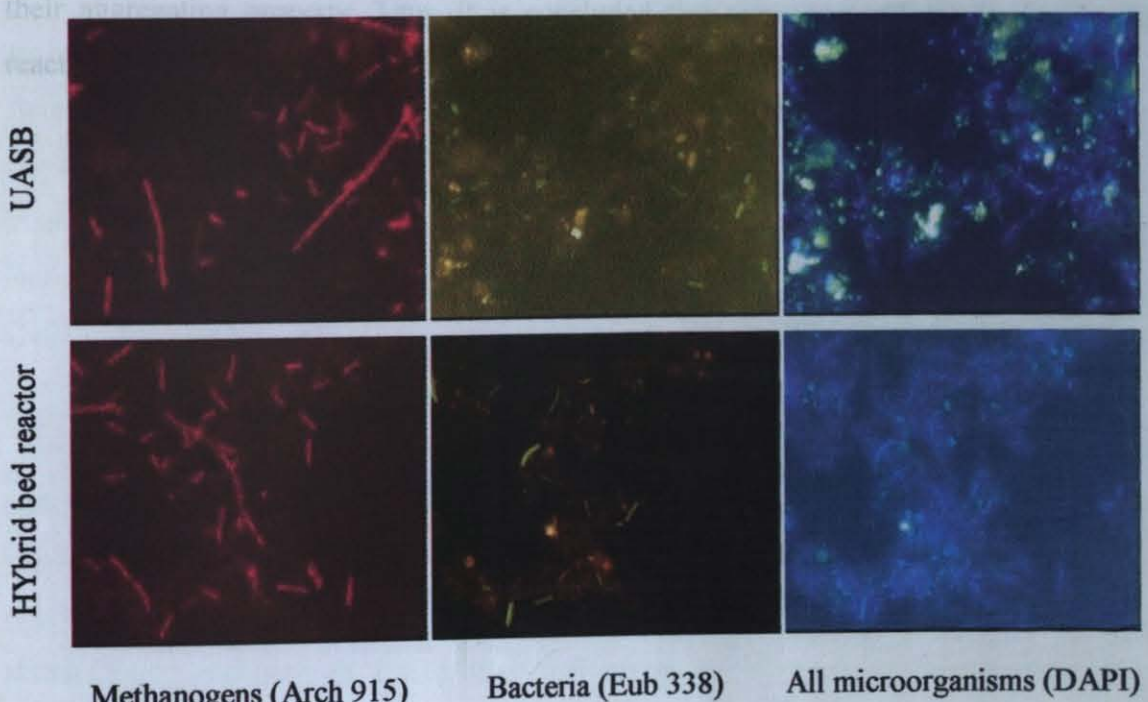

Figure 4.15. DAPI staining and FISH (Arch 915-Eub 338) photomicrographs at Day 982

4.4. Discussions

The results obtained in this long-term anaerobic treatability study have indicated that, the young landfill leachate containing total ammonia concentrations as high as 2700 mg/l can be treated successfully by using either an UASB or a hybrid bed reactor if the pH is kept below a critical level above which free ammonia inhibition is possible. Consequently, COD removal efficiency only depends on the biodegradable portion of the leachate. Stability of the reactors with low levels of acetate was also supported by the abundance of *Methanosaeta* population identified with DGGE, FISH and morphological analysis. However, after the termination of pH adjustment both of the reactors became unstable parallel to the jump in free ammonia concentration up to 400 mg/l and COD removal efficiencies have dropped significantly down to 42 and 48% in hybrid bed and UASB reactors respectively. Although, it is defined as an ammonia sensitive methanogen in literature, many *Methanosaeta* cells were identified after two free ammonia inhibitions. Their occurrence may be explained by their massive intensity before inhibitions and short durations of high free ammonia periods. However, it was observed that long filamentous morphologies of *Methanosaeta* cells have shifted to shorter filaments consequently loosing their aggregating property. Thus, it is concluded that temporary pH monitoring in the reactor influent is necessary when high ammonia concentrations are experienced.

5. EFFECTS OF HIGH FREE AMMONIA CONCENTRATIONS ON THE PERFORMANCES OF ANAEROBIC REACTORS

5.1. Introduction

Ammonia nitrogen is a very common metabolic end product in anaerobic digestion of protein-containing wastes. Although it is an important buffer in anaerobic reactors and an essential nutrient for anaerobic microorganisms, high concentrations can be a major cause of operational failure. Two species of ammonia nitrogen, the gaseous form free ammonia and the aqueous form ammonium exists together in equilibrium as indicated in the following equation.

$$NH_3 + H_2O \leftrightarrow NH_4^+ + OH^- \qquad (5.1)$$

The relative abundance of the phases depends upon both pH and temperature of liquid phase of the anaerobic reactor. As pH increases, equilibrium shifts to the left and ammonium is converted to more inhibitory free ammonia form (Sprott *et al.*, 1984; De Baere *et al.*, 1984; Koster and Koomen, 1988; Angelidaki and Ahring, 1993). Increasing pH from 7 to 8 actually leads to an eight-fold increase in free ammonia fraction of total ammonia. Concentration of free ammonia was calculated by using the formula given in 2.2 and 2.3 (Henrichs *et al.*, 1990; Poggi-Varaldo *et al.*, 1991; Angelidaki and Ahring, 1993; Gallert *et al.*, 1998).

In literature, there are reports of various wastes containing high ammonia concentrations near or above inhibition levels for anaerobic treatment. These are, organic fraction of municipal solid waste (Kayhanian, 1994; Poggi-Varaldo *et al.*, 1997; Gallert *et al.*, 1998) waste activated sludge (Lay *et al.*, 1997), wastewaters originating from potato starch (Koster and Lettinga, 1984), dairy (Vidal *et al.*, 2000) and seafood processing (Soto *et al.*, 1991; Omil *et al.*, 1995) industries, young landfill leachate (Borzacconi *et al.*, 1999; Inanc *et al.*, 2000) and animal manures (van Velsen, 1979; Wiegant and Zeeman, 1986; Angelidaki and Ahring, 1993; Borja *et al.*, 1996; Krylova *et al.*, 1997). However,

noticeably different inhibition threshold concentrations were reported in these studies in consequence to differences in parameters which directly affect the form of ammonia such as pH and temperature and the adaptation level of anaerobic sludge used.

In one of the basic studies in literature ammonia inhibition was reported to occur above pH 7.4 in the range of 1500 to 3000 mg/l TAN, whereas at concentrations in excess of 3000 mg/l, ammonia was claimed to be toxic irrespective of pH (McCarty, 1964). Likewise, in another study ammonia inhibition was reported to take place at 1700 mg/l TAN at pH 7.5 (Koster and Lettinga, 1984). These studies have been carried out using anaerobic digester sludge, in which TAN concentrations were typically below 1000 mg/l, as a seed material. By using the seed sludge taken from a swine manure digester acclimated to 2400 mg/l TAN; methane production occurred even at 5000 mg/l TAN without any sign of inhibition. This was also verified when the methanogenic activity, even with 10%, was maintained at 11800 mg/l TAN after acclimation of the granular sludge which initially first completely inhibited at 1900 mg/l (Koster, 1986).

The inhibitory effects of ammonia, as far as is known, influence mainly the methanogenesis phase in anaerobic reactors (van Velsen, 1979; Koster, 1986; Blomgren *et al.*, 1990; Angelidaki and Ahring, 1993). Other sensitive bioreactions, such as those performed by the acetogenic bacteria, may also be either directly or indirectly affected by accumulation of volatile fatty acids or hydrogen in the system (van Velsen, 1979; Koster and Lettinga, 1984; Wiegant and Zeeman, 1986). Within two distinct methanogenic groups, acetate consuming methanogens were in most cases found to be more sensitive than hydrogen utilizing ones (Koster and Lettinga, 1984; Poggi-Varaldo *et al.*, 1997; Lay *et al.*, 1997). Although some pure culture experiments supported this result (Sprott and Patel, 1986) in some studies under thermophilic conditions, hydrogen utilizing methanogens were defined as the sensitive group (Wiegant and Zeeman, 1986; Fujishima *et al.*, 2000).

Two different mechanisms were attributed to ammonia inhibition on methanogens. According to the first mechanism, activities of methane synthesizing enzymes are directly inhibited by free ammonia. In the second one, hydrophobic free ammonia molecule diffuses passively into the cell and is rapidly converted to ammonium owing to the

intracellular pH conditions (Fig. 2.3). Ammonium accumulated inside the cell may become toxic by altering the intracellular pH (Sprott *et al.*, 1984; Kadam and Boone, 1996).

In Chapter 3 and 4, results of the long-term anaerobic treatability study on Istanbul, Kömürcüoda Landfill leachate, which frequently contains TAN concentrations above 3000 mg/l, were represented regarding COD removal performances of the reactors and variations in the diversity of anaerobic microorganisms. Furthermore it was concluded that, by reducing the influent pH to 4.5 to keep FAN below inhibitory level, satisfying COD removal efficiencies were achieved without obvious adverse effects on anaerobic microorganisms. Nevertheless, in the start-up in which diluted leachate was fed, and in the periods after the termination of pH adjustment, FAN in anaerobic reactors exceeded inhibitory levels several times. Consequently, investigations were focused on the impacts of high FAN on the composition and activity of anaerobic microorganisms in the reactors. This was actually difficult because of unexpected perturbations in raw leachate characteristics in the form of sudden jumps and drops in biodegradability, pH, VFA and TAN concentrations. Thus, to stabilize and simplify the conditions for the evaluation of free ammonia inhibition and adaptation mechanisms, five upflow anaerobic reactors seeded with different sludges were fed with a synthetic wastewater. Organic loading rates were kept constant under gradually increasing FAN concentrations.

5.2. Experimental Set-up and Methods

In this study, five laboratory scale identical upflow anaerobic reactors inoculated with different seed sludges were operated for 450 days under a constant organic loading rate of about 1.2 kg COD m^3/day and gradually increasing influent TAN concentration from 1000 to 6000 mg/l.

Each reactor had an effective volume of 4.3 liters. Their diameters and effective heights were 10 cm and 55 cm, respectively. There were 3 sampling ports located along the height of the reactors. Reactors were fed from the influent funnels connected to the bottom and located above the effluent port level. Synthetic wastewater in the feed tank was pumped to these funnels with peristaltic pumps providing 4 days hydraulic retention time

in each reactor. To homogenize the solution and prevent precipitation, feed tank was intermittently stirred with a mechanical mixer. Meanwhile, a constant temperature room was used to maintain the temperature at 35±2 °C. Configurations and dimensions of the reactors and the details of the experimental set-up are shown in Figure 5.1.

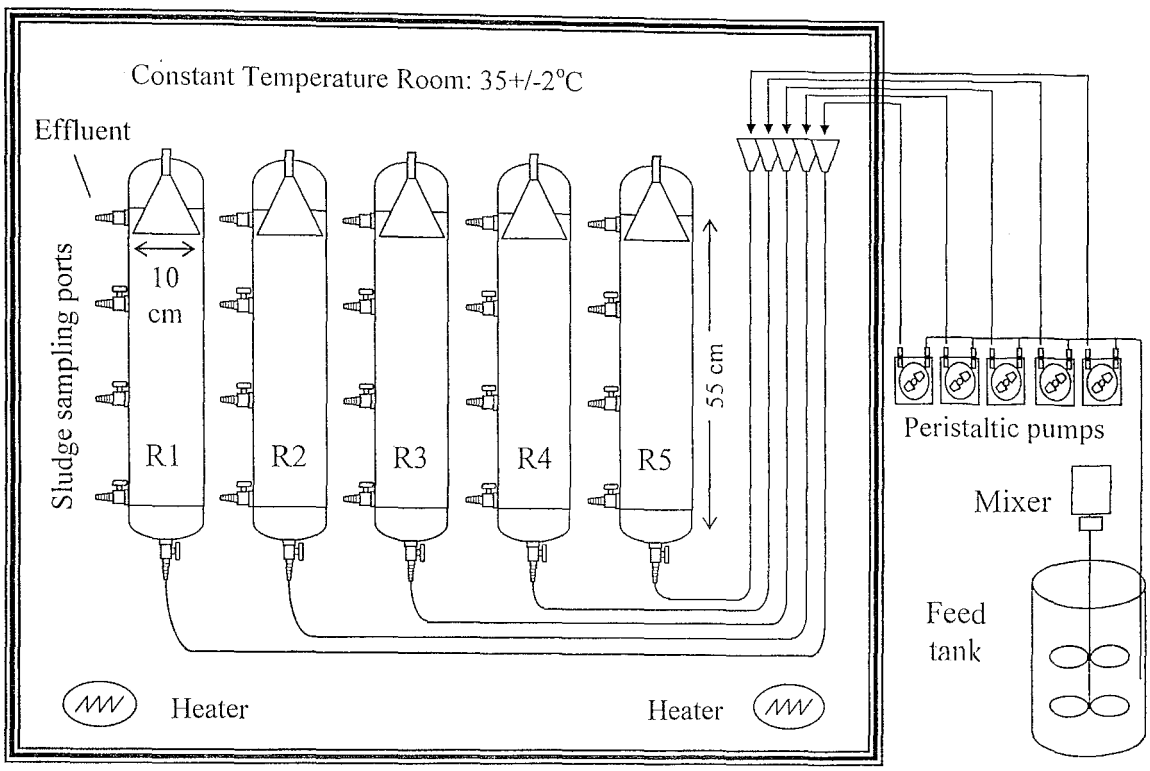

Figure 5.1. Experimental set-up consisting of five upflow anaerobic reactors

Three of the seed sludges inoculated to the reactors were taken from full scale anaerobic reactors treating corn processing, alcohol distillery and snack food processing wastewaters. The fourth one was the laboratory scale UASB reactor treating Kömürcüoda Landfill Leachate mentioned in Chapter 3 and 4. The fifth seed sludge was obtained by concentrating the biomass in one of the leachate samples taken from Kömürcüoda Landfill. Sources, physical forms and volatile to total suspended solids (VSS/TSS) ratios of seed sludges are given in Table 5.1.

A simple synthetic wastewater was fed to the reactors which was adapted from a dilution solution used in methanogenic activity assays (Valcke and Verstraete, 1983). It

was composed of 3230 mg/l acetic acid, 570 mg/l propionic acid, 550 mg/l butyric acid, 150 mg/l $CaCl_2.2H_2O$, 120 mg/l $Na_2S.9H_2O$ and 200 mg/l yeast extract and prepared with dechlorinated tap water to supply some of the trace elements that anaerobic microorganisms require. As the ammonium source, 25% NH_4OH solution was used and added in proper amounts parallel to gradually increasing concentrations. The pH of the feed was adjusted to 7.7 with HCl (4.5%) and H_3PO_4 (12.5%) mixture instead of sole HCl to prevent chloride inhibition in the reactors. Furthermore, pH of the feed was elevated up to 8.1 to increase the FAN concentration in addition to increasing TAN loads.

Table 5.1. Seed sludges and their characteristics

Reactor	Source	Form	VSS/TSS
R1	Full scale EGSB reactor treating corn processing wastewater	Granular (broken)	0.83
R2	Lab scale UASB reactors treating Kömürcüoda Landfill leachate	Flocculent	0.39
R3	Full scale UASB reactor treating alcohol distillation wastewater	Granular (broken)	0.94
R4	Concentrated biomass from Kömürcüoda Landfill leachate	Flocculent	0.17
R5	Full scale UASB reactor treating snack food processing wastewater	Granular (tiny)	0.54

In the influent and effluents of the reactors pH, total alkalinity, COD and TAN were monitored three times in a week, while volatile fatty acids (VFAs), TSS and VSS were measured twice and soluble COD once a week. Meanwhile, sludge concentrations in the reactors were monitored once a month by taking samples from bottom sampling ports. All analyses were carried out in accordance with the Standard Methods (APPHA/AWWA and WEF, 1998)

5.3. Experimental Results

All five reactors were fed with the synthetic wastewater having a COD concentration of about 5300 mg/l and operated for 450 days under almost constant loading rates in the range of 0.9 to 1.4 kgCOD/m^3/day. In the meantime, influent TAN concentration was step by step increased from 1000 to 6000 mg/l with 500 mg/l increments (Fig. 5.2a). Influent pH was adjusted to 7.7 until Day 302 and then increased gradually up to 8.1 to elevate the FAN concentration in the reactors (Fig. 5.2b).

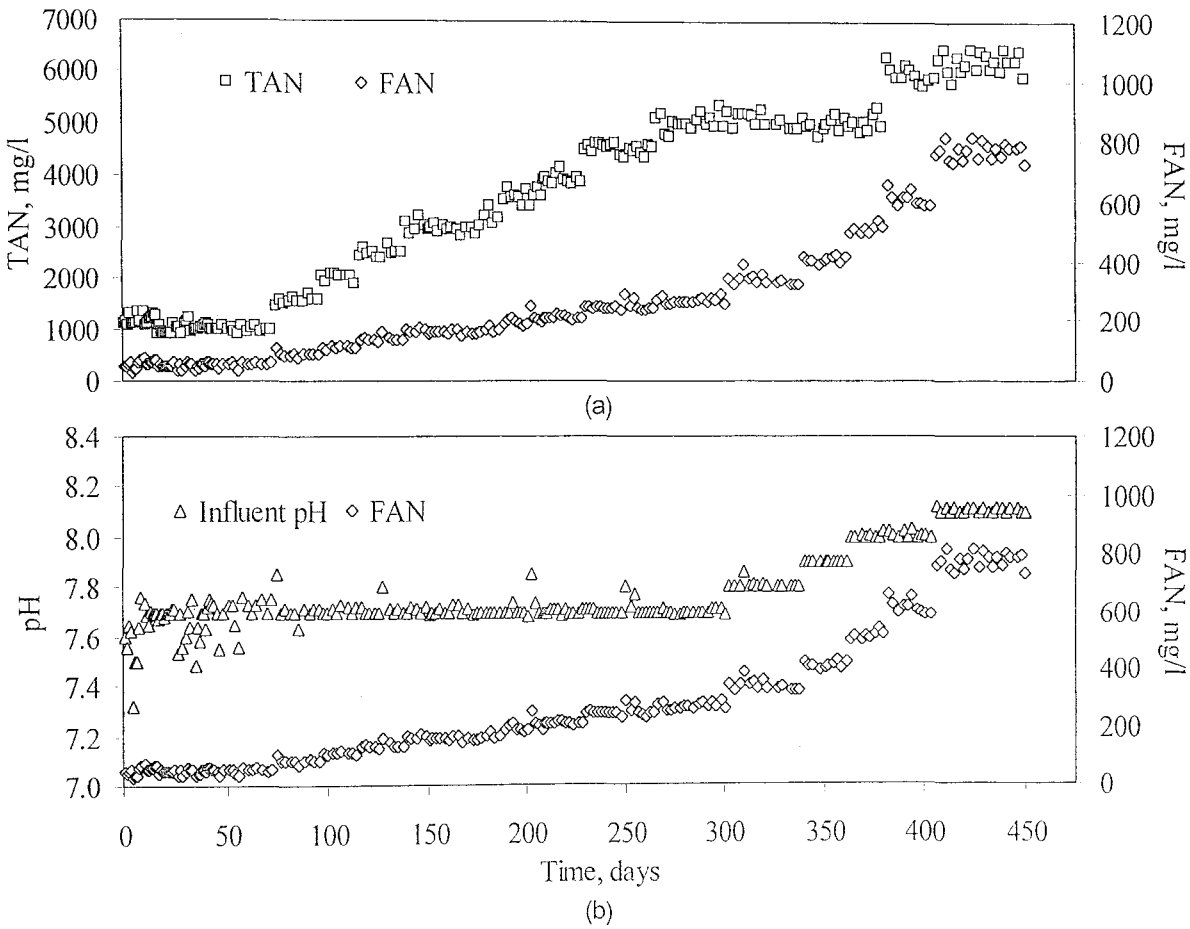

Figure 5.2. FAN concentrations increasing parallel to pH and TAN

In the start-up period until Day 75, performances of the reactors were unstable therefore influent TAN concentration was kept around 1000 mg/l not to elevate the FAN concentration above 50 mg/l (Fig. 5.2a). In this period after Day 50, COD removal

efficiencies of about 95% were observed in R2 and R4. 80% efficiency in R3 and 70% efficiencies in R1 and R5 at Day 50 reached to 85% at Day 75 and 90% at Day 98, respectively (Fig. 5.3). Meanwhile, reductions in acetic and propionic acid concentrations in the effluents corresponding to improvement in reactor performances indicated the termination of the start-up period (Fig. 5.3).

Influent TAN concentration was elevated to 2000 mg/l at Day 98, to 2500 mg/l at Day 117 and to 3000 mg/l at Day 140. Thus, FAN concentration in the reactors reached to 160 mg/l (Fig. 5.2a). Although this level doubles the minimum free ammonia inhibition threshold indicated by Koster and Lettinga (1984), detrimental effects were not observed on the performances of reactors. In contrast, 90% COD removal efficiency in R5 at Day 98 increased up to 95% at Day 150 and reached to the performances of R2 and R4 (Fig. 5.3e).

By increasing the influent TAN to 3500 mg/l at Day 189, to 4000 mg/l at Day 210 and to 4500 mg/l at Day 230, FAN in the reactors exceeded 160 mg/l and elevated up to 240 mg/l (Fig. 5.2a). FAN concentrations attained in this period were reported as completely inhibitory and responsible for unbalancing in the reactors (McCarty and McKinney, 1961). In addition to high FAN levels, problems in feeding pumps resulted in a shock organic load at Day 210. Consequently, acetic acid concentration in the effluent of R1 exceeded 500 mg/l at Day 212 which was usually around 100 mg/l. Thus, COD removal efficiency in R1 dropped from 91% to below 85%. Corresponding drops were from 96% to 92% in R2 and from 89% to 81% in R3. In spite of the shock organic load and FAN concentrations of about 240 mg/l, R4 and R5 achieved 94 and 96% COD removal efficiencies, respectively without any decline in performance (Fig. 5.3).

Until Day 267, influent TAN concentration was kept at 4500 mg/l to provide an adaptation period for R1, R2 and R3 which were inhibited after the previous jump in FAN level. In this 37 day period, COD removal efficiencies of about 92% and 83% were restored back to 96% and 90% in R2 and R3, respectively. At Day 267, influent TAN was increased to 5000 mg/l which resulted in 280 mg/l FAN in the reactors.

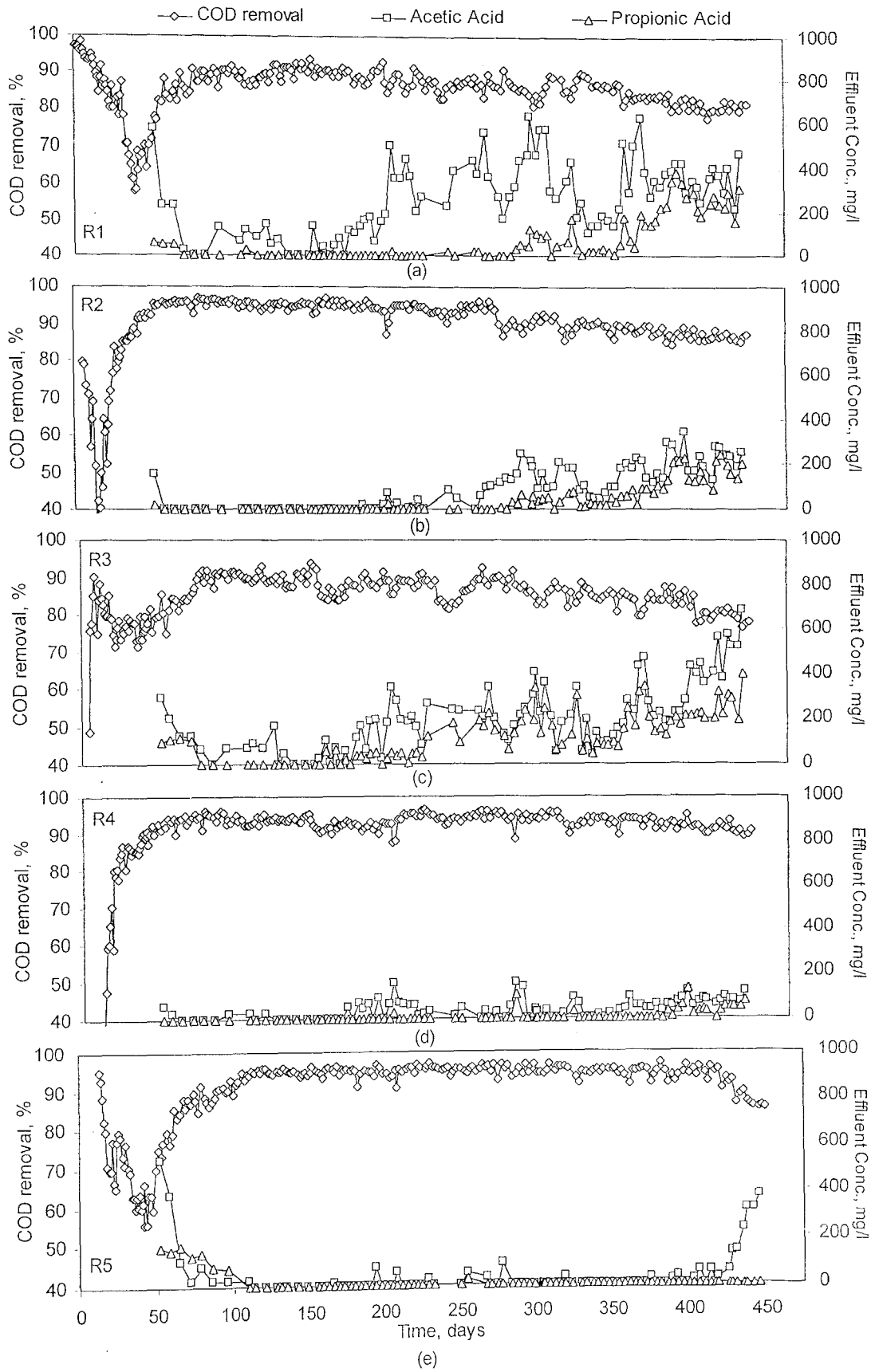

Figure 5.3. COD removal efficiencies and effluent VFA concentrations of the reactors

However, until Day 275, TAN concentration in the feed could not be kept at 5000 mg/l and fluctuated around 4800 mg/l. Therefore, the increase in FAN was tolerated until Day 285 in R2 and until Day 289 in R1 and R3. At Day 287, COD removal efficiency in R2 suddenly dropped from 95% to 86% within 3 days and acetic acid concentration in the effluent jumped to 130 mg/l at Day 292 and to 250 mg/l at Day 299 which was usually below 30 mg/l until then. Reductions in COD removal efficiencies in R1 and R3 were not severe as in R2 while R4 and R5 were still very efficient in this period (Fig. 5.3).

At Day 303, influent pH was adjusted to 7.8 which had been kept at 7.7 until that day. After that, FAN level in the reactors reached to 340 mg/l on average. In this period, COD removal efficiency in R1 fluctuated between 81% and 89% due to the presence of acetic acid in the effluent at concentrations as high as 600 mg/l. COD removal efficiency in R2 exhibited an increase up to 92% at Day 322 after the unexpected inhibition at Day 287, however it again dropped to 85% at Day 329 and nevermore exceeded 90%. Above 200 mg/l propionic acid and about 400 mg/l acetic acid in the effluent of R3 were indications of inhibition and thus COD removal efficiency dropped from 91% to 84%. Although R1, R2, and R3 were more or less affected from FAN concentration of about 340 mg/l in this period, once more R4 and R5 had satisfactory COD removal of above 93% (Fig. 5.3).

By increasing the influent pH to 7.9 at Day 340 and to 8.0 at Day 364, FAN concentrations in the reactors were elevated up to 537 mg/l (Fig. 5.2b). Consequently, effluent COD concentrations exceeded 900 mg/l and 600 mg/l in R1 and R2, respectively. Moreover, parallel to the increase in acetic and propionic acid concentrations of up to 470 and 340 mg/l respectively, COD concentrations as high as 1085 mg/l were measured in R3 at Day 376. Astonishingly, R4 and R5 tolerated even these increments in FAN and achieved 93% and 95% COD removal efficiencies, respectively with very low total VFA concentrations in the effluents of less than 50 mg/l (Fig. 5.3).

At Day 383, TAN concentration in the feed was elevated from 5000 mg/l to 6000 mg/l which resulted in about 620 mg/l FAN in the reactors (Fig. 5.2). Hence, in the effluent of R1, both acetic and propionic acid concentrations approximately doubled and reached to 400 mg/l at Day 404, 21 days after the increase. The same trend was observed only in R2

among the other reactors. In R3, acetic and propionic acid concentrations in the effluent dropped back to 200 mg/l level and about 85% COD removal rate was provided once more (Fig. 5.3).

Finally at Day 406, pH was increased to 8.1 to increase the FAN concentration to 750 mg/l on average (Fig. 5.2). In literature, usually severe or complete inhibition was reported at similar or lower FAN concentrations and most of these studies were conducted at thermophilic conditions. However, methanogenic activities were reported at FAN concentrations as high as 1100 mg/l in thermophilic digestion of different animal manures (Angelidaki and Ahring, 1993; Hansen *et al.*, 1998).

After Day 406, R1 and R2 exhibited constant COD removal efficiencies of 82 and 87%, respectively until the end of the experimental period. Acetic acid of about 400 mg/l and propionic acid around 300 mg/l were measured in the effluents of R1 while in R2, concentrations were almost half of these. R3 exhibited a sudden drop in COD removal efficiency from 85% to 78%. The most resistant reactor R5 was affected drastically at this FAN level and 96% removal decreased to 85% progressively within 40 days associated with the increase in effluent acetic acid concentration up to 370 mg/l. However, very low propionic acid concentrations of less than 10 mg/l in the effluent of R5 indicated the presence of propionate degraders that are resistant to these extremely high FAN levels. In R4, reduction in methanogenic activity was still inconsiderable, consequently 90% COD removal efficiency was obtained with acetic and propionic acid concentrations of less than 100 mg/l in the effluent.

In addition to effluent COD and VFA parameters, VSS concentrations in sludge samples taken from bottom sampling ports of the reactors exhibited interrelated results between inhibition and adaptation events. After start-up, biomass increased to significant amounts in R4 and R5 which gradually adapted to elevated FAN concentrations and achieved high COD removal efficiencies. Nevertheless, even though no sludge wastage was observed, VSS concentrations in R1 and R3 remained almost constant. Continuously growing biomass in R2 started to decline after the sudden drop in COD removal efficiency from 94% to 87% at Day 282 (Fig. 5.4).

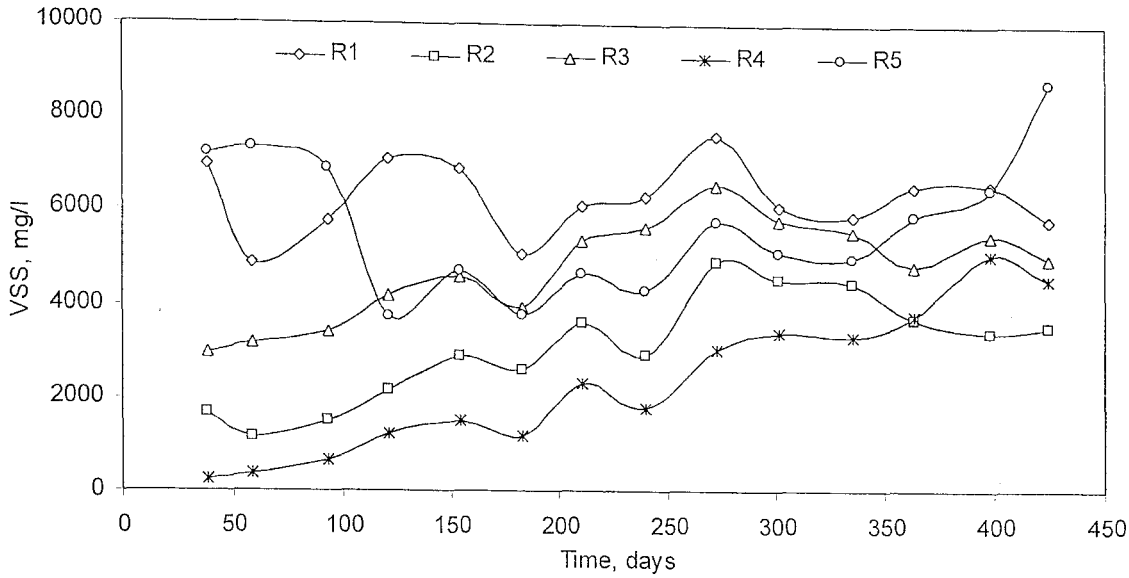

Figure 5.4. VSS concentrations in the bottom sampling port of reactors

However, it is really difficult to understand the microbial behavior under extremely high free ammonia by using only the conventional VSS parameter. To define the adaptation phenomena as modification of the appropriate enzymes or as selection and domination of resistant microorganisms, microbial diversity and activity analyses should also be conducted in addition to the measurement of VSS. In this manner, recent molecular microbiology techniques should be used for identification of anaerobic microorganisms in their natural habitats, since especially methanogens are difficult to study by cultivation-based techniques. Therefore, methanogenic populations in the sludge samples taken monthly were identified and results will be discussed in the next chapter.

5.4 Discussions

As FAN concentrations gradually rose up to 800 mg/l throughout the 450 days experimental period, COD removal efficiencies in the range of 78 to 96% were observed in five identical upflow anaerobic reactors seeded with different sludges. High removal efficiencies indicated that seed sludges taken from anaerobic reactors treating also considerably low ammonia containing wastewaters may be successfully adapted to

elevated free ammonia concentrations. Variations in the efficiency of reactors may be explained with the divergence in the amount of free ammonia resistant microorganisms in seed sludges.

The results obtained in this study indicate that, like acetoclastic methanogens, propionic acid degrading acetogens are also sensitive to high free ammonia concentrations. Moderately high propionic acid concentrations in the effluents of R1, R2 and R3 pointed out to this conclusion. However, low propionic acid concentrations of less than 10 mg/l in the effluent of R5 indicated the presence of propionate degraders resistant to extremely high free ammonia levels. On the other hand, there was no problem in the conversion of butyric acid at any free ammonia level in all reactors revealing the resistance of butyrate degraders.

Granulated sludges are known to be resistant to environmental factors and toxic substances did not show the same resistance to high free ammonia concentrations in this long term study. Besides, the lowest removal efficiencies were observed in R1 and R3 which were seeded with granular sludges. This revealed that, microbial content is more significant than physical structure of the sludge against ammonia inhibition. Thus, to elucidate the inhibitory effects and adaptation phenomena accurately, changes in the methanogenic community under increasing ammonia levels were investigated and the results will be given in the next chapter.

6. METHANOGENIC COMMUNITY CHANGES IN ANAEROBIC REACTORS EXPOSED TO INCREASING LEVELS OF AMMONIA

6.1. Introduction

To successfully start-up and operate an anaerobic bioreactor, a proper balance should be maintained in the biomass between fermenting bacteria and methanogenic archaea through volatile fatty acid production and consumption, respectively. As the reactions in fermentation give a greater energy yield than in methane formation, the fermenting bacteria grow relatively rapidly than the methanogens. For this reason, more slowly growing methanogens tend to be rate-limiting in anaerobic degradation of soluble substrates. Therefore, accumulation of volatile fatty acids should not be allowed for a proper balance in the reactor, especially during start-up, by controlling the organic loading rate and buffering capacity (Rittmann and McCarty, 2001). Since, methanogens have very slow doubling times; duration of the start-up period may be longer if the seed sludge contains only small numbers of active methanogens or methanogens unadapted to recent conditions. Thus, to decrease the start-up period, seeding from an active anaerobic reactor treating a similar waste is actually beneficial.

As any biological treatment process, anaerobic treatment systems should be regularly monitored to ensure a successful operation. Furthermore, owing to sensitivity of slow growing methanogens, anaerobic reactors should be more strictly monitored. Imbalance in anaerobic reactors may be caused by both hydraulic and organic overloads, and introduction of inorganic and organic toxic substances. Some of the more commonly used indicators for monitoring include; pH, alkalinity, volatile fatty acid concentrations, biogas production rate and composition, volatile solids or COD reduction. In general, as they supply complementary information, combinations of these indicators are monitored together (Switzenbaum *et al.*, 1990). Meanwhile, the best operation is achieved by regular monitoring and applying corrective actions before the process gets out of control.

Nevertheless, a desirable monitoring analysis for anaerobic reactors should be directly associated with the active population of microorganisms especially of the populations responsible for the critical steps including methanogens. At this point, microbial identification tools for such analyses are not available for routine use due to the limitations of traditional microbiological techniques, including selective enrichments, pure culture isolations, most probable number estimates, and phenotypic characterizations. However, through the 16S rRNA/rDNA based molecular methods, it is now possible to identify microorganisms present in anaerobic reactors - regardless of their cultivation capabilities (Raskin *et al.*, 1994; 1996; Rocheleau *et al.*, 1999; Santegoeds *et al.*, 1999; Sekiguchi *et al.*, 1999). Therefore, such monitoring analyses are becoming progressively more essential in the control of anaerobic treatment systems (Fernandez *et al.*, 1999; 2000; McMahon *et al.*, 2001; Chan *et al.*, 2001; Angenent *et al.*, 2002). On the other hand, even though methanogenic population dynamics in anaerobic treatment systems have been studied widely in more recent studies by using molecular methods, our understanding is still limited under stress conditions in the form of elevated concentrations of inhibitory compounds. Since the susceptibility of methanogens to toxic compounds is much greater than that of fermenting bacteria, the presence of toxic compounds in the reactor can easily disrupt the balance between fermentation and methanogenesis. Therefore, this imbalance may be the start of a fatal upset of the anaerobic treatment process.

Ammonia nitrogen is one of the most common toxic substances experienced during anaerobic treatment of protein-containing wastes. Although it is an important buffer in the process and an essential nutrient for methanogens, high concentrations can be a major cause of operational failure. The inhibitory effects of ammonia, as far as is known, influence mainly the phase of methanogenesis in anaerobic reactors (van Velsen, 1979; Koster & Lettinga, 1984; Heinrichs et al., 1990; Angelidaki & Ahring, 1993; Lay et al., 1998). Other sensitive reactions, such as those performed by the syntrophic acidogenic fermenters, may also be either directly or indirectly affected by accumulation of volatile fatty acids or hydrogen in the system (van Velsen, 1979; Koster, 1986; Koster and Lettinga, 1988; Heinrichs *et al.*, 1990). Within two distinct methanogenic groups, acetate consuming methanogens were usually found to be more sensitive than hydrogen utilizing ones (Koster and Lettinga, 1984; Angelidaki and Ahring, 1993; Schnürer *et al.*, 1994; Varaldo *et al.*, 1997). Sprott and Patel (1986) supported this with some pure culture

experiments. However, in some of the studies hydrogen utilizing methanogens were in contrast defined as the sensitive group (Wiegant and Zeeman, 1986; Fujishima *et al.*, 2000).

As methanogenic population adapts to elevated ammonia levels in anaerobic reactors, there are obviously differing threshold concentrations for inhibition in the literature. In studies conducted with unadapted biomass, ammonia inhibition was reported to occur above 1500 mg/l TAN (total ammonia nitrogen) at pH of about 7.5 (McCarty, 1964; Koster and Lettinga, 1984). By using adapted sludge, methane production without any significant inhibition was reported even at 5000 mg/l TAN (van Velsen, 1979). Sprott and Patel (1986) postulated that adaptation results from the selection of resistant methanogens already present in seed sludge rather than an alteration through genetic changes of initially sensitive methanogens, as proposed by Koster (1986).

This study was conducted to increase our understanding on the inhibition of methanogens at elevated free ammonia concentrations. Furthermore, to elucidate the aforementioned postulates about the adaptation phenomena, changes in methanogenic populations in anaerobic reactors exposed to gradually increasing ammonia levels were monitored by using cloning, denaturing gradient gel electrophoresis (DGGE) and fluorescent in-situ hybridization (FISH) techniques and the collected population data were linked with reactor performances.

6.2. Material and Methods

In this study, five laboratory scale identical upflow anaerobic reactors inoculated with different seed sludges were operated for 450 days under constant organic loading rates of about 1.2 $kgCODm^3/day$ (HRT = 4 days) and gradually increasing influent ammonia concentrations from 1000 to 6000 mg/l TAN. Approximately 5300 mgCOD/l synthetic feed was composed of acetate (3450 mgCOD/l), propionate (860 mgCOD/l), and butyrate (1000 mgCOD/l) as substrate. Details about the experimental set-up and seed

sludges are given in Chapter 5. Operational plan and sludge sampling days are in given Figure 6.1.

To investigate the changes in methanogenic communities, sludge samples were monthly taken from the bottom sampling ports of the reactors and stored at -20 °C before DNA isolation.

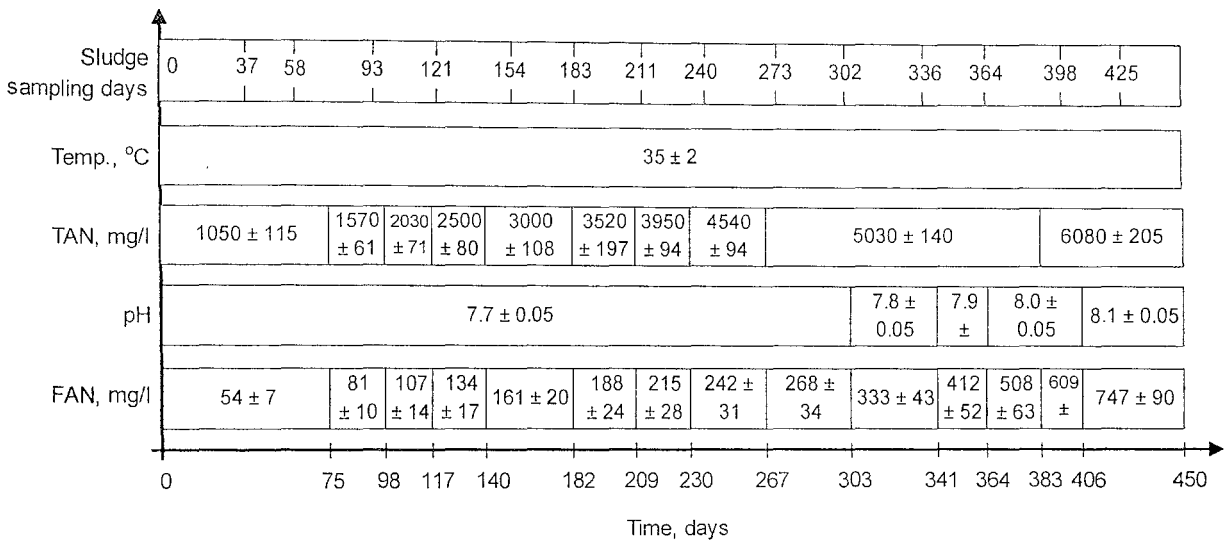

Figure 6.1. Time schedule for sludge sampling days and changes in TAN, pH and FAN

6.2.1. DNA Isolation and PCR Amplification

To extract and isolate DNA, initially 1 ml of sludge sample was mechanically bead beaten with zirconia/silica beads (BioSpec Products) in Tris/HCl buffered phenol. After the mechanical disruption, released DNA was purified with phenol/chloroform/isoamyl alcohol (25:24:1) and chloroform/isoamyl alcohol (24:1) extraction according to the procedure outlined in Oude Elferink *et al.*, 1997. Afterward, for DGGE analysis, V6 to V8 region of the 16S rDNA gene of the isolated DNA was amplified using polymerase chain reaction (PCR). The primer set specific to *Archaea* domain was A109 for (5' AC(G/T) GCT CAG TAA CAC GT-3') (Grosskopf *et al.*, 1998) and GC-515 rev (5' CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC

GCG GCT GCT GGC AC-3') (Lane *et al.*, 1991). For amplification of the 16S-rDNA gene for cloning, the primer set specific to *Archaea* domain A-109 for and 1510 rev (5' GTG AAG CTT ACG G(C/T)T ACC TTG TTA CGA CTT-3') (Lane *et al.*, 1991) was used. PCR programs were performed in a Progene thermocycler (Techne). In the PCR for total archaeal 16S-rDNA and for DGGE the following program was used: pre-denaturation (94°C, 5 min), 34 cycles of annealing (52°C, 40 sec), elongation (68°C, 1 min), and denaturation (94°C, 30 sec), a final annealing (52°C, 40 sec) and post elongation (68°C, 5 min). The reactions were subsequently cooled to 4°C. PCR products were examined on ethidium bromide-stained agarose gels, and subsequently used for DGGE analysis and cloning.

6.2.2. Denaturing Gradient Gel Electrophoresis (DGGE) and Cloning

DGGE of the PCR amplified partial 16S rDNA was performed with D-Code Universal Mutation Detection System (BioRad) in accordance with Nübel *et al.*, 1996. The polyacrylamide concentrations used in the analysis of DGGE were 8% and the denaturing gradients were 32–50%. The denaturing gradient gel was electrophoresed at 85 V and 60°C for 16 hours after a voltage of 200 V was applied for 5 min. At the end, gels were silver stained according to procedure given in Sanguinetti *et al.*, 1994. Community shifts in the reactors were described as changes in the DGGE profiles of the partial 16S rDNA amplicons.

As DGGE gels contain many bands in one lane because of the microbial complexity of sludge samples, cloning and sequencing techniques were used to find out which band corresponded to which species. For cloning, the amplified archaeal 16S rDNA products were purified with a QIAquick PCR purification kit (Qiagen) and cloned in *E. coli* JM109 by using the pGEM®-T Easy vector system (Promega) with ampicillin selection and blue/white screening, according to the manufacture's manual. The inserts were screened by Restriction Fragment Length Polymorphism (RFLP) analysis with the enzyme *MspI* (Fermentas) and by mobility comparison on DGGE. Plasmids of selected transformants were purified using the Wizard Plus SV miniprep DNA purification kit (Promega).

Sequencing analysis was carried out in a private sequence laboratory. A similarity search, in the GenBank database, with the derived partial (app. 800 bp) 16S rDNA sequences from the clones, was performed by using the NCBI sequence search service, available on the internet.

6.2.3. Fluorescent In-situ Hybridization (FISH)

The sludge samples were fixed overnight in 4% formaldehyde-PBS solution at 4°C. Fixed cells were spotted on Teflon-coated multiwell glass slides and allowed to dry (Raskin *et al.*, 1994). Hybridizations were performed at 46°C for 2 h with 10 µl hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 8.0, 0.01% SDS) and 1 µl labeled probe (30 ng/well) (MWG Biotech, Ebersberg). A list of the oligonucleotide probes used in FISH is given in Table 6.1. Formamide was added to the final concentrations of about 20% for Arch 915 and 35% for the other probes to ensure the optimal hybridization stringency. After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer containing the same components of the hybridization buffer except the probes. Slides were subsequently incubated at 48 °C for 10 min with washing buffer. Then, washing buffer was removed by rinsing the slides with distilled water. For detection of all DNA, the sludge samples were additionally stained with 4,6-diamidino-2-phenylindole (DAPI; 1 µg/ml) for 10 min in the dark, finally again rinsed with distilled water and immediately air-dried. Vectashield (Vector Laboratories) was used to prevent photo bleaching. The slides were examined with an Olympus BX50 microscope. Digital images of the slides were taken with a digital camera.

6.3. Results

Five upflow anaerobic reactors seeded with different seed sludges were operated for 450 days under nearly constant loading rates in the range of 0.9 to 1.4 kg COD m^3/day. To investigate the methanogenic population dynamics during adaptation to elevated free ammonia levels, influent TAN concentrations were step by step increased from 1000 to

6000 mg/l. In addition, influent pH which was constant at 7.7 until Day 302 increased gradually up to 8.1 to elevate the FAN in the reactors.

Table 6.1. Oligonucleotide probes used in fluorescent in-situ hybridization

Probe name	Label	Sequence	Target group	Ref
Arch 915	Cy3	GTGCTCCCCCGCCAATTCCT	Archaea domain	Stahl *et al.*, 1991
Mx 825	Cy3	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta*	
Ms 821	Cy3	CGCCATGCCTGACACCTAGCGAGC	*Methanosarcina*	
MsMx860	Cy3	GGCTCGCTTCACGGCTTCCCT	*Methanosarcina* & *Methanosaeta*	Raskin *et al.*, 1994
Mb 1174	Cy3	TACCGTCGTCCACTCCTTCCTC	*Methanobacteriales*	
Mc 1109	Cy3	GCAACATAGGGCACGGGTCT	*Methanococcales*	
Mg 1200	Cy3	CGGATAATTCGGGGCATGCTG	*Methanogenium*-relatives	

During the start-up until Day 75, influent TAN was adjusted to about 1000 mg/l resulting in FAN of about 54 mg/l (Fig 6.1). In this interval, within the five reactors, R2 and R4 reached steady state conditions more rapidly until second sludge sampling at Day 58 with almost no acetate and propionate in the effluent (Fig 6.2 b and d). Since the sludges seeded in these reactors have been adapted to TAN levels above 2000 mg/l, relatively shorter start-up periods seem consistent. However, as the DGGE banding patterns were compared, significant changes in methanogenic community were observed in both of the reactors conflicting with this consistency (Fig. 6.3). Because, intensive *Methanosaeta* related DGGE bands in the seed sludge of R2 represented by clones CM6, CM21 and CM42 faded in this period and completely disappeared until Day 121. At Day 58, abundant methanogens in R2 were a *Methanosarcina*-like (CM80) acetoclastic species and a hydrogenotrophic uncultured archaeon clone ASDS11 that belongs to order *Methanobacteriales* (CM14). Methanogenic population shift in R4 was more rapid and momentous. As the low volatile solids (17%) inoculum seeded to this reactor was obtained by concentrating the biomass in the leachate taken from a municipal solid waste landfill,

the quantity of methanogens was very low. Therefore, among the very few faint bands on DGGE gel, only a *Methanoculleus* related species (CM20) could be identified which was also represented as the dominating archaea in another landfill leachate with similar characteristics (Huang *et al.*, 2002). Hydrogenotrophic *Methanoculleus* species in R2 disappeared just after one month (Day 37) and parallel to rapid improvement in the reactor performance, clone CM80 (*Methanosarcina barkeri* strain SAR, 98% similarity) dominated in the system as in R2. Additionally, another hydrogenotrophic methanogen (clone CM85) related to (98% similarity) the uncultured archaeaon LK33 was also identified at Day 58 which was almost invisible in the seed. Fascinatingly, this *Methanospirillium*- like uncultured archaeon was also recovered from the same aforementioned leachate (Huang *et al.*, 2002). Sequencing and similarity search results of the clones matching with the bands on DGGE gels are given in Table 6.2.

As revealed in Figure 6.2, VFA sampling and analysis in the effluents were started after Day 54. However, the decreasing trends in acetate and propionate levels at the beginning of VFA analysis period indicate that reactors were exposed to elevated concentrations of acetate and propionate in the start-up. In particular, high acetate levels in this period promote the presence of *Methanosarcina*- related species instead of *Methanosaeta* as the main acetate utilizing methanogens in the reactors. In the literature, it is generally accepted that at high acetate concentrations species of *Methanosarcina* has a competitive advantage against *Methanosaeta* with relatively higher maximum growth rate (μ_{max}) and half saturation constant (K_S) values (Jetten *et al.*, 1992; Raskin *et al.*, 1994).

Contrary to short start-up phases in R2 and R4, instability continued until Day 90 in R1 and R3 and until Day 115 in R5. Relatively lower COD removal efficiency in R5 in this period primarily originated from slightly high and gradually decreasing acetate and propionate concentrations in the effluent. As acetate and propionate were reduced to very low levels, COD concentrations of about 200 mg/l were determined in the effluent of R5 after Day 115 and it was kept stable at this level for a long time (Fig 6.2 e). In addition to quite high effluent VFA concentrations, flocs escaping from the reactors resulted in relatively high COD concentrations in the effluents of R3 and R5. As the disintegration of granular seed sludges was also continued after the start-up in these reactors, effluent COD concentrations never decreased below 400 mg/l in average (Fig 6.2 a and c).

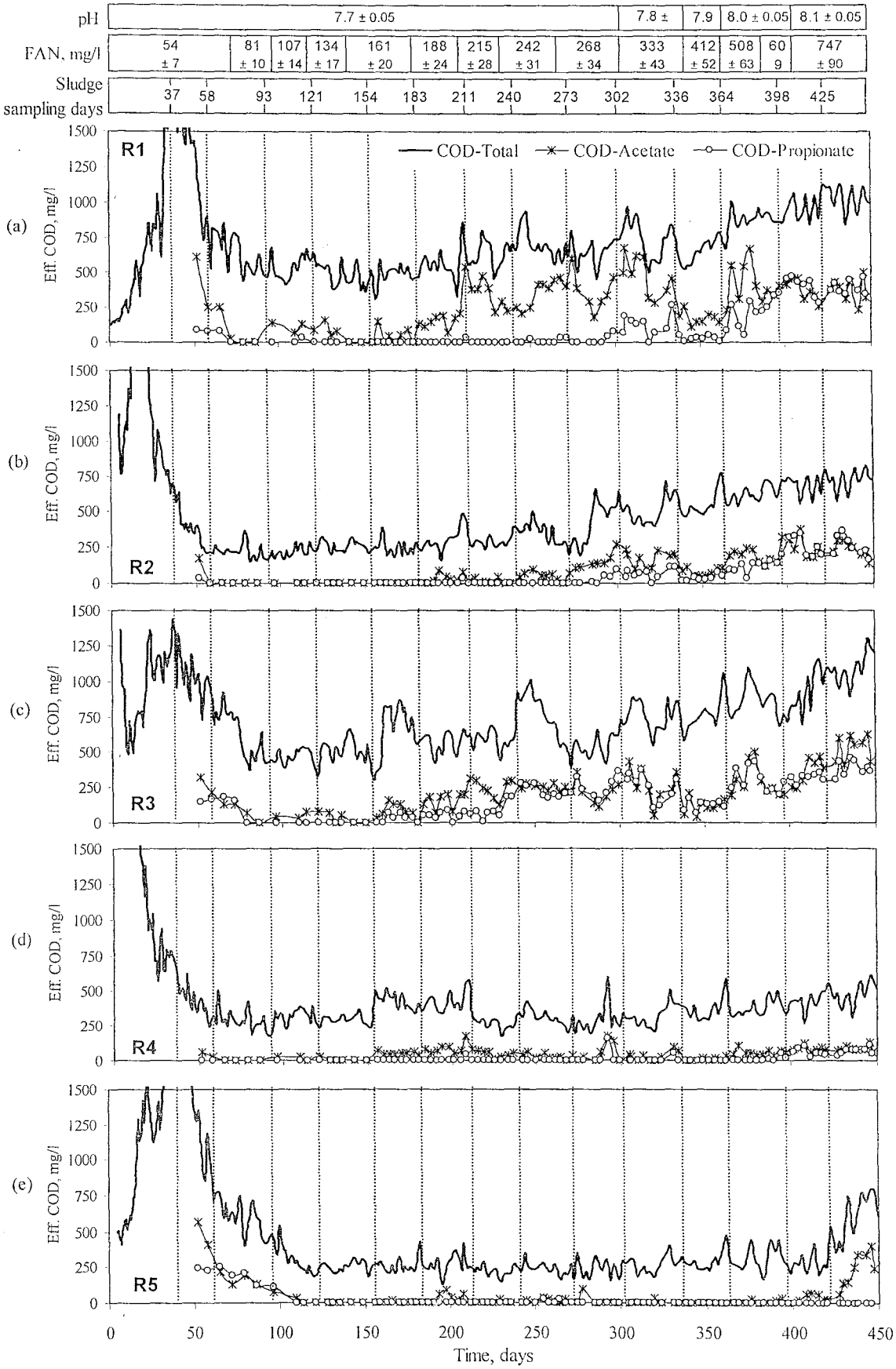

Figure 6.2. Effluent acetate, propionate and total COD concentrations

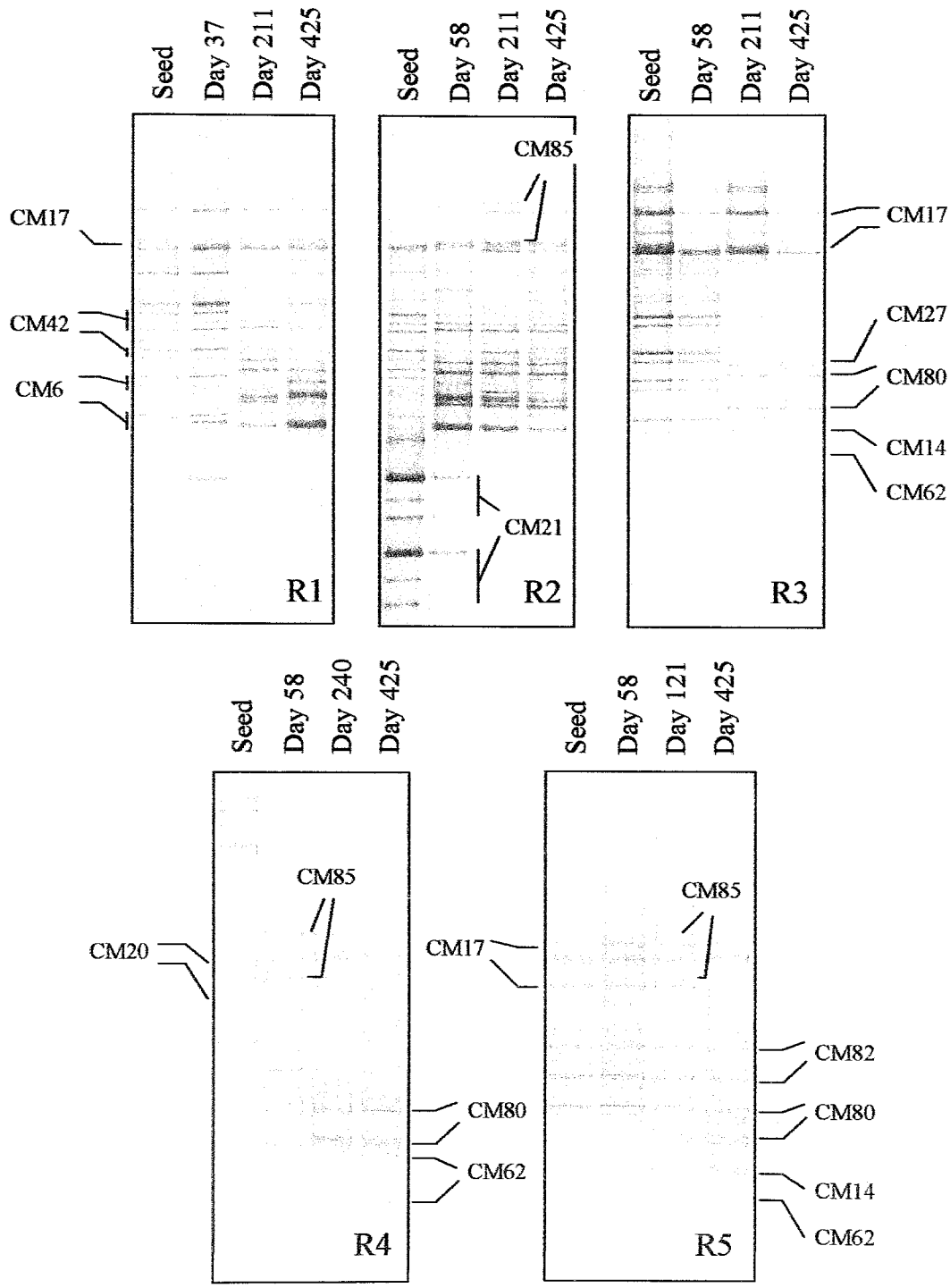

Figure 6.3. DGGE profiles and correspondent position of matching clones

According to DGGE analysis, granular sludges seeded to R1 and R3 were very similar to each other and composed of clones CM6, CM17 and CM42 which were identified as *Methanosaeta concilii*, uncultured archaeon clone Vadin DC06 and *Methanosaeta sp.* with very high similarities (>99%), respectively (Fig. 6.3). Clone CM17

representing a hydrogenotrophic *Methanobacterium*-like species was also identified in R5. In addition to clone CM17, there was another *Methanobacterium*-related species specific to seed sludge of R5. It was (CM82) closely related to (>99%) uncultured archaeon clone A3 detected in an expanded granular sludge bed reactor treating oleic acid (Pereira *et al.*, 2002).

Table 6.2. Sequencing and similarity results of the selected clones.

Clone	Sequence Length	Similarity results	Source Reactor
CM6	572 bp	*Methanosaeta concilii* (99%)	R1, R2, R3
CM14	835 bp	Uncultured archaeon clone ASDS11 (95%)	R1, R2, R3, R5
CM17	831 bp	Unidentified archaeon clone Vadin DC06 (99%), *Methanobacterium formicicum* (94%)	All
CM20	585 bp	Methanoculleus sp. (97%)	R4
CM21	834 bp	Methanosaeta sp. clone A1 (98%)	R2
CM27	835 bp	Uncultured *Methanosaeta* sp. KuA1 (96%)	R1, R2, R3
CM42	829 bp	*Methanosaeta* sp. AMPB-Zg (99%)	R1, R2, R3
CM62	833 bp	*Methanosarcina mazei* Goe1 (99%)	R3, R4, R5
CM80	832 bp	*Methanosarcina barkeri* Sar (98%), *Methanosarcina acetivorans* C2A (97%)	All
CM82	568 bp	Uncultured archaeon clone A3 (99%), *Methanobacterium* sp. DSM 11103 (96%)	R5
CM85	572 bp	Uncultured archaeon LK33 (98%), *Methanospirillium* sp. clone A2 (95%)	R1, R2, R4, R5

During start-up, changes in methanogenic community in R1, R3 and R5 were quite mild and slow parallel to delay in adaptation of sludges seeded to these reactors. For instance, DGGE banding patterns of the inoculums and sludge samples taken at Day 58 were very similar to each other in these reactors (Fig. 6.3). However, after Day 93, methanogens (CM80 and CM14) previously dominated R2 and R4 also started to be

abundant in R1, R3 and R5. Moreover, clone CM62 closely related to (99%) *Methanosarcina mazei* strain Goe1 was also identified in R3. Nevertheless, despite the prevailing methanogens in the flocculent, sludges seeded to R2 and R4 almost completely disappeared during start-up, inoculum methanogens persisted in R1, R3 and R5 for a long time as a result of granular structure.

With 500 mg/l increments at Day 75, 98, 117 and 140, influent TAN concentration was elevated from 1000 to 3000 mg/l (Fig. 6.1). Thus, after Day 140, FAN reached to 161 ± 20 mg/l in the reactors (Fig. 6.1). In literature, generally free ammonia inhibition thresholds for mesophilic anaerobic treatment were reported in the range 50 to 150 mg/l (Koster and Lettinga 1984; De Baere *et al.*, 1984; Soubes *et al.*, 1994). However, although FAN level exceeded these thresholds, none of the reactors were affected severely until Day 150. After Day 152, a slight increase in VFA concentrations especially in the form of acetate were observed in R4 reflected as a small jump in the effluent COD (Fig. 6.2 b). The response of R3 was more remarkable ever since the jump in effluent COD concentration was up to 800 mg/l and it was kept on until Day 180 (Fig. 6.2c).

By increasing the influent TAN to 3500 mg/l at Day 182 and to 4000 mg/l at Day 209, FAN concentrations exceeded 200 mg/l in the reactors (Fig. 6.1). Consequently, at Day 212 effluent acetate concentrations which were formerly less than 200 mgCOD/l exceeded 500 mgCOD/l in R1 and 300 mgCOD/l in R3 (Fig. 6.2 a and c). Additionally, effluent propionate concentrations of about 80 mgCOD/l were also measured in R3 (Fig 6.2 c). In spite of FAN concentrations exceeding 200 mg/l, R2, R4 and R5 achieved total COD removal efficiencies as high as 95%, without any decline in their performances (Fig. 6.2 b, d and e).

At Day 211, according to their performances under 215 mg/l FAN, reactors could be classified as highly (R2, R4 and R5) and moderately (R1 and R3) efficient reactors with 95 and 85% total COD removal efficiencies, respectively. At this point, abundant methanogens in R1 and R3 were two *Methanobacterium* (CM14, CM17) and a *Methanosarcina*- related (CM80) species. Moreover, there was an uncultured *Methanosaeta* sp. (CM27) represented by a faint band on DGGE gel which was not present in the seed. In a previous study, we characterized the same clone (CM27) in an anaerobic

reactor treating moderately high ammonia bakers' yeast wastewater. In addition to species in R1 and R3, a *Methanospirillium* -like (CM85) hydrogenotrophic methanogen was also prevailing in R2 at Day 211. On the other hand, in R1 and R3 intensive *Methanosaeta* -related seed sludge bands represented by clones CM6 and CM42 completely disappeared (Fig. 6.3). Likewise, in a previous study investigating the ammonia toxicity in pure cultures of methanogens, Sprott and Patel (1986) found that *Methanosaeta concilii* was completely inhibited at 560 mg/l TAN, while *Methanosarcina barkeri* was not inhibited at 2800 mg/l. Furthermore, a similar conclusion was also given in a more recent study on anaerobic digestion of high ammonia swine waste (Angenent *et al.*, 2002).

Since, the gaseous form of free ammonia is considered to be more toxic than ammonium ion (Angelidaki and Ahring, 1993; Sprott *et al.*, 1984; Koster and Koomen, 1988) inhibiting effect of ammonia nitrogen occurs after the passive diffusion of hydrophobic free ammonia molecule into the cell. *Methanosarcina* species consist of large spherical cells and forms big clusters, thus they have a much higher volume-to-surface ratio than rod shaped *Methanosaeta* sp. which is normally interconnected end to end in long filaments. Hence, per unit mass the free ammonia diffusion will be less for *Methanosarcina* cells than *Methanosaeta* (Wiegant and Zeeman, 1986). This was also similar for diffusion of free acetic acid that explains why *Methanosarcina* sp. has higher minimum threshold values for acetate than *Methanosaeta* sp. (Jetten *et al.*, 1992). From another point of view, *Methanosarcina* packets are more resistant since the cells inside the packets are protected from inhibitory compounds by comparison with single cells (Macario *et al.*, 1999).

After 500 mg/l increments in TAN concentration of synthetic feed at Day 230 and 267, TAN and FAN levels in the reactors were elevated to 5000 mg/l and 268 mg/l, respectively (Fig. 6.1). Moreover, by increasing the influent pH from 7.7 to 7.8 at Day 303, FAN concentration reached to approximately 335 mg/l in the reactors. In this period, R3 was the most inefficient reactor after Day 233 with acetate and especially propionate concentrations in the effluent as high as 300 mgCOD/l equivalent (Fig. 6.2c). Similar increases in effluent propionate concentrations were also observed in R1 and R2 after Day 295. Between Day 335 and 365, even though there was not any noteworthy methanogenic community shift in the reactors, substantial improvements in VFA removal were observed

in R1, R2 and R3. The only change was fading of DGGE band representing clone CM85 (uncultured archaeon LK33, 98% similarity) in R2. On the other hand, despite R1, R2, and R3 were more or less affected from FAN concentrations above 300 mg/l, satisfactory total COD removals above 93% were again achieved in R4 and R5 under these conditions.

By increasing the influent pH to 7.9 at Day 340 and to 8.0 at Day 364, FAN concentrations were elevated up to 510 mg/l (Fig. 6.1). Consequently, effluent total COD concentrations exceeded 900 mg/l in R1 and 600 mg/l in R2 (Fig. 6.2a and b). Moreover, parallel to jumps in effluent acetate and propionate levels, COD concentrations as high as 1085 mg/l were determined in R3 at Day 376 (Fig. 6.2c). Astonishingly, R4 and R5 tolerated even these increments in FAN and achieved 93 and 95% COD removal efficiencies, respectively with very low total VFA concentrations in the effluents corresponding to less than 50 mgCOD/l (Fig. 6.2d and e).

Finally at Day 383, influent TAN and at Day 406, influent pH were elevated from 5000 to 6000 mg/l and from 8.0 to 8.1, respectively (Fig. 6.1). In this way, as a final point, FAN in the reactors reached to approximately 750 mg/l. In the literature, usually severe or complete inhibitions were reported for similar or lower free ammonia concentrations in the aforementioned studies. However, Hansen *et al.* (1998) have reported relatively low but constant methane formation at FAN concentrations as high as 1100 mg/l in thermophilic swine manure digestion.

The final increases in FAN resulted in noticeable drops in COD removal efficiencies except R4 although the preceding methanogenic populations (CM80, CM17 and CM14) were still abundant in the reactors. Additionally, in R3 and R5, *Methanosarcina* -related clone CM62 and in R5 the seed sludge specific hydrogenotrophic uncultured archaeon A3 (CM82) were also present. While graphs representing FAN concentration versus acetate and propionate removal efficiency, were drawn as in figure 6.4, the influence of increasing free ammonia was more easily recognized on propionate oxidation than on acetate removal. In the calculation of acetate removal efficiency, butyrate and propionate amounts converted to acetate were also taken into account.

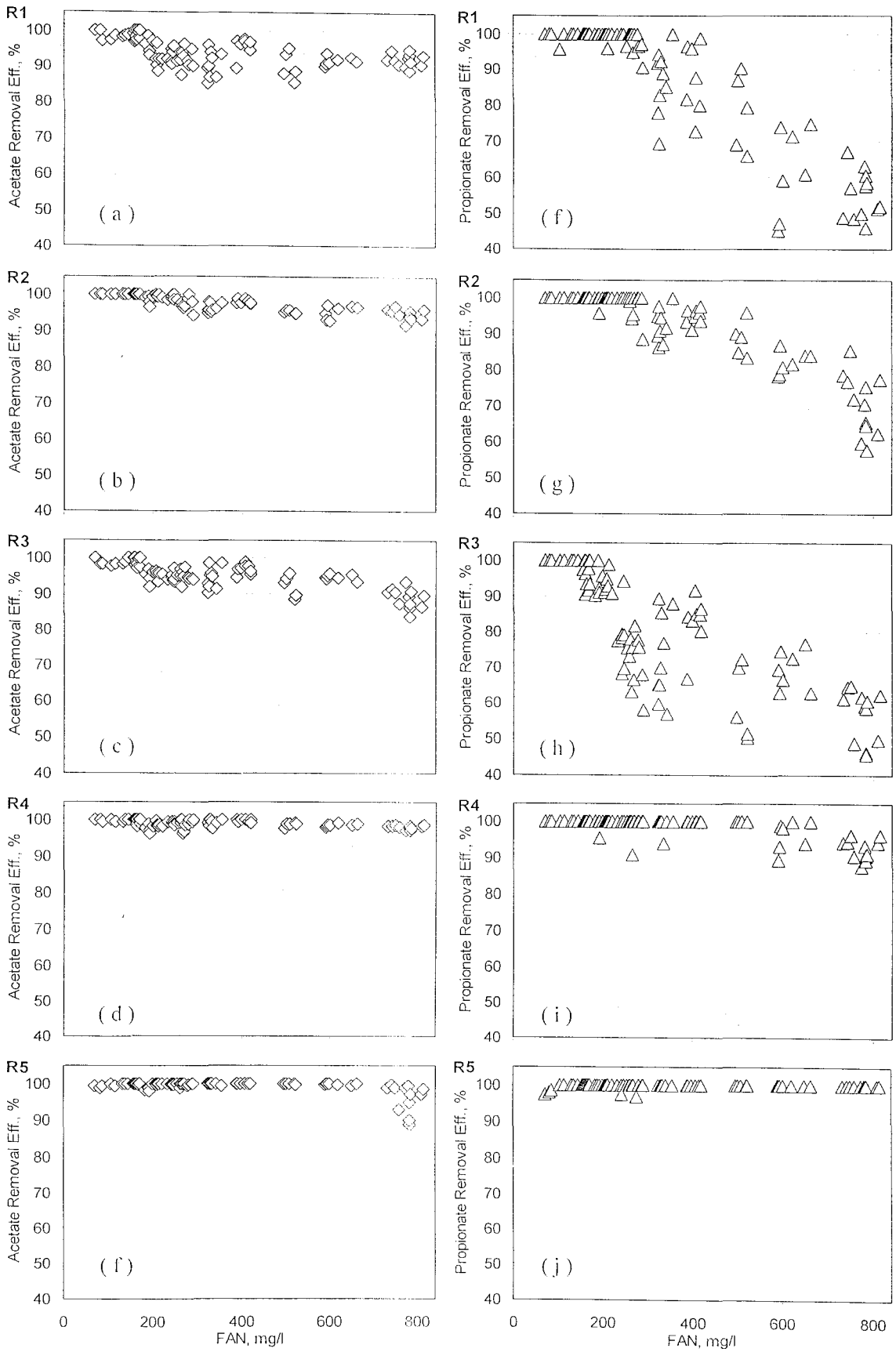

Figure 6.4. Effects of increasing FAN levels on acetate and propionate removal efficiency

In all of the reactors, while methanogenic acetate removal never dropped below 80%, syntrophic propionate oxidation was considerably affected by FAN above 290 mg/l in R1 and above 220 mg/l in R3 (Fig. 6.4f and h). In the literature, mainly methanogenesis was reported as the most sensitive phase in anaerobic treatment systems under high ammonia levels (Koster and Lettinga, 1988; Bhattacharya and Parkin, 1989; Heinrichs *et al.*, 1990). However, direct or indirect inhibition of acetogenic bacteria was also reported (van Velsen, 1979; Wiegant and Zeeman 1986; Koster and Lettinga, 1988; Heinrichs *et al.*, 1990; Fujishima *et al.*, 2000).

Meanwhile, after Day 398, as an indication of inhibition in propionate oxidation, effluent propionate concentrations above 400 mgCOD/l were observed in R1 and R3 (Fig 6.2a and c). These remarkable results indicated that in addition to methanogenic population shifts, variations in syntrophic propionate degraders should also be investigated for more satisfying conclusions on ammonia inhibition. As revealed in figure 6.4g, the ammonia inhibition threshold for propionate degradation was as high as 600 mg/l for R2. Therefore, since the FAN level elevated up to 750 mg/l at Day 406, the influence on R2 was slighter than in R1 and R3 and after Day 429, effluent VFA concentrations started to decrease gradually (Fig. 6.2b). In R4, related with the stable methanogenic community (CM62 and CM80) for more than 350 days (Fig. 6.3), substantially high acetate and in addition propionate removal efficiencies were still available in this period with minor VFA concentrations in the effluent (Fig. 6.2d). On the other hand, R5, the most resistant and efficient reactor until Day 427, was significantly affected at this FAN level. Almost 100% acetate removal efficiency dropped to 90% (Fig. 6.4e) within 20 days associated with the increase in effluent acetate concentrations up to 390 mgCOD/l (Fig. 6.2e). However, in contradiction to diminishing propionate removal efficiencies in R1, R2 and R3, very low propionate concentrations of less than 10 mg/l were obtained in the effluent of R5 (Fig. 6.2e). This indicates that, in addition to methanogens, some propionate degraders can also resist to FAN levels as high as 750 mg/l after acclimation.

At this point, to confirm the results of cloning and DGGE analysis and in addition to determine the activities of presenting methanogens FISH experiments were conducted by using the genus specific oligonucleotide probes given in Table 6.1. The rRNA based in-situ

hybridization provided valuable information about the abundance of active methanogens and gave opportunity to investigate the inhibitory effects of ammonia on structural forms. Resembling to the DGGE results, *Methanosaeta*-like cells (Mx 825) in filamentous forms were detected as the prevailing methanogens in R1, R2 and R3 just after the start-up (Fig 6.5a). Thereafter, parallel to elevation in ammonia level, *Methanosaeta* species have gradually lost their activities as their filamentous forms deteriorated until Day 121. In R2 and R4, after Day 37 and in R1, R3 and R5 after Day 93, *Methanosarcina*-related cells (Ms 821) were detected in abundance (Fig 6.5b). Moreover, there was a superior correlation between reactor performances and abundance of *Methanosarcina* cells. Therefore, until Day 398, highly active *Methanosarcina* cells were identified in large multicellular packets in R2, R4 and R5 (Fig. 6.5c-f). There are also similar results in the literature revealing the abundance of *Methanosarcina*-like cells under extremely high ammonia levels (De Baere *et al.*, 1984; Angelidaki and Ahring, 1993). In this period, mainly *Methanosarcina*-like cells were also dominant in R1 and R3; however they were in quite disturbed, fragile structures (Fig. 6.5g and h). When the FAN level exceeded 700 mg/l, disintegration of large *Methanosarcina* clusters were observed in R2 and R5 as well. Nevertheless, in spite of extremely high FAN levels, rigid *Methanosarcina* packets were detected in R4 (Fig. 6.6a and b). As this reactor was seeded with the biomass concentrated from a landfill leachate, it contained high inorganic constituents which might reduce the inhibitory effects of high free ammonia levels. In a previous study, addition of phosphorite ore was found to prevent ammonia inhibition in anaerobic digestion of poultry manure, supposedly by either immobilizing methanogens on the mineral grains, which increase the buffering capacity of the medium, or by exchanging ammonium ion for cations such as K, Ca and Mg (Krylova *et al.*, 1997).

Microscopic results supporting the first hypothesis were obtained in R4 during DAPI staining and FISH. Well developed *Methanosarcina* packets embedded in inorganic particles were abundantly present in R4 even at Day 425 under 750 mg/l FAN. On the other hand, despite some *Methanobacterium* and *Methanospirillium* -related methanogens were identified with DGGE analysis and cloning, only very few and weak hybridization signals were obtained with probes Mb 1174 and Mg 1200. The best hybridization signals with Mb 1174 were obtained at Day 93 in R3 and at Day 58 in R5 at rather low FAN levels.

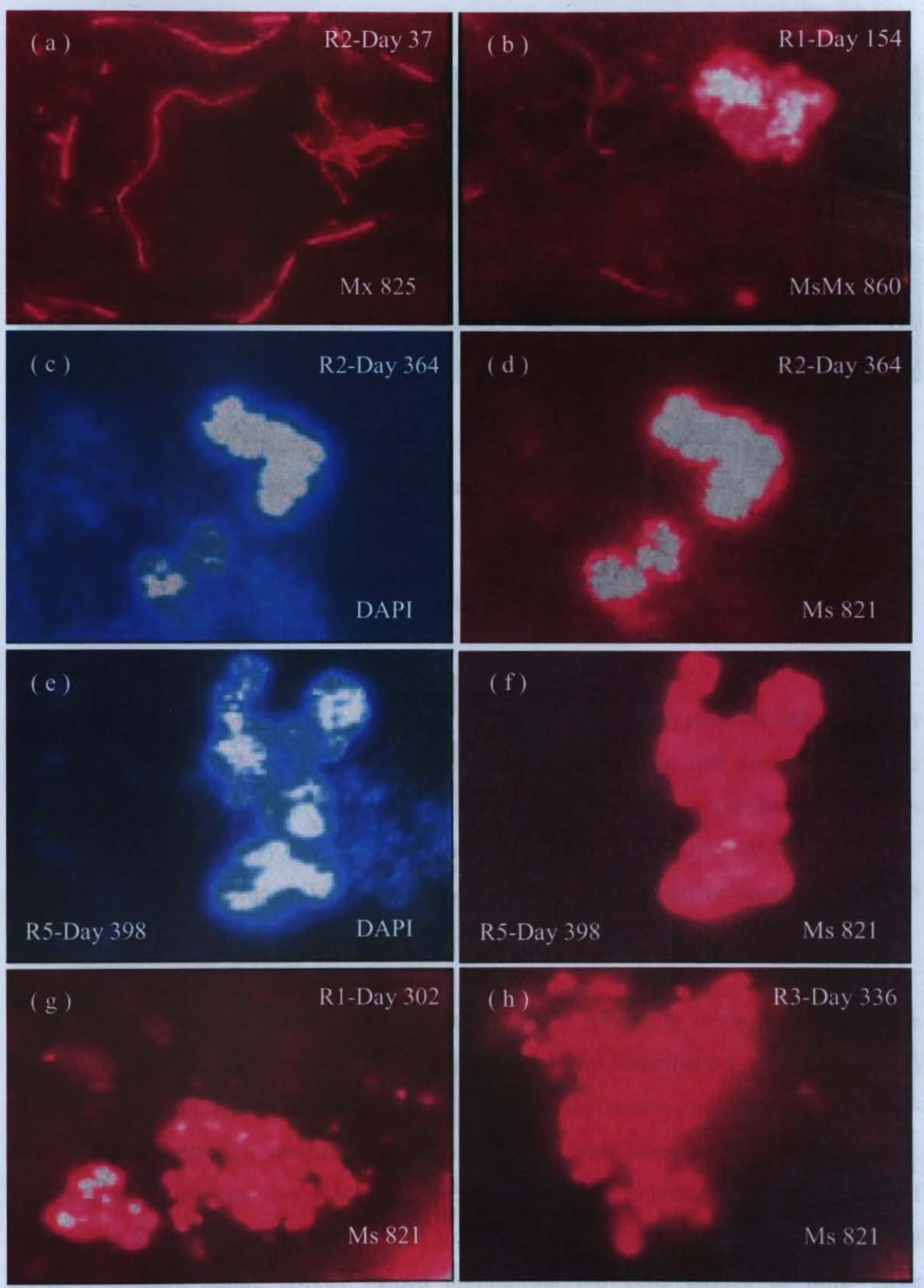

Figure 6.5. Fluorescent in-situ hybridization and DAPI staining photomicrographs; sludge samples stained with DAPI and hybridized with Cy3 labelled (red) Mx 825, MS 821 and MsMx 860 probes. Couples in e-f, and g-h represent the same field of the view.

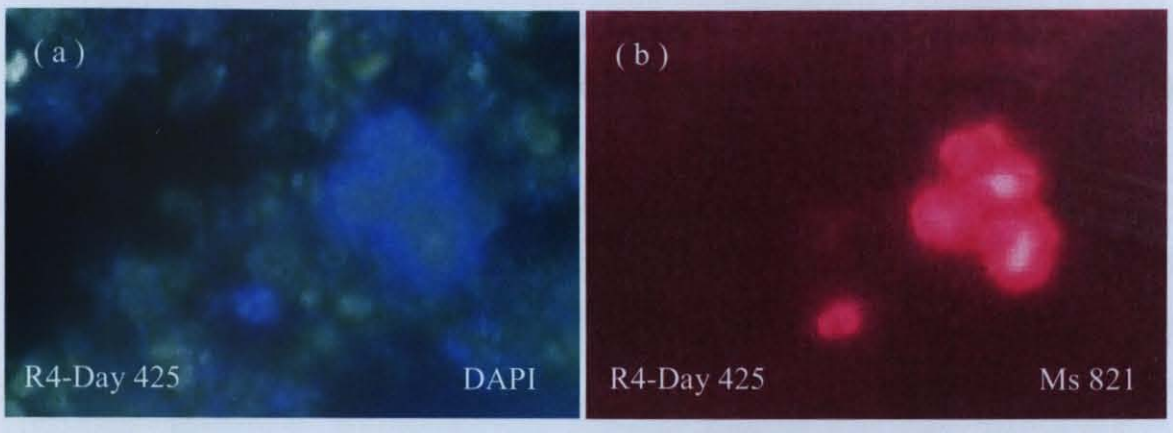

Figure 6.6. DAPI stained (blue) and Cy3 labelled (red) *Methanosarcina* clusters embedded in inorganic particles (green)

6.4. Discussions

After a gradual adaptation in 406 days, moderately high COD removal efficiencies in the range of 77 to 90% were obtained above 700 mg/l FAN in five upflow anaerobic reactors seeded with different sludges. In three of these reactors, propionate degradation was affected more severely than acetate removal and degradation efficiency dropped below 50% in R1 and R3. Meanwhile, acetate removal in R4 and propionate degradation in R5 was in no way inhibited even at FAN level as high as 750 mg/l.

During adaptation, *Methanosaeta*-related species prevailing in seed sludges have noticeably lost their activities and their filamentous forms deteriorated when FAN level exceeded 100 mg/l. Afterward, although some *Methanobacterium* and *Methanospirillium*-related hydrogenotrophic methanogens were identified with cloning and DGGE analysis, mainly *Methanosarcina*- like acetoclastic methanogens were abundantly detected in all of the reactors by using activity based FISH technique. Following the increase in FAN level above 150 mg/l, single coccus shaped *Methanosarcina* cells tended to form large multicellular packets. Subsequently, at higher FAN levels, reactor performances were strongly correlated with the stringency of these clusters rather than any shifts in methanogenic community. When the FAN level exceeded 600 mg/l, disintegration of large

Methanosarcina clusters were observed in R2 and R5. However, inorganic particles originated from seed sludge provided a good support for *Methanosarcina* clusters in R4. Consequently with this special feature, R4 successfully resisted to FAN levels as high as 750 mg/l.

7. CONCLUSIONS

Results of the long-term characterization and anaerobic treatability studies on Istanbul Kömürcüoda Landfill leachate were presented in chapter 3. High VFA concentrations and BOD_5/COD ratios above 0.6 determined in the leachate were indications of high acidogenic activity in the landfill. In contrast, relatively elevated pH (8.1) and extraordinarily high TAN (total ammonia nitrogen) (3250 mg/l) values were also determined in acidogenic Kömürcüoda Landfill leachate. These high pH and TAN levels increased the possibility of free ammonia inhibition during anaerobic treatment. By adjusting the influent pH to 4.5 to keep the free ammonia concentrations below inhibitory level, COD removal efficiencies above 90% were achieved with laboratory scale anaerobic reactors, namely, anaerobic filter, upflow sludge blanket and hybrid bed reactors.

As influent pH was adjusted to 4.5, stability of the reactors and low levels of acetate in the effluent were supported with the abundance of *Methanosaeta* community identified with DGGE, cloning, FISH and morphological analysis. However, after the termination of pH adjustment, the reactors became unstable parallel to the jump in FAN (free ammonia nitrogen) concentration up to 400 mg/l and COD removal efficiencies have dropped significantly down to 42 and 48% in hybrid bed and UASB reactors respectively. Although, it is defined as an ammonia sensitive methanogen in literature, many *Methanosaeta* cells were identified after two free ammonia inhibitions. Their occurrence may be explained by their massive intensity before inhibitions and short durations of high free ammonia periods. However, it was observed that long filamentous morphologies of *Methanosaeta* cells have shifted to shorter filaments consequently loosing their aggregating property. Thus, it is concluded that pH adjustment should be applied in the reactor influent to prevent free ammonia inhibition when high pH values and TAN concentrations are experienced.

Nevertheless, sudden and unexpected fluctuations in the characteristics of leachate resulted in complications in understanding of the inhibitory effects of ammonia on

microbial diversity and activity. Therefore, to investigate the methanogenic population dynamics during adaptation to free ammonia, five mesophilic anaerobic reactors seeded with different sludges were operated for 450 days under gradually increasing free ammonia levels and more stable COD loading rates by feeding with a synthetic wastewater.

As FAN gradually rose up to 800 mg/l throughout the 450 days experimental period, COD removal efficiencies in the range of 78 to 90% were observed in all of the anaerobic reactors. High removal efficiencies indicated that seed sludges also taken from anaerobic reactors treating considerably low ammonia containing wastewaters may be successfully adapted to elevated free ammonia concentrations.

In three of the reactors, propionate degradation and in one of them acetate removal was inhibited more severely. In no way, neither of the phases was inhibited in R4 which was seeded with the biomass concentrated from Kömürcüoda Landfill leachate. *Methanosaeta* mainly prevailing in seed sludges were substituted for *Methanosarcina*-related methanogens when FAN exceeded 100 mg/l. Afterward, although some *Methanobacterium* and *Methanospirillium*- related hydrogenotrophic methanogens were identified with cloning and DGGE analysis, mainly *Methanosarcina*- like acetoclastic methanogens were abundantly detected in all of the reactors by using activity based FISH technique. As FAN level elevated, rather than any shifts in the methanogenic community, single coccus shaped *Methanosarcina* cells started to form stringent multicellular packets. However, when the FAN level exceeded 600 mg/l, disintegration of *Methanosarcina* clusters was observed. In contrast, inorganic particles originated from seed sludge provided a good support for *Methanosarcina* clusters in R4 and with this special feature, it successfully resisted to FAN levels as high as 750 mg/l.

The results obtained in this study indicate that, like acetoclastic methanogens, propionate degrading acetogens are also sensitive to high free ammonia concentrations. Moderately high propionic acid concentrations in the effluents of R1, R2 and R3 pointed out to this conclusion. However, low propionic acid concentrations of less than 10 mg/l in the effluent of R5 indicated the presence of propionate degraders resistant to extremely high free ammonia levels. On the other hand, there was no problem in the conversion of

butyric acid at any free ammonia level in all reactors revealing the resistance of butyrate degraders.

Granulated sludges known to be resistant to environmental factors and toxic substances did not show the same resistance under high free ammonia concentrations. Besides, the lowest removal efficiencies were observed in R1 and R3 which were seeded with granular sludges. Fascinatingly, despite granules couldn't tolerate high free ammonia levels, inorganic particles intensively present in R4 reduced the inhibitory effects by supporting the formation of large *Methanosarcina* clusters.

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