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IDENTIFICATION OF PREVALENT MICROBIAL COMMUNITIES IN MUNICIPAL SOLID WASTE LANDFILL LEACHATE

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ABSTRACT

IDENTIFICATION OF PREVALENT MICROBIAL COMMUNITIES IN MUNICIPAL SOLID WASTE LANDFILL LEACHATE

Landfilling is the most economical and commonly used method for disposal of municipal solid wastes. A better understanding of landfill microbiology is very important to accelerate the stabilization of landfilled waste.

In this study, a total of fourteen leachate samples originated from the different sections of Istanbul Kemerburgaz Sanitary Landfill were used for investigation of the prevalent microorganisms in municipal solid waste leachate. Besides chemical characterization molecular microbiology techniques such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), cloning and sequencing, and fluorescence in situ hybridization (FISH) were applied to the biomass inhabiting the samples.

The results suggested that there were significant dissimilarities in microbial diversity between different sections of the landfill at different ages. High BOD_5: COD and TKN: NH_3-N ratios of young age leachates were good indicators of the acidogenesis phase as well as the high VSS concentrations. Acetate-utilizing *Methanosarcina* was found predominantly in acidogenic leachates by both cloning and sequencing and FISH analyses. On the contrary, mature landfill leachates with very low organic pollutant concentrations were dominated by H_2-utilizing *Methanomicrobiales.* All samples contained gamma-*Proteobacteria* as the most abundant bacterial group. However, no bacterial composition change was observed between the young and mature leachates.

June, 2005 **Selçen DURMAZ**

ÖZET

KATI ATIK DEPO SAHASI SIZINTI SULARINDAKİ BASKIN MİKROORGANİZMA TÜRLERİNİN TESPİTİ

Düzenli depolama, kati atiklarin bertarafi için en ekonomik ve yaygin olarak kullanilan yöntemdir. Depolanmış atıkların stabilizasyonunu hızlandırmak için depo sahası mikrobiyolojisinin iyi anlaşılması çok önemlidir.

Bu çalışmada, sızıntı suyundaki baskın mikroorganizmaların incelenmesi amacıyla İstanbul Kemerburgaz Sıhhi Depo Sahası'nın farklı kısımlarından kaynaklanan ondört sızıntı suyu numunesi kullanılmıştır. Numunelere, kimyasal karakterizasyonun yanısıra, PCR (Polymerase Chain Reaction), DGGE (Denaturing Gradient Gel Electrophoresis), klonlama ve dizi analizi, ve FISH (Fluorescence In Situ Hybridization) gibi moleküler mikrobiyoloji teknikleri uygulanmıştır.

Sonuçlar, depo sahasının farklı stabilizasyon safhalarındaki kısımlarında bulunan mikrobiyal çeşitlilikler arasında önemli farklılıklar olduğunu ortaya koymuştur. Genç sızıntı sularında ölçülen yüksek UAKM konsantrasyonlarının yanısıra yüksek $BOİ_5$: KOİ ve TKN: NH_3-N oranları asit üretim safhasının göstergeleridir. Asetat kullanan *Methanosarcina*'nın asit üretim safhasına ait sızıntı sularında baskın tür olarak bulunduğu hem klonlama ve dizi analizi hem de FISH ile tespit edilmiştir. Çok düşük organik kirletici konsantrasyonlarına sahip yaşlı sızıntı sularında ise H_2 kullanan *Methanomicrobiales*'in baskın olduğu belirlenmiştir. Numunelerin tümünde gamma-*Proteobacteria*'nın en baskın bakteri grubu olduğu bulunmuştur. Bununla birlikte, genç ve yaşlı sızıntı suları arasında bakteriyel kompozisyon değişimi gözlenmemiştir.

Haziran, 2005 **Selçen DURMAZ**

ABBREVIATIONS

MSW : Municipal Solid Waste
BOD : Biochemical Oxygen Demand
COD : Chemical Oxygen Demand
SS : Suspended Solids
VSS : Volatile Suspended Solids
VFA : Volatile Fatty Acids
TKN : Total Kjeldahl Nitrogen
DGGE : Denaturing Gradient Gel Electrophoresis
FISH : Fluorescent In Situ Hybridization
PCR : Polymerase Chain Reaction

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Landfilling is one of the most common method for disposal of municipal solid wastes. In a landfill, like a bioreactor, enhanced microbiological processes take place to transform and stabilize the readily and moderately decomposable organic waste constituents within few years up to more than thirty years. Landfills have to be well-engineered facilities that are located, designed, operated, monitored, closed, cared for after closure, cleaned up when necessary, and financed to ensure compliance with regulations. A better understanding of landfill microbiology is very important to accelerate the stabilization of landfilled waste. As stabilization is accelerated, the requirement for leachate treatment, ambient air and groundwater monitoring reduces while generation rate and energy content of landfill gas increase significantly.

In Turkey, especially in crowded cities, the generation of municipal solid wastes has increased rapidly with increasing industrialization and urbanization after 1980s. Therefore, effective management of increasing amounts of these solid wastes has become a major social and environmental concern. The most important problem was that municipal solid waste was dumped mostly in uncontrolled dumping sites that were not properly regulated. In 2003, average municipal solid waste generation reached up to about 0.95 kg/capita-day in Turkey (Metin et al., 2003). Nevertheless appropriate removal processes such as landfilling and composting are not carried out sufficiently, yet. While the number of landfills especially in large cities and touristic regions is increasing, the amount of landfilling is still less than 50% in overall (Metin et al., 2003). Even though this proportion has been increasing in recent years,

the lack of sufficient landfills requires new initiatives to increase the number of landfills.

Istanbul, the most crowded metropolis in Turkey, had similar problems associated with the management of municipal solid wastes until the construction of two landfills in 1995 and the central composting plant in 2001. Currently, metropolitan municipality in Istanbul manage about 10,000 tons of solid waste generated from residential and commercial sources everyday containing about 60% biodegradable organic fraction (İSTAÇ, 2003).

In the literature, only very few studies are available on identification of landfill microorganisms because of sampling difficulties and problems in conventional cultivation dependent microbiological methods. For representative sampling, solid waste samples drilled from different points and depths of the landfill (Boon *et al*., 2000; Chen *et al*., 2003), leachate samples collected with perforated pipes (Huang *et al*., 2002; Boothe *et al*., 2001) and landfill gas condenses or filtrates (Kim, 2003) were used. Since landfill is a heterogeneous system, each sampling method has its own benefits and disadvantages. However, we believe that leachate samples are more likely to have a homogeneous content because of their formation by seeping through the layers of the solid waste within the landfill.

As in most of the environmental samples, only a small fraction of the microorganisms in the landfill is cultivable (Huang *et al*., 2002). Thus, culture independent molecular microbiology techniques are very important in investigation of landfill microorganisms.

In this study, to identify the prevalent microbial communities in municipal solid waste leachate, leachate samples were collected from Kemerburgaz-Odayeri Landfill. Leachate samples were taken from different collection pipes coming from different parts of the landfill at different stabilization phases. In this manner, samples were more representative. In identification of microorganisms in leachate samples 16S rRNA and *mcr*A gene based molecular microbiology techniques such as fluorescence in-situ hybridization (FISH), polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and cloning and sequencing were used.

CHAPTER II

GENERAL BACKGROUND

II.1 LANDFILLING: A METHOD FOR THE MANAGEMENT OF MUNICIPAL SOLID WASTE

II.1.1 Overview of sanitary landfills

Solid wastes arise from human and animal activities which are discarded as useless. The rapid population growth and the changes in consumption habits have caused a considerable increase in solid waste production and the amounts of refuse destined for final disposal rapidly consume landfill capacity. The final disposal of solid waste in cities, across the world, has become a serious environmental problem.

The production and management of municipal solid waste (MSW) can be thought to be a cycle with many very closely related stages. The cycle begins with the production of goods and continues with generation, storage, collection and final disposal of waste.

The composition of solid waste is a crucial element for defining waste management strategies. Knowledge on the composition of waste is essential for implementing the most appropriate waste reduction policies and for choosing the adequate waste treatment and disposal processes. There are many factors affecting the variability of MSW such as socio-economic conditions, weather, fuels combusted etc. Typically, landfilled wastes are dominated by organics comprising

of 50% cellulose, 15% lignin, 10 % hemicellulose, 5% protein as well as starch, pectin and other soluble sugars **(**Barlaz *et al.,*1992).

Sanitary landfilling is the preferred method of MSW disposal due to its favorable economics. Sanitary landfills are engineered land burial facilities for the disposal of solid waste which are located, designed, constructed, and operated to contain and isolate the waste so that it does not pose a substantial present or potential hazard to human health or the environment (Tchobanoglous *et al*., 1993). Prior to placement of waste in the ground, a site is typically excavated to increase the disposal volume-to-surface area ratio. The lowest component of a landfill is the liner system, which includes layers to minimize the migration of leachate to the groundwater and to collect leachate for treatment. Liners usually consist of layers of compacted clay and geomembrane material (Tchobanoglous *et al.,* 1993).

As solid waste is deposited in the landfill, waste is compacted and covered daily to minimize the attraction of rodents, wildlife, and disease carrying insects and contamination of storm water run-off. Once waste has reached the final design level, a final cover is applied. The goals of the final cover are to minimize rainfall and snowfall infiltration, to limit the uncontrolled release of landfill gases, to suppress the proliferation of disease vectors, to limit the risk of fire, and to provide suitable conditions for use of the site after landfill closure.

II.1.2. Stabilization Phases of a Landfill

Stabilization of organic solids in a landfill is dynamic, complex, biologically mediated process, which is primarily influenced by waste characteristics, availability of moisture and nutrients, and prevailing operation circumstances. When refuse is placed in a landfill, biological decomposition does not occur immediately. A period ranging from months to years is necessary for the proper growth conditions and the required microbiological system to become established.

Landfill investigation studies have shown that the stabilization of waste proceeds in five sequential and distinct phases (Pohland and Harper, 1985). Movement through the phases is reflected by significant changes in leachate and gas quality. (Fig.II.1)

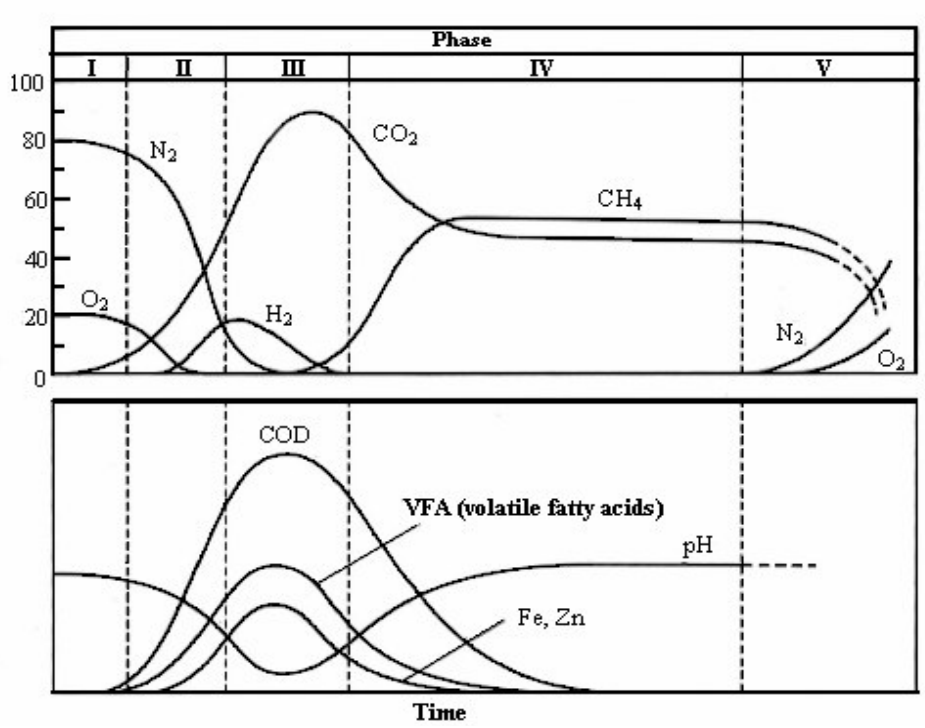


Figure II.1. Generalized Phases in the Generation of Landfill Gases (Pohland, 1991)

II.1.2.1. Phase I - Initial Adjustment Phase

Phase I is characterised by rapid breakdown of complex organics in the presence of water and oxygen into simple sugars – often termed hydrolysis. Some components, such as lignin, are only effectively broken down during this phase. This phase is characterised by the depletion of O_2 and N_2 due to the production of large quantities of CO_2. Since only a finite quantity of oxygen is buried within the waste, and there are limitations on air transport into the landfill, aerobic decomposition is responsible for only a small portion of biodegradation within the landfill (Lu *et al*., 1985; McBean *et al*., 1995). It is accompanied by a dramatic drop in redox potential and increase in leachate ionic strength. The large release of energy is also reflected by a rapid rise in landfill temperature.

II.1.2.2. Phase II - Transition Phase

In the transition phase, the field capacity is often exceeded, and a transformation from an aerobic to an anaerobic environment occurs, as evidenced by the depletion of oxygen trapped within the landfill media. A trend toward reducing conditions is established in accordance with shifting of electron acceptors from oxygen to nitrates and sulfates, and the displacement of oxygen by carbon dioxide.

By the end of this phase, measurable concentrations of COD and volatile organic acids (VOA) can be detected in the leachate.

II.1.2.3. Phase III - Acid Formation Phase

The continuous hydrolysis of solid waste, followed by the microbial conversion of biodegradable organic matter results in the production of intermediate VOAs, ammonia, hydrogen, and CO_2 at high concentrations throughout this phase. Acid phase anaerobic biodegradation processes are carried out by a mixed anaerobic population, composed of strict and facultative anaerobes (Lu *et al*., 1985). Facultative anaerobes aid in the breakdown of materials and reduce the redox potential so that methanogenic bacteria can grow. A decrease in pH values is often observed, and is accompanied by metal species mobilization resulting in a chemically aggressive leachate. The highest concentrations of BOD, COD, and specific conductance occur during the acid formation phase (McBean *et al*., 1995). Viable biomass growth associated with the acid formers (acidogenic bacteria), and rapid consumption of substrate and nutrients are the predominant features of this phase.

II.1.2.4. Phase IV - Methane Fermentation Phase

During Phase IV, intermediate acids are consumed by methane-forming consortia and converted into methane and carbon dioxide. Reducing conditions corresponding to this phase will influence the solubility of inorganics, resulting in precipitation or dissolution of these constituents. For example, sulfate and nitrate are reduced to sulfides and ammonia, respectively. COD and BOD concentrations decline since much of these materials are converted to gas (McBean *et al*., 1995). A small portion of the original refuse organic content (e.g. lignin-type aromatic compounds) is not degraded to any extent anaerobically and remains in the landfill material. The pH level consequently supports the growth of methanogenic archaea. Heavy metals are removed by complexation and precipitation. Methanogens work relatively slowly but efficiently over many years decomposing any remaining degradable organics.

II.1.2.5. Phase V - Maturation Phase

Once available organic matter is degraded CO_2 and CH_4 production ceases and air diffuses back into the landfill. Since available substrates become limiting the biological activity shifts to relative dormancy. However, the slow degradation of resistant organic fractions may continue with the production of humic-like substances.

In a landfill, like a bioreactor, enhanced physical, chemical, and biological processes take place to transform and stabilize the readily and moderately decomposable organic waste constituents within few years up to more than thirty years. Solid waste and water are the major inputs, landfill gas and leachate are the principal outputs of that bioreactor.

II.1.3 Landfill Gas

Landfill gas is a product of anaerobic decomposition of biodegradable organic waste. For any MSW landfill site, the gas produced will consist primarily of methane (CH_4) and carbon dioxide (CO_2). In theory, the microbial process of waste degradation should yield equal amounts of methane and carbon dioxide. However, a small proportion of carbon dioxide dissolves in aqueous solution, resulting in typical concentrations of around 50-55% methane and 45% carbon dioxide. The quantity and composition of landfill gas produced is site-specific and will depend on:

- the composition of the waste;
- the age of the landfill;
- site design and engineering (including presence and type of gas collection system);
- site operation and management;
- factors affecting the rates of aerobic and anaerobic degradation of biodegradable matter (e.g. moisture content and temperature).

These gases can have a wide range of effects, including impacts on public health and damage to ecosystems. In addition, both methane and carbon dioxide are greenhouse gases, with methane being far more potent in this respect.

Landfill sites produce a small but highly variable yield of other gases (e.g. hydrogen, oxygen, nitrogen, ammonia and hydrogen sulphide) (USEPA 1995, Tchobanoglous *et al.,* 1993). In addition there are up to 200 minor constituents

present in trace amounts, mainly organic compounds. The amounts of these minor constituents varies from site to site and with time. They depend on similar factors to the main gases (e.g. waste composition, biodegradation processes, etc.). The most important of these pollutants include:

- Heavy metals;
- Organic compounds, including; BTX (benzene, toluene, xylene) compounds, including some carcinogens; Poly-aromatic hydrocarbons (PAHs), including some carcinogens; Organo-sulphur compounds (notably mercaptans); Halocarbons (including chlorinated hydrocarbons).

Landfill gas has to be controlled to reduce atmospheric emissions, to minimize the release of odorous emissions, to minimize subsurface gas migration, and to allow for the recovery of energy from methane. Landfill gas is controlled either with a passive control system in which the the driving force is the pressure of the gas generated, or with an active control system that uses a partial vacuum to collect it. The captured gas is either flared off or piped to nearby facilities for use as fuel in heating buildings or generating electricity.

Typically, 200 – 300 m^3 of gas is produced per tonne of waste deposited (Swarbrick, G.E.,2001). The rate of gas production commonly peaks at about 5 – 15 years after closure and is negligible after about 40 – 60 years.

II.1.4 Landfill Leachate

Leachate is produced when moisture enters the refuse in a landfill, extracts contaminants into the liquid phase, and produces a moisture content sufficiently high to initiate liquid flow. Sources of moisture entering the landfill include liquid present in the refuse at placement, precipitation falling on refuse at placement and infiltrating after cover application, and intrusion of groundwater from outside into the landfill.

As water percolates through the landfill, contaminants are leached from the solid waste. Effective pollution control through the proper design of landfill and leachate management facilities requires an understanding of leachate quality.

II.1.4.1 Composition of Leachate

As a result of the broad spectrum of solid waste characteristics, leachate composition will vary tremendously. Leachate composition is primarily a function of the age of the landfill and the degree of waste stabilization. It contains organic and inorganic compounds and decomposition products, some of them arising from incomplete decomposition. The comparison between the typical leachate characteristics of young and mature landfills are shown below.(Table II.1)

Table II.1. Typical Leachate Characteristics of New and Mature Landfills (Tchobanoglous et al., 1993)

Constituent, mg/L [a]	New Landfill (less than 2 years)	Mature Landfill (greater than 10 years)
BOD_5	2,000 – 30,000	100 – 200
COD	3,000 – 60,000	80 – 160
Total suspended solids	200 – 2,000	100 – 400
Organic nitrogen	10 – 800	80 – 120
Ammonia nitrogen	10 – 800	20 – 40
Nitrate	5 – 40	5 – 10
Total phosphorus	5 – 100	5 – 10
Ortho phosphorus	4 – 80	4 – 8
Alkalinity	1,000 – 10,000	200 – 1,000
pH	4.5 – 7.5	6.6 – 7.5
Total hardness	300 – 10,000	200 – 500
Calcium	200 – 3,000	100 – 400
Magnesium	50 – 1,500	50 – 200
Potassium	200 – 1,000	50 – 400
Sodium	200 – 2,500	100 – 200
Chloride	200 – 3,000	100 – 400
Sulfate	50 – 1,000	20 – 50
Total iron	50 – 1,200	20 – 200

[a] Except pH which has no unit

Since the BOD test is predominately a biological test, it generally reflects the biodegradability of the organic matter in leachate. The BOD to COD ratio is an indicator of the proportion of biologically degradable organic matter to total organic matter and it decreases as the landfill ages and more degradation products are leached from deposited residues (Copa *et al*., 1995; Westlake, 1995).

The class of organic compounds found at highest concentration in leachates is generally volatile fatty acids (e.g. acetic, propionic, and butyric) produced during the decomposition of lipids, proteins, and carbohydrates (Swarbrick, 2001). Aromatic hydrocarbons, including benzene, various xylenes, and toluene, are also

frequently found at lower concentrations. Oman and Hynning (1993) observed that a total of 150 different organic compounds have been identified in multiple studies, however only 29 were identified in more than one. They therefore concluded that leachate composition was quite site specific.

A variety of heavy metals are frequently found in landfill leachates including zinc, copper, cadmium, lead, nickel, chromium, and mercury (Lu, *et al*, 1985). These metals are either soluble components of the refuse or are products of physical processes such as corrosion and complexation. Heavy metal release is a function of characteristics of the leachate such as pH, flow rate, and the concentration of complexing agents. Metal solubilities generally decrease with increasing pH.

II.1.4.2 Factors Affecting Leachate Quality

Chemical composition of the leachate is highly variable depending mainly on the age of landfill. The variation in leachate quality can be attributed to many interacting factors such as the composition and depth of waste, the availability of moisture and oxygen, landfill design and operation, and waste age.

1.a Waste Composition

Municipal waste has great variation in composition and characteristics. The waste composition of refuse determines the extent of biological activity within the landfill. Rubbish, food and garden wastes, and crop and animal residues contribute to the organic material in leachate (Pohland and Harper, 1985). Inorganic constituents in leachate are often derived from ash wastes and construction and demolition debris (Pohland and Harper, 1985). Chen and Bowerman (1974) found that increased quantities of paper in solid waste resulted in a decreased rate of waste decomposition. Lignin, the primary component of paper, is resistant to anaerobic decomposition which is the primary means of degradation in landfills.

Codisposal of municipal solid waste and sludge from municipal wastewater treatment plants (WWTP) can have a significant impact on leachate quality. Codisposal can accelerate leachate formation and the rate of biological stabilization through the addition of moisture, microbes, and nutrients provided by the sludge (Lu *et al*., 1985). Codisposal of ash with municipal solid waste may provide a means to attenuate the toxic species within the ash (Westlake, 1995). Poon and Chu (1990) found that leachate from codisposal sites contained more chemical oxygen demand

(COD), sulfide, and low molecular weight organic acids (acetate and formate). The greater amounts of acetate and formate generated were expected to depress pH and enhance metal mobility. However, sulfide would tend to immobilize metals due to the low solubility of most metal sulfides. Poon and Chu (1990) also determined that codisposal generated more lead and cadmium in the particulate form.

1.b Moisture

Water is the most significant factor influencing waste stabilization and leachate quality. Moisture within the landfill serves as a reactant in the hydrolysis reactions, transports nutrients and enzymes, dissolves metabolites, provides pH buffering, dilutes inhibitory compounds, exposes surface area to microbial attack, and controls microbial cell swelling (Noble and Arnold, 1991). Lu *et al*. (1985) stated that high moisture flow rates can flush soluble organics and microbial cells out of the landfill and in such cases microbial activity plays a lesser role in determining leachate quality. Also, high moisture application rates can remove the majority of waste contaminants early in the life of the fill. Under low flow rate conditions, anaerobic microbial activity is the significant factor governing leachate organic strength (McBean *et al*., 1995). The quantity of moisture is important because it directly affects stabilization rates within the landfill. Sulfita *et al*. (1992) and Miller *et al*. (1994) both noted the important role of moisture in supporting methanogenic fermentation of solid waste when examining samples removed from operating landfills. Relatively dry landfills (i.e. 20 to 40 percent water) have very slow stabilization rates because there is only a small quantity of moisture to support biological degradation. Recommended moisture content reported in the literature ranges from a minimum of 25 percent (wet basis) to optimum levels of 40 to 70 percent (Barlaz *et al*., 1990; Chen and Bowerman, 1974).

1.c Oxygen

The quantity of free oxygen in a landfill indicates the type of decomposition (i.e. anaerobic or aerobic). Aerobic decomposition occurs during initial placement of waste, while oxygen is available. Aerobic degradation may continue to occur at, and just below, the surface of the fill (McBean *et al*., 1995). Aerobic decomposition products differ greatly from those produced during anaerobic degradation (Bagchi, 1990). During aerobic decomposition, microorganisms degrade organic matter to CO_2, H_2O, and partially degraded residual organics, producing considerable heat.

High concentrations of organic acids, ammonia, hydrogen, carbon dioxide, methane, and water are produced during anaerobic degradation (McBean *et al*., 1995). Phase changes occur in a landfill as a result of reductions in the quantity of oxygen in the landfill. For example, a transitional change takes place when oxygen is depleted and an anaerobic environment develops.

1.d Temperature

Landfill temperature is an uncontrollable factor influencing leachate quality. It has been shown to fluctuate with seasonal ambient temperature variations (Lu *et al*., 1985). Temperature affects bacterial growth and chemical reactions within the landfill. Each microorganism possesses an optimum growth temperature, and any deviation from that temperature will decrease growth due to enzyme deactivation and cell wall rupture. Solubility of many salts (e.g. $Ca_3(PO_4)_2$ and NaCl) increases with temperature. However, a number of compounds in leachate, such as $CaCO_3$ and $CaSO_4$, show a decrease in solubility with increasing temperature (Lu *et al*., 1985).

1.e Landfill Age

Leachate quality is greatly influenced by the length of time which has elapsed since waste placement. The quantity of chemicals in the waste is finite and, therefore, leachate quality reaches a peak after approximately two to three years followed by a gradual decline in ensuing years (McBean et al., 1995; Lu et al., 1985). Generally, leachate from new landfills will be high in BOD and COD and will then steadily decline, leveling off after about 10 years (Akyurek, 1995). All contaminants do not peak at the same time. Due to their initially biodegradable nature, organic compounds decrease more rapidly than inorganics with increasing age of the landfill. Inorganics are only removed as a result of washout by infiltrating rainwater (Qasim and Chiang, 1994). Organic compounds, however, decrease in concentration through decomposition as well as washout.

II.2. SOLID WASTE MANAGEMENT IN TURKEY

The majority of the household waste in Turkey is organic in nature. Although natural gas has been becoming the major source of energy used for household heating in big cities slag and ash still constitute an important fraction. Organic components can be assumed to be 50–55%, whereas recyclable and others (ash and slag, dust etc.) can be assumed to be 20–25% (Metin et al., 2003).

Tablo II.2 Municipal Solid Waste Composition in Major Cities of Turkey, (%, in weight)

	İstanbul	**Bursa**	**İzmir**	**Adana**
Organic	43	53.1	46	64.4
Recyclable	33.9	36.4	31	25.2
Paper/board	7.8	18.4	12	14.8
Plastics	14.2	11.6	12	5.92
Metal	5.8	3	3	1.4
Glass	6.2	3.4	4	3.08
Others	23.1	10.5	23	11.4

In Turkey, 81 provinces have a total of 3215 municipalities, 16 of which are metropolitan municipalities. Generally, 33% of wastes are disposed in 13 sanitary landfills, 1% is being composted in 3 composting plants and the rest is being disposed using non-conventional methods like dumping, burning etc. (Fig.II.2).

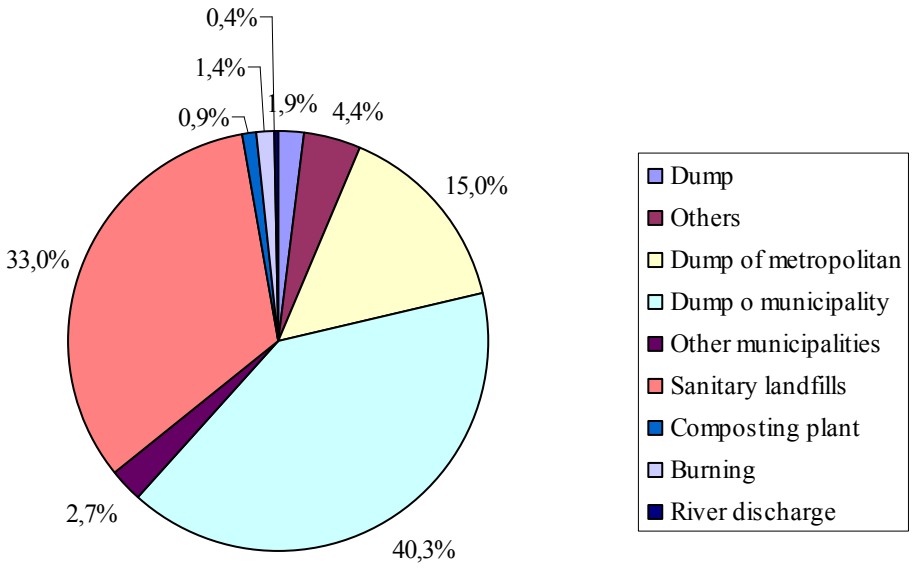


Figure II.2 Distribution of Solid Waste Management Applications in Turkey (SIS, 2001)

II.3 MICROBIOLOGY OF LANDFILL

Landfills are extremely heterogenous as a result of the nature of the waste buried in. The development and growth of bacterial populations within landfilled waste is a very complex and dynamic phenomenon. The bacteria that flourish in this environment are naturally occurring aerobes and anaerobes and begin their degradation process as soon as waste is disposed.

The major objective of most biological conversion processes is the conversion of the organic matter to a stable end product. The organic fraction of MSW typically contains adequate amounts of nutrients (both organic and inorganic) to support the biological conversion of the waste. In a landfill, the majority of organisms are chemoorganotrophs which use organic compounds and chemical pathways to generate energy (Blackall and Silvey, 1994). Since redox reactions tend to produce more energy than other types of reactions, microorganisms, which utilize these reactions, flourish within the landfill. As energy is released microorganisms utilize some of this energy internally in the form of adenosine tri-phosphate (ATP), an intracellular compound used for further metabolism and reproduction. Like all chemical reactions, microorganisms are bound by the principles of thermodynamics.

This, and the fact that they derive their energy from chemical reactions, is strongly reflected in typical landfill degradation behavior (Swarbrick, 2001).

Initially the reactions that release the highest amounts of energy are favored. Initially oxidation by oxygen, followed by oxidation by nitrate, manganese, iron & sulfate and then methanogenesis by fermentation (Fig.II.3). The energy released by methanogenesis is only slightly more than that required for the formation of ATP. Consequently growth of methanogens is very slow compared to other microorganisms utilising more energetically favourable reactions.

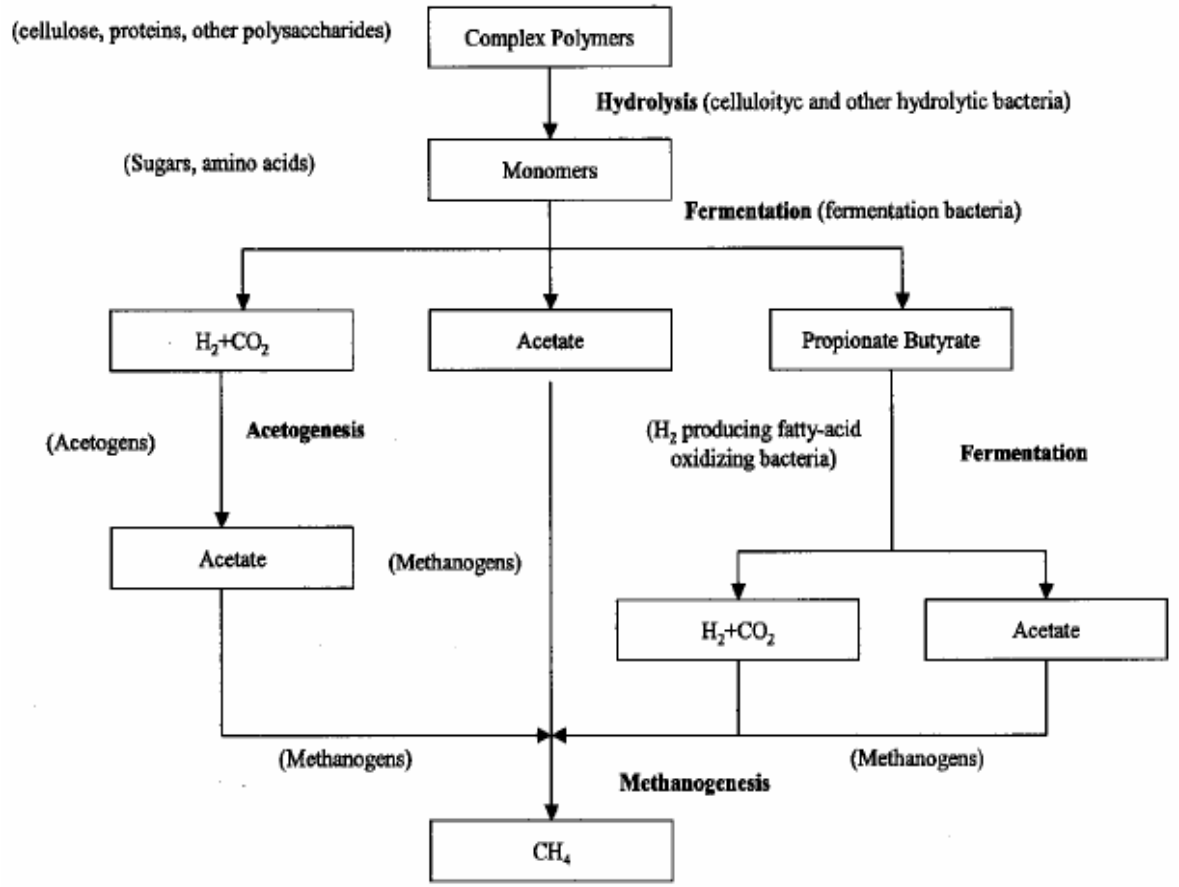


Figure II.3 – Overall Process of Anaerobic Decomposition (Barlaz et al., 1996)

Hydrolysis is an important process through which the solid and complex dissolved organic matters are broken down into smaller, soluble components required for subsequent microbial conversions (e.g. carbohydrates into simple sugars, proteins into amino acids and lipids into glycerol and long chain fatty acids). The extra-cellular enzymes produced by the fermentative bacteria promote hydrolysis.

Acid fermentation process involves the fermentation of insoluble long chain sugars to long chain fatty acids like propionate, butyrate, valerate etc. and alcohols

resulting in a high concentration of VFAs. Acidogenic bacteria are responsible from that process.

Acetogenesis is achieved by the acetogenic group of bacteria which converts the longer chain volatile fatty acids (propionate, butyrate, isobutyrate, valerate, isovalerate and caproate) and alcohols into acetate (the shortest chain fatty acid), hydrogen, and carbon dioxide.

In methanogenesis, subsequently methane is produced by the methanogenic archaea. There are two main groups of methanogens those that convert acetate directly to methane and those that combine hydrogen and carbon dioxide. The methanogens that consume acetate are termed *acetophilic,* those that metabolise hydrogen are termed *hydrogenophilic.* Both groups are strictly-anaerobic and require a condition of low redox potential.

II.3.1. Bacterial Diversity In Landfills

Bacteria population in landfill environment mostly consist of fermentative and acetogenic bacteria. Fermentative bacteria perform hydrolysis and organic acid fermentation. They are a large heterogeneous group of strictly-anaerobic and facultative-anaerobic bacteria. Acetogenic bacteria are also heterogeneous bacteria that convert the products derived from the above fermentation into acetic acid.

Chen et al. (2003b) developed a modified Fluorescence In Situ Hybridization (FISH) technique to count numbers of bacteria and archaea directly from the soil samples of a landfill site. Bacterial cells accounted for 37 and 62% of total cells in 1 and 3 m depth buried samples, respectively. In contrast, direct counting indicated that archaeal cells accounted for only 1 and 0.3% of total cells at 1 and 3 m depth samples, respectively.

Sulfate reducing bacteria (SRB) are a diverse group of anaerobic bacteria that have the ability to use sulfate as a terminal electron acceptor in the consumption of organic matter, with the concomitant production of H_2S. In landfills, while the key terminal process is methanogenesis, SRB can compete with methanogenic bacteria for available electron donors such as acetate and H_2, and have the potential to inhibit the methanogenic decomposition of waste organic matter, resulting in the increased production of H_2S.

Methanotrophic bacteria are a physiologically unique group of microorganisms distinguished by their ability to use methane as sole carbon and energy source. Landfills produce large amounts of CH_4 due to the methanogenic activity under anaerobic conditions. Very high rates of CH_4 oxidation and large methanotrophic populations have been reported in the oxic portion of landfill cover soils (Jones et al., 2003; Whalen et al., 1990). Chen et al. (2003b) evaluated the methane oxidizing and producing activities of cover soil (10, 30 cm depth) and burial waste (1,3 m depth). The study revealed that top cover soil (10 cm) had the highest methane oxidizing activity while 1 m depth buried waste showed the highest methane producing potential. In another study, members of the genera *Methylocystis* and *Methylocaldum* were found to be the dominant members in landfill simulating lysimeters (Stralis-Pavese et al., 2004).

Huang et al. (2005) performed a study on the leachate of a closed MSW landfill to determine the bacterial constituents. According to the phylogenetic analysis, *low G + C gram-positive bacteria* (38.4%), *Proteobacteria* (35.5%) and *Cytophaga Flexibacter Bacteroides* (11.6%) were the dominating groupes. They also gathered similar results with another study about the leachate of a full-scale recirculating landfill (Huang et al., 2004). This time, low G + C gram-positive bacteria (53%), *Chlamydiae/Verrucomicrobia* (21%) and *Cytophaga Flexibacter Bacteroides* (19%) were the most abundant groups. Low G + C group is related to the genus *Clostridium* which is assumed to be responsible for the anaerobic degradation of cellulose. However, most of the bacterial sequence types were not closely related to known cultivated species, indicating that the majority of bacterial species associated with the landfill leachate remained to be identified.

In the study of Van Dyke and McCarthy (2002), primer sets specific for 16S rRNA genes were designed for four phylogenetic groups of clostridia known to contain mesophilic cellulolytic species. Specific amplification of these groups from landfill leachate DNA extracts demonstrated the widespread occurrence of clostridia from the *Clostridium thermocellum* and *C. leptum* groups. Burrell et al. (2004) operated an anaerobic landfill leachate bioreactor in fed-batch mode to identify microbial consortium responsible for cellulose hydrolysis. The findings were consistent with the previous investigations. The majority of the clones fell into one of five lineages of clostridia.

There is a unique research conducted by Kim (2003) in the literature which includes the investigation of microbial content of the landfill gas and gas

condensate. The study revealed that bacteria dominated in the landfill populations. The relative abundance of bacteria found by hybridization was approximately 86-87%.

II.3.2. Archaeal Diversity In Landfills

The anaerobic microorganisms capable of methanogenesis belong to the kingdom Euryarchaeota in the domain of Archaea. They are characterized by their ability to produce methane under anaerobic conditions. This particular metabolism has provided the methanogens with a unique set of enzymatic pathways, as well as certain coenzymes and cofactors that are not found in other organisms (Daniels, 1993). The methanogens are responsible for the final step of the anaerobic degradation of organic matter. Their natural habitats are anaerobic niches, such as intestines of ruminants, the human digestive system, rice paddies, lake or marine sediments, hot pools and salt ponds, sewage digestors and landfills. The methanogens are found at temperatures from 20°C and up to as much as 98°C (Sowers, 1995; Boone *et al*., 1993). Phylogenetically the methanogens are divided into five orders, the *Methanobacteriales*, the *Methanococcales*, the *Methanosarcinales*, the *Methanopyrales*, and the *Methanomicrobiales*.

When it concernes methanogenesis, there are two groups of methanogens: those that convert acetate directly to methane and those that combine hydrogen and carbon dioxide.

The methanogens that metabolise hydrogen are termed hydrogenophilic and do so according to:

$$4\,H_2 + CO_2 \Longrightarrow CH_4 + 2\,H_2O$$

$$HCOOH + 3\,H_2 \Longrightarrow CH_4 + 2\,H_2O$$

Hydrogenophilic methanogens produce slightly more energy thermodynamically and are therefore more favorable when hydrogen is available. They include members of the order *Methanomicrobiales.*

The other main group of methanogens that generate acetate are termed acetophilic and do so by facilitating reactions similar to:

$$CH_3COOH \Longrightarrow CH_4 + CO_2$$

This reaction releases relatively small amounts of free energy. This is just enough to sustain cell growth and the increase in this group of methanogens is accordingly slow. 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).

There are various researches on the characterization of archaeal namely methanogenic diversity in landfills or landfill simulating reactors. Methanogenic microorganisms play an important role in the degradation of solid waste in landfills (Christensen et al., 2001). However, little information is available on methanogen distribution in landfill sites because the conventional methods for enumerating these organisms require long cultivation times (Grosskopf et al., 1998a). Limitations of the traditional cultivation-based techniques have restricted our knowledge of the microbiology in landfills. In recent years, molecular techniques based on 16S rRNA gene sequence (rDNA) have been widely used to elucidate such microbial features (Amann et al., 1995), providing a powerful tool for advancing our understanding of landfill microbial ecology.

Qian and Barlaz (1996) enumerated the acetate and $H_2 - CO_2$ utilizing methanogenic archaea present on grass, leaves, branches, food waste, whole waste and two landfill cover soils by the most probable number (MPN) technique. The highest methanogen populations were measured in leaves (6.3×10^3) and one of two fresh refuse samples (10^5) indicating that the yard waste is an important source for methanogens as well as diapers and pet wastes.

Huang et al. conducted two different studies for the characterization methanogenic archaea in the leachate of a closed municipal solid waste landfill (2003) and a full-scale recirculating landfill (2002). In both studies cultivation-independent molecular approaches were the tools. The sequencing and phylogenetic analysis results of the recirculating landfill leachate samples showed clone distribution as follows: *Methanoculleus* (68.6%), *Methanosarcina* (15.7%), *Methanocorpusculum* (4.3%), *Methanospirillum* (1.4%) and *Methnogenium* (1.4%). The leachate of closed landfill had the similar results except the dominant hydrogenotrophic methanogen was *Methanomicrobiales* (35.1%) instead of *Methanoculleus*. *Methanosarcinales* and *Methanobacteriales* were the followers with the percentages 29.8% and 1.3%, respectively. The findings of those studies are in contradiction with Mori, *et al.* (2003)'s suggestions which includes the majority of methane emitted from the landfill site originated from the acetate utilizing *Methanosaeta*.

Chen et al. (2003a) and Chen et al. (2003b) investigated the archaeal community within the old and young age landfill samples respectively. Chen et al. (2003b) has concluded that H_2-utilizing methanogens were the dominant archaeal population in the young age landfill sample, which is similar to the results obtained in the old age sample. The members of the genus *Methanothermobacter* predominated in archaeal populations in the old age landfill samples. However, members of the genus *Methanosarcina* seemed to predominate in the young age sample, was minor constituent in the old age sample. These observations suggest that major methanogenic archaeal members could be significantly different in young age and old age samples, as well as in different landfills in different locations.

Methanogenic Archaea may be studied more specifically using a characteristic `functional' marker gene. A specific marker for methanogens is the methyl coenzyme-M reductase (MCR), the key enzyme of methanogenesis (Ermler et al., 1997). Unlike other enzymes in methanogenic metabolism (Chistoserdova et al., 1998), MCR appears to be unique to methanogens (Thauer, 1998). MCR catalyses the reduction of methyl-coenzyme M, which leads to the release of methane (Ellermann et al., 1988). The *mcrA* gene is highly conserved (Lehmacher and Klenk, 1994; Springer et al., 1995; Nölling et al., 1996), which renders it suitable for use in molecular ecology studies. Environmental, PCR-amplified *mcrA* sequences have been retrieved from a variety of different habitats, such as termite guts (Ohkuma et al., 1995), peat bogs (Lloyd et al., 1998; Nercessian et al., 1999), rice field soil

(Lueders et al., 2001) and marine sediments (Bidle et al., 1999), specifically to detect methanogens and study their diversity. There is a study on the phylogenetic analysis of methanogen populations with aid of mcrA gene in a landfill conducted by Luton et al. (2002). Analysis revealed a far greater diversity in the methanogen population within landfill material than has been seen previously.

II.4 IDENTIFICATION OF LANDFILL MICROORGANISMS

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't be cultured are often difficult to identify. Environmenatal 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). 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 of low growth rates, unknown growth requirements and obligate anaerobiosis.

Since cultivation dependent methods have many limitations, culture independent methods, such as rRNA based molecular techniques, have become important tools for examination 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. 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.

In the present study, PCR-DGGE technique, hybridization with oligonucleotide probes, cloning and sequencing were used to describe the prevalent microbial communities in the landfill leachate. The details of each technique are described next.

II.4.1 Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) was invented by Kary Mullis in 1983. This invention was awarded with a Nobel Prize for Chemistry in 1993. The basic principle of PCR is to amplify a specific region on DNA enzymeticaly through a repetitive process. The region to be amplified on DNA is specified by primers which are single stranded short chains of nucleotides that will, under the right conditions, bind to a specific complementary sequence of nucleotides in single-stranded DNA.

There are three basic steps in PCR. First, the target genetic material must be denatured-that is, the strands of its helix must be unwound and separated-by heating to 90-96°C. The second step is hybridization or annealing, in which the primers bind to their complementary bases on the single-stranded DNA. The third is DNA synthesis by a polymerase. Starting from the primer, the polymerase can read a template strand and match it with complementary nucleotides very quickly. The result is two new helixes in place of the first, each composed of one of the original strands plus its newly assembled complementary strand. This process is repeated many times (usually 20 –30 cycles) resulting in an exponential increase in the amount of target DNA.

Rapid automated PCR has been the key to the extraordinary upsurge in its applications throughout the life sciences. And the key to the process's automation has been *Taq* polymerase. *Taq* is a short name for *Thermus aquaticus*, a bacterium that survives and reproduces in an environment that is lethal to other organisms: hot springs. Unlike other polymerases, the enzyme extracted from *Taq* (and made in commercial quantities by genetically engineered bacteria) is stable at high temperatures.

II.4.2 Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a genetic fingerprinting technique that is used to separate individual sequences from a complex mixture. DNA sequences with differing base composition have different melting properties when passed through an acrylamide gel containing an increasing gradient of chemical or thermal denaturant. The melting temperature of a double stranded DNA fragment is influenced by hydrogen bonds formed between complementary base pairs and also by the attraction between neighbouring bases on the same strand (known as stacking interactions). The order

of bases on a strand determines the degree of stacking. A DNA molecule may therefore have several melting domains with characteristic melting temperatures (Tm) determined by the nucleotide sequence. Changes in base sequence as small as a single base may alter the stacking significantly enough to modify the Tm by over 1°C. When separated by electrophoresis through a gradient of increasing temperature (TGGE) or chemical denaturant (DGGE — usually formamide and urea), the mobility of the molecule is retarded at the concentration at which the DNA strands dissociate, forming a partially single stranded molecule with no further movement in the gel. Complete denaturation is prevented by the presence of a high melting domain, which is usually artificially created at one end of the molecule by incorporation of a GC clamp. This is produced by using a primer with a 5' tail consisting of a GC rich sequence of around 30-50 base pairs in the PCR. In principle, this means that DNA fragments of the same length are separated on the basis of differing sequences, even if only by a single base. The separation of the DNA fragments is then visualised by staining. The most sensitive method of detection is silver staining, however this prevents any further analysis of bands by techniques such as sequencing or hybridisation analysis. Alternative staining methods are ethidium bromide or SYBR green.

As with all PCRbased analyses, DGGE is not without its limitations. In general, for optimal resolution the fragment size of the PCR product generated prior to DGGE analysis is limited to a maximum of around 500bp. This means that definitive species identification from databases may not be possible. Difficulties have also been reported in attempting to resolve fragments that differ by only two or three bases, yet co-migration of non-related sequences has also been reported. Microheterogeneity in rRNA encoding genes present in some species must also be taken into consideration where multiple bands for a single species can subsequently lead to an overestimation of community diversity (Muyzer & Smalla, 1998).

One of the main advantages of DGGE is that it allows the simultaneous analysis of multiple samples, making it feasible to monitor shifts in populations over periods of time or different environmental conditions. Before the arrival of DGGE this was more commonly achieved by cloning and sequencing - an approach that is both labour intensive and relatively costly, especially when dealing with numerous samples. The DGGE method was first used to profile communities of a microbial mat and bacterial biofilms (Muyzer et al.., 1993) and since then has been used to

analyse microbial communities from extremely diverse microbial environments too numerous to mention.

II.4.3 Fluorescent In Situ Hybridization

Fluorescence in situ hybridisation (FISH) with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments. Hybridization techniques rely on the specific binding of nucleic acid probes to such signature regions on the rRNA. By definition these probes are single strands of nucleic acids which have the potential of carrying detectable marker molecules highly specifically to complementary target sequences. There are several reasons which make rRNA targeted oligonucleotides particularly suitable for the in situ identification of microorganisms. Firstly, rRNA molecules occur in high copy numbers of usually more than 1,000 in any living cell. Secondly, rRNA sequences can be today routinely retrieved from the samples of interest without prior cultivation. Thirdly a directed probe design, considering a priori nontarget organisms, is possible due to the huge sequence collections available especially for the 16S rRNA.

The in situ hybridization procedure, shortly, the permeabilization of microorganisms is a prerequisite for successful in situ hybridization with oligonucleotide probes. This is, in most cases, achieved by fixation of the sample with paraformaldehyde (for gram-negative bacteria) or ethanol (for gram-positive bacteria). Samples are then immobilized on microscobic slides coated with gelatin (or alternatively with poly-L-lysine) to improve adhesion to glass surface, and dehyrated in an ethanol dilution series. Hybridization is done in a moisture chamber at an appropriate hybridization temperature by convering the slides with hybridization solution containing the fluorescently labeled probes. After a stringent washing step to remove unbound or unspecifically bound probes the slides are air dried, mounted in antibleaching buffer, and examined with an epifluorescence microscope. Using suitable filter sets individual bacterial cells can be identified and their morphology, spatial distribution, and number can be determined.

II.4.4 Cloning and Sequencing

The terms "recombinant DNA technology," "DNA cloning," "molecular cloning,"or "gene cloning" all refer to the same process: the transfer of a DNA fragment of interest from one organism to a self-replicating genetic element such as a bacterial plasmid. The DNA of interest can then be propagated in a foreign host cell. This technology has been around since the 1970s, and it has become a common practice in molecular biology labs today.

Scientists studying a particular gene often use bacterial plasmids to generate multiple copies of the same gene. Plasmids are self-replicating extra-chromosomal circular DNA molecules, distinct from the normal bacterial genome. Plasmids and other types of cloning vectors are used by many researchers to copy genes and other pieces of chromosomes to generate enough identical material for further study.

To "clone a gene," a DNA fragment containing the gene of interest is isolated from chromosomal DNA using restriction enzymes and then united with a plasmid that has been cut with the same restriction enzymes. When the fragment of chromosomal DNA is joined with its cloning vector in the lab, it is called a "recombinant DNA molecule." Following introduction into suitable host cells, the recombinant DNA can then be reproduced along with the host cell DNA.

Plasmids can carry up to 20,000 bp of foreign DNA. Besides bacterial plasmids, some other cloning vectors include viruses, bacteria artificial chromosomes (BACs), and yeast artificial chromosomes (YACs). Bacteria (e.g *E.coli*) are most often used as the host cells for recombinant DNA molecules, but yeast and mammalian cells also are used.

Cloning and sequencing techniques can identify the origin of the DGGE-bands. Microbial species can be classified with sequencing techniques. A DGGE-gel with environmental samples often contains many bands in one lane. To find out which band is corresponding to which species, cloning and sequencing techniques are used. The first step is the ligation of the purified PCR product into a vector. Then the plasmid is built into *E.coli* JM 109 high efficiency competent cells. This process is called transformation. To get pure cultures the transformed cells are plated on selective plates and placed in an incubator overnight. To identify the different clones a restriction enzyme is used. This is called screening. The different

clones can be found with PCR-DGGE techniques as well. The identification of the bacterial and archaeal species is done by sequencing and blast search.

CHAPTER III

MATERIALS & METHODS

III.1. MUNICIPAL SOLID WASTE LANDFILLS

III.1.1. Sampling Site: Istanbul Kemerburgaz Landfill

Istanbul is the biggest metropolitan in Turkey with a population over 10 million and it has to manage 10,000 tons of solid waste everyday. The composition of the solid waste changes with seasons. 42.97% organics, 7.76% paper, 4.8% plastic, 9.37% nylon, 6.2% glass, 5.8% metal, and 9.28% ash constitute the average values (ISTAC, 2003).

Kemerburgaz Municipal Sanitary Landfill was constructed in the European Side of the city and has been under operation since 1995. It has an area of 25 ha and 6000 tons/day storage capacity.

In this study leachate samples were collected from five different leachate collection pipes which come from different parts of the Kemerburgaz landfill (Fig.III.1). 3 groups of samples are present. First group denoted by K was collected in 2003 and used in the MS thesis of Emine Girgin (2004), second group denoted by YK collected in December, 2004, and the last group (YYK) was collected in March, 2005. The sections, where leachate collection pipes belong to, are written below:

Pipe-1 - Landfill section has been used since 2003;

Pipe-2 - Lanfill section used between 2000-2002

Pipe-3, 5- Landfill section used between 2002-2003

Pipe-4 - Landfill section used between 1995-2000



Fig.III.1 Sampling Site in Kemerburgaz Sanitary Landfill

III.2. CHEMICAL CHARACTERIZATION OF LEACHATE SAMPLES

For the chemical characterization of the landfill leachate samples was the determination of chemical oxygen demand (COD), 5-days biochemical oxygen demand (BOD_5), pH, alkalinity, ammonia-nitrogen (NH_3-N), total kjeldahl nitrogen (TKN), suspended solids (SS), volatile suspended solids (VSS), conductivity, color, and chloride (Cl^-). All analyses were carried out according to the Standard Methods (APHA , 1998). The analytical methods are given in Table III.1

Table III.1. Parameters and Methods Used to Determine the Characteristics of Leachate

Parameter	Method	Reference
pH	Electrode	Standard Methods 4500-H B
COD	Closed reflux - Calorimetric	Standard Methods 5220 D
BOD	5 Day BOD test	Standard Methods 5210 B
NH_3-N	Nesslerization	Standard Methods 4500-NH_3 C
TKN	Dissociation + nesslerization	HACH method 8075
Alkalinity	Titrimetric	Standard Methods 2320 B
SS	Filtration + evaporization	Standard Methods 2540 D
VSS	Filtration + burning	Standard Methods 2540 E
Color	Spectrophotometric	HACH method 8025
Cl^-	Argentometric Method-1	Standard Methods 4500-Cl^- B

III.3. MICROBIAL COMMUNITY ANALYSIS OF LEACHATE SAMPLES

III.3.1 Sample Preparation

Initially, leachate samples were concentrated by centrifuging for half an hour at 7,000 rpm. DNA extractions and fixation part of the FISH experiment were performed immediately after centrifugation. Extracted DNA samples and fixed cells were stored at - 20 °C.

As soon as the samples became ready for analysis, DNA extraction, PCR amplification, DGGE, cloning and sequencing and FISH analysis were employed to find the prevailent microorganisms in the Kemerburgaz Landfill leachate. The summary of how these analysis are connected to each other is given in Figure.III.2.

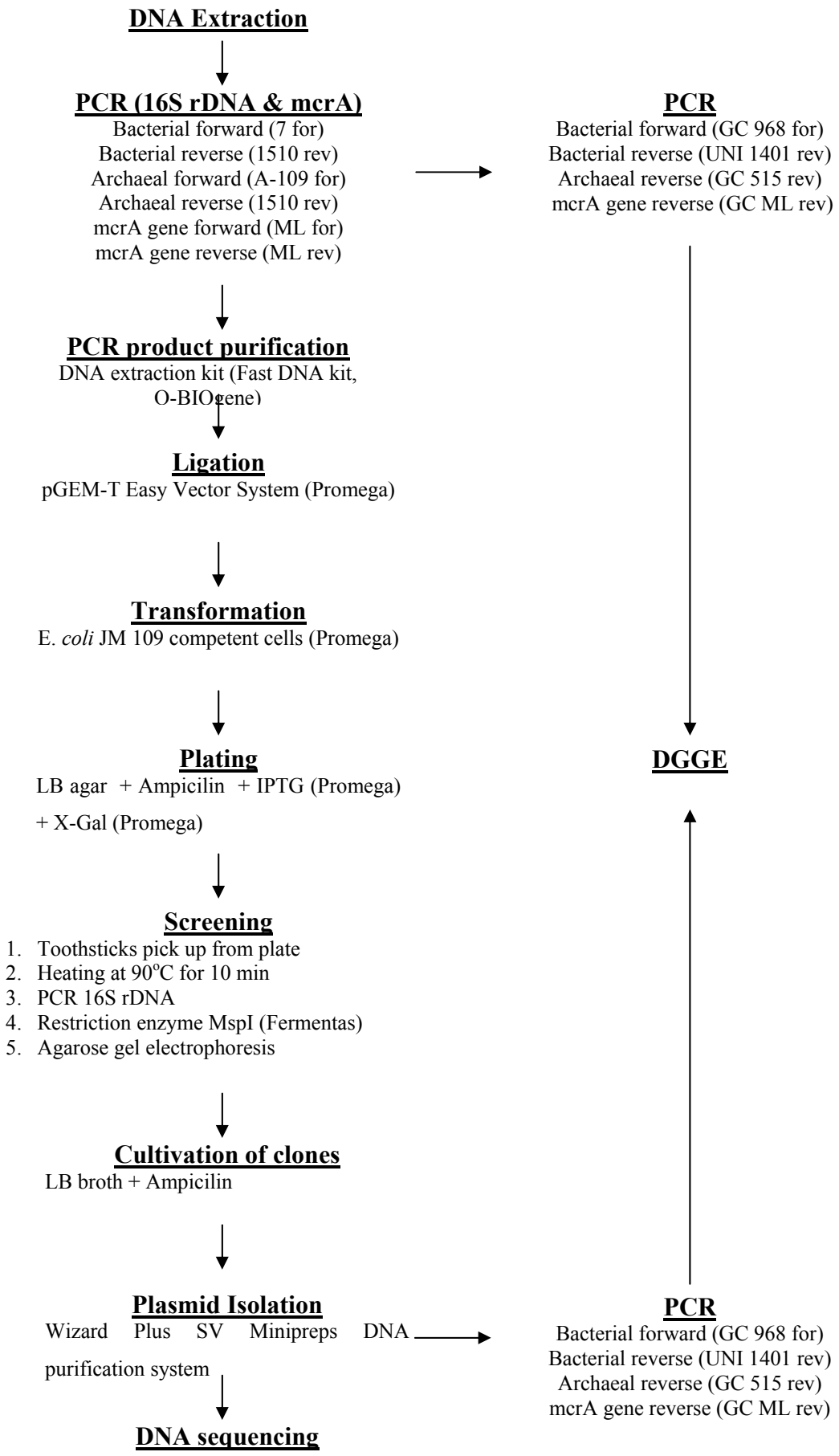


Figure.III.2 Summary of the Analyses Used in This Study

III.3.2. DNA Extraction

There are quite a lot of DNA isolation protocols varying from one research to another. That's because of the differences in the nature of the samples used. However, all those protocols share the same configuration which involves lysis of the cells either by chemically or mechanically to release the DNA and isolation and collection of DNA in water or a proper buffer.

It is necessary to adapt the DNA isolation protocol to the specific samples. Therefore, we have found the following procedure to give superior results with our leachate samples.

- 1 mL of concentrated sludge sample is taken into a 2 mL microcentrifuge tube,
- 0.3 mL of phenol, 0.2 mL of TE buffer (at pH = 8.0) and 2 spoon full of 0.1 mm sized zirconia beads are added to the sample tube,
- The tube is beat-beaten for 10 sec at 2500 rpm using Mini Bead-beater-8 (Biospec Products),
- As soon as beat-beating is over sample containing tube is centrifuged at 14,000 rpm for 10 min,
- 0.6 mL of supernatant is taken into a clean 1.5 mL microcentrifuge tube and gently mixed with 0.6 mL of Binding matrix of Fast DNA SPIN kit, allowed to settle for 5 min at room temperature,
- The mixture is centrifuged for 1 min and the supernatant is discarded,
- 0.5 mL of SEWS-M Solution (Salt Ethanol Wash Solution – Fast DNA SPIN kit) is added and mixed gently,
- After 1 min centifugation at 14,000 rpm, the supernatant is removed,
- For further removal from ethanol the tube is centrifuged for additional 10 sec and the supernatant is discarded with the help of a pipet,
- As the final step, 100 µL of DES (DNA Elution Solution – Fast DNA SPIN kit) which is pure water is added to the tube, mixed gently and incubated for 2 min at room temperature,
- After centrifugation at 14,000 rpm for 1 min, supernatant which contains dissolved DNA is transffered to a new tube,
- DNA extraction results are contolled using agarose gel electrophoresis.

III.3.2.1 Agarose Gel Electrophoresis

DNA extracts and PCR products were examined on ethidium bromide-stained agarose gels before using for DGGE analysis. The procedure is below:

- To prepare 0.8% agarose gel, 0.32 g of agarose (Boehringer Mannheim, Germany) is added into 40 mL of 1xTAE buffer,
- It is stirred on a hot plate until agarose is dissolved and the solution becomes clear,
- Before removing the solution from hot plate 2 µL of 10 mg/mL ethidium bromide is added,
- Gel tray is prepared by sealing the ends with tape and placing the comb,
- Gel solution is poured into the tray and allowed to solidify at room temperature,
- As soon as the gel solidifies the comb is removed gently and the tray is placed in the electrophoresis tank which contains 1xTAE buffer,
- 5 µL of DNA or PCR solution is mixed with 1 µL of 6x loading dye (Fermentas) on a petrifilm and the mixture is loaded in a well of the gel,
- GeneRuler 100bp DNA Ladder Plus (Fermentas) is loaded at least one well ,
- After all samples are loaded into the wells 4 µL of 10 mg/mL ethidium bromide is added to the buffer in electrophoresis tank,
- Gel is electrophoresed at 150 volts for 40 min,
- UV-light is used to visualize the result.

III.3.3. Polymerase Chain Reaction (PCR)

In this study, PCR methods were examined for denaturing gradient gel electrophoresis (DGGE) and cloning. The variable regions of the ribosomal DNA gene and mcrA gene for methanogens were amplified by PCR from DNA extracts. PCR primers used in this study are shown in Table III.2.

Table III. 2 PCR Primers Used in This Study

Primer	Base Length	Sequence (5' – 3')	Reference
A 109 for	17	ACK GCT CAG TAA CAC GT	Grosskopf et al., 1998
1510 rev	19	GGT TAC CTT GTT ACG ACT T	Ficker et al., 1999
GC 515	65	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC	Lane, 1991
27 for	20	AGA GTT TGA TCC TGG CTC AG	Ficker et al., 1999
GC 968 for	57	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC	Nubel et al., 1996
1401 rev	17	CGG TGT GTA CAA GAC CC	Nubel et al., 1996
MCR for	17	TAY GAT CAR ATH TGG YT	Lueders et al., 2001
MCR rev	17	ACR TTC ATN GCR TAR TT	Lueders et al., 2001
ML for	32	GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC	Luton et al., 2002
ML rev	23	TTC ATT GCR TAG TTW GGR TAG TT	Luton et al., 2002
GC ML rev	63	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GTT CAT TGC RTA GTT WGG RTA GTT	Luton et al., 2002
T7 for	18	AAT ACG ACT CAC TAT AGG	Zoetendal et al., 1998
SP6 rev	18	ATT TAG GTG ACA CTA TAG	Zoetendal et al., 1998

III.3.3.1 Archaeal 16S rDNA PCR

A109 for – 1510rev primer pair was used for the amplification of full length of the archaeal 16S rDNA gene. PCR program (Pereira *et al*., 2002) involves 34 cycles of denaturation at 94 ^{o}C, annealing at 52 ^{o}C, and elongation at 68 ^{o}C as depicted in Table III.3.

Table III.3 PCR Program & Premix for Primer Pair A 109 for – 1510 rev

PCR program for A 109 for – 1510 rev			
Pre-denaturation	94 °C	5 min	
Annealing	52 °C	40 sec	34 cycles
Elongation	68 °C	1 min	
Denaturation	94 °C	30 sec	
Final annealing	52 °C	40 sec	
Post elongation	68 °C	7 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/ $MgCl_2$	10 µL
	12 µL
10 mM dNTPs	2 µL
A 109 for (10µM)	2 µL
1510 rev (10µM)	2 µL
Taq (5U/µL)	0.8 µL

III.3.3.2 Archaeal DGGE PCR

For the amplification of V2 – V4 region of archaeal 16S rDNA gene, A 109 for and GC 515 rev primers were employed and PCR products were used in the DGGE analysis. The PCR program and premix used lies below (Table III.4).

Table III.4 PCR Program and Premix for Primer Pair A 109 for – GC 515 rev

Primer pair: A 109 for – GC 515 rev			
Pre-denaturation	95 °C	2 min	
Denaturation	95 °C	30 sec	34 cycles
Annealing	52 °C	40 sec	
Elongation	72 °C	1.5 min	
Post elongation	72 °C	5 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/ $MgCl_2$	10 µL
	12 µL
10 mM dNTPs	2 µL
A 109 for (10µM)	2 µL
GC 515 rev (10µM)	2 µL
Taq (5U/µL)	0.5 µL

II.3.3.3 Bacterial 16S rDNA PCR

Two different PCR programs were utilized with primers 27 for and 1510 rev (Table III.5and Table III.6). Major differences between those two programs were elongation temperatures and the number of cycles. Premixes used were the same. However, program-2, which was adopted from Ficker *et al.* (1999), gave superior results compared to the program-1.

Table.III.5 PCR Program-1 & Premix-1 for Primer Pair 27 for – 1510 rev

Primer pair: 27 for – 1510 rev			
Pre-denaturation	94 °C	5 min	
Denaturation	94 °C	30 sec	25 cycles
Annealing	52 °C	20 sec	
Elongation	68 °C	40 sec	
Post elongation	68 °C	7 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/	10 µL
$MgCl_2$	12 µL
10 mM dNTPs	2 µL
27 for (10µM)	1 µL
1510 rev (10µM)	1 µL
Taq (5U/µL)	0.8 µL

Table.III. 6 PCR Program-2 & Premix-2 for Primer Pair 27 for – 1510 rev

Primer pair: 27 for – 1510 rev			
Pre-denaturation	95 °C	5 min	
Denaturation	95 °C	1 min	30 cycles
Annealing	52 °C	1.5 min	
Elongation	72 °C	1.5 min	
Post elongation	72 °C	10 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/	10 µL
$MgCl_2$	12 µL
10 mM dNTPs	2 µL
27 for (10µM)	1 µL
1510 rev (10µM)	1 µL
Taq (5U/µL)	0.8 µL

III.3.3.4 Bacterial DGGE PCR

Primer pair GC 968 and 1401 rev were used for bacterial DGGE PCR with the program depicted below (Table III.7). To find the optimum conditions for this PCR, primer and Taq polimerase amounts were changed. The best results were obtained with 0.2 µM primer consentration and 4 U of Taq per 100 µL of premix.

Table.III.7 PCR Program & Premix for Primer Pair GC 968 – 1401 rev

Primer pair: GC 968 – 1401 rev			
Pre-denaturation	94 °C	5 min	
Denaturation	94 °C	30 sec	35 cycles
Annealing	56 °C	20 sec	
Elongation	68 °C	40 sec	
Post elongation	68 °C	7 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/	10 µL
$MgCl_2$	12 µL
10 mM dNTPs	2 µL
GC 968 for (10µM)	2 µL
1401 rev (10µM)	2 µL
Taq (5U/µL)	0.8 µL

III.3.3.5 Archaeal *mcr*A gene PCR

Two different primer sets were employed; MCR for – MCR rev and ML for - ML rev. However, trials with the former primar pair gave discouraging results. Two programs were tried for primer pair MCR for – MCR rev. One was adopted from Lueders *et al*. (2001), the other from Springer *et al*. (1995) (Table III.8). While using those programs, different primer and Taq polimerase concentrations were also tested. However, optimization couldn't be achieved.

Table.III. 8 PCR Programs for Primer Pair MCR for – MCR rev

PCR program from Lueders *et al.* (2001)			
Pre-denaturation	94 °C	3 min	30-35 cycles
Denaturation	94 °C	45 sec	
Annealing	50 °C	45 sec	
Elongation	72 °C	1.5 min	
Post elongation	72 °C	5 min	
	4 °C	10 min	

PCR program from Springer et al. (1995)			
Pre-denaturation	94 °C	3 min	35 cycles
Denaturation	94 °C	1 min	
Annealing	37 °C	30sec	
Elongation	55 °C	1 min	
Post elongation	55 °C	5 min	
	4 °C	10 min	

On the other hand, the trials with primer set ML for and ML rev gave more satisfying results. Program adopted from Sheppard *et al*. (2005) was used for amplification. Many trials with changing concentrations of primers, Taq and DNA template were done and premix shown below (Table III.9) was chosen as the most superior.

Table.III.9 PCR Program & Premix for Primer Pair ML for – ML rev

Primer pair: ML for – ML rev			
Pre-denaturation	95 °C	5 min	35 cycles
Denaturation	95 °C	45 sec	
Annealing	54 °C	45 sec	
Elongation	72 °C	2 min	
Post elongation	72 °C	7 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/	10 µL
$MgCl_2$	12 µL
10 mM dNTPs	2 µL
ML for (10µM)	5 µL
ML rev (10µM)	5 µL
Taq (5U/µL)	0.5 µL

III.3.3.6 PCR of Isolated Plasmids

In the cloning procedure, which is described in detail on section III.3.6, plasmid isolation is necessary for sequencing. In this respect cloned samples were amplified with the primer pair T7 for and Sp6 rev using the premix and thermocycle program shown in Table.III.10.

Table.III.10 PCR Program & Premix for Primer Pair T7 for – Sp6 rev

Primer pair: T7 for – Sp6 rev			
Pre-denaturation	95 °C	5 min	
Denaturation	95 °C	30 sec	30 cycles
Annealing	45 °C	30 sec	
Elongation	72 °C	40 sec	
Post elongation	72 °C	5 min	
	4 °C	10 min	

Constituents for 100 µL premix	
Buffer w/	10 µL
$MgCl_2$	12 µL
10 mM dNTPs	2 µL
T7 for (10µM)	5 µL
Sp6 rev (10µM)	5 µL
Taq (5U/µL)	0.8 µL

III.3.4. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was performed with a DCode denaturing gel electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The procedure of DGGE technique is given in the following:

- First glass plates, in which the gel is poured, spacers (160 mm long, 1 mm thick) and the comb (depicted in Fig.III.3) are cleaned with ethanol,



Figure III.3 Assembly of Gel Chamber

- The gel bond (Pharmacia, Biotech) film has to be placed between glass plates exactly on the same level as the bottom line of the glass. Then, the glass plates

and spacers are placed together with the sandwich clamps in the casting stand in which a rubber strip is placed at the bottom to prevent leakage,

- Buffer tank is filled with 0.5xTAE buffer, and heated till the temperature reaches to 60 °C,
- Proper gel gradients are created using 0 and 100% denaturant stock solutions, Temed (13.5 µL), and APS (50 µL). The 0% denaturant stock is prepared by adding 50 ml of 40% acrylamide:bisacrylamide (37.5:1) solution (Boehringer Mannheim, Germany) and 5 mL of glycerol (Merck, Germany) and 2.5 ml of 50x Tris-Acetic acid-EDTA (TAE) buffer to 181 ml of distilled water. The 100% denaturant stock solution is prepared by mixing 50 ml of 40% acrylamide:bisacrylamide (37.5:1) solution, 5 mL of glycerol and 2.5 ml of 50x TAE buffer with 100 ml of formamide and 105.4 g of molecular biology grade urea. After the gel chamber is filled with the mixture the comb is placed which creates slots for sample loading,
- Both low and high denaturant solutions are drawn into the syringes making sure of the removal of air from the them. These two syringes are attached to the proper places on the gradient wheel and the gel is poured slowly and consistently by turning the wheel (Fig.III.4),
- As soon as the gel is poured the comb, which will inturn make the gel slots for sample loading, is placed on top of the gel between the plates,



Figure III.4 Gel Chamber Filling Slowly

- Gel is allowed to polymerise for at least 1 h. After polymerization comb is removed, the slots are rinsed with distelled water to remove non-polymerized

acrylamide. The gel chamber is attached to the central core, then the core is immersed into the pre-heated buffer tank,

- 10 µl DGGE-PCR product is mixed with 2 µl loading dye and the mixture is applied to the gel slots,
- The recirculating tube is attached to the upper reservoir of the Dcode unit, and the gel is electrophoresed at 60 °C for 16 h at 85 V.

III.3.4.1 Silver Staining

The photochemically derived silver stain was originally described for protein staining and later applied to nucleic acids. Silver nitrate reacts with biopolymers under acidic conditions and subsequently silver ions are selectively reduced to metallic silver by formaldehyde under alkaline conditions. The silver stain detects nucleic acids in polyacrylamide gels very sensitively and therefore small amounts of PCR products are needed for DGGE analysis.

Steps in the procedure:

- The power of the electrophoresis machine is switched off. The gel and gel bond are taken out of the gel chamber and placed in the fixation container,
- Fixation solution (20 ml 96% ethanol, 1 ml acetic acid, and 179 ml deionized distilled water) is added and the container is rocked for 3 min,
- The solution is stored for the later application,
- Staining solution (0.45 g Silver Nitrate, 200 ml fixation solution) is added to the container and rocked exactly for 10 min,
- The gel is rinsed thoroughly with distilled water and immersed in developer solution (0.02 g sodium borohydrate, 750 µl formaldehyde to 250 mL 1.5% NaOH) for 45 min,
- After developer solution is removed, previously used fixation solution is added and rocked for 5 min,
- Then it is replaced with preservation solution and rocked for at least 7 min,
- Finally, a conventional sized piece of cellophane foil (Pharmacia, Biotech) is prewetted in the preservation solution and the gel is covered with it preventing any air bubble's existence,
- The gel is dried at 40 ^{o}C overnight.

III.3.5. Fluorescent In Situ Hybridization (FISH)

Fluorescence in situ hybridisation (FISH) with rRNA-targeted oligonucleotide probes facilitates the rapid and specific identification of individual microbial cells in their natural environments. FISH consists of basicly two steps: Fixation and Hybridization. Since the cells are exposed to elevated temperature, detergents, and osmotic gradient during hybridization fixation is essential for maintaining the morphological integrity of the cells. A variety of fixatives have been evaluated. Among them formaldehyde solution is distinctive by its effect of minimizing the autofluorescence.

III.3.5.1 Preparation of Paraformaldehyde Fixative

- 2 g of paraformaldehyde and 1 drop of 1 M NaOH solution are added into 33 mL of double distelled water and the solution is heated to 60 ^{o}C,
- When the solution clarifies it is removed from the heat,
- 16 mL of 3x PBS (phosphate-buffered saline) is added and the solution is cooled on ice,
- pH is adjusted to 7.2 using HCl,
- The solution is filtered through a 0.2 μm filter and stored at 4 ^{o}C at most for 5 days.

III.3.5.2 Fixation

- 1 mL of cell suspensions obtained by centrifugation of leachate samples are transferred to 1.7 mL of eppendorf tubes, centrifuged at 14,000 rpm for 3-5 minutes, and the supernatant is discarded,
- Cells are washed with 1x PBS several times until the supernatant clarifies. Each washing step involves addition of 1 mL of 1x PBS, vortexing, centrifuging and the removal of the supernatant. It is important to vortex the cells thoroughly during washing to avoid clumbing,
- Cell pellets are resuspended in 750 μL of paraformaldehyde fixative, vortexed for 1min and incubated for 3 h or overnight at 4 ^{o}C,
- After incubation the suspension is centrifuged for 2 min at 14,000 rpm, and the supernatant is removed,
- Cells are washed with 1x PBS,

- In the final step, 750 µL of ice cold ethanol (100%) is added, the solution is mixed well and stored at -20 ^{o}C or directly spotted on glass slides.

III.3.5.3 Application of Cells to The Microscope Slides

- 4µL of the fixed cell suspension is spreaded on glass slides,
- The slide is incubated in an oven at 46 ^{o}C for 10 min,
- The cells are dehydrated by successive immersions in 50%, 80% and 100% ethanol for 3 min in each,
- The slide is air-dried. (After drying slides can be stored at room temperature for maximum 1 month or hybridization can be proceeded.)

III.3.5.4 Hybridization

- The oligonucleotide probe is thawed and its concentration is adjusted to 50 ng/µL,
- Hybridization buffer which contains 5 M NaCl, 1 M Tris/HCl (pH 8.0), 10 % SDS, formamide and double distelled water is prepared (Formamide concentration is specific to the probe used and can be seen in Table III.10),
- 10 µL of hybridization buffer and 1 µL of the probe are added in each well on the slide,
- A hybridization tube is prepared by folding a piece of tissue in a 50 mL Falcon tube and pouring the rest of the hybridization buffer onto it,
- The slide is transferred into the hybridization tube and incubated in the oven at 46 ^{o}C for 2.0 hours (Tube should be sealed well to prevent evaporation of the hybridization buffer and in turn unspecific binding of the fluorescent probe).

III.3.5.5 Washing

- 5 M NaCl, 1 M Tris/HCl (pH=8.0), 0.5 M EDTA (pH=8.0), 10% SDS (w/v) and distilled water containing washing buffer (50 mL) is prepared and pre-heated to 48 ^{o}C,
- Immidiately after hybridization, the slide is placed in washing buffer to remove unbound oligonucleotides and incubated at 48 ^{o}C for 20 min,
- At the end of 20 min, slide is taken out of the washing buffer and dried quickly.

III.3.5.6 DAPI staining

- DAPI (diamidineophenylindole) solution (2.5 mg/mL) is thawed,
- 25 µL of DAPI is added to 50 mL of distilled water,
- The slide is first soaked in pre-cooled distilled water for 3-5 sec, then stained with DAPI solution for 7min, and rinsed with distilled water.

III.3.5.7 Visualization

Before visualizing the slides with microscope Vectashield (Vector Laboratories) is added to prevent photo bleaching (Calli et al., 2003). The slides are examined with Leica DM-LB fluorescent microscope and digital images of the slides are captured with Leica DC350F digital camera.

Table III.11 The Oligonucleotide Probes Used in FISH and Their Target Groups

Probe	Label	Formamide conc. (%)	OPD name	Target group	References
EUB338	FLUOS	20	CTGCTGCCTCCCGTAGGAGT	*Bacteria* domain	*Amann et al. 1990*
ARC915	Cy3	20	GTGCTCCCCCGCCAATTCCT	*Archaea* domain	*Stahl and Amann 1991*
MX825	Cy3	35	TCGCACCGTGGCCGACACCTAGC	*Methanosaetaceae*	
MS821	Cy3	35	CGCCATGCCTGACACCTAGCGAGC	*Methanosarcina*	
MSMX860	Cy3	35	GGCTCGCTTCACGGCTTCCTT	*Methanosarcinales*	
MB310	Cy3	35	CTTGCTTCAGGTTCCATCTCCG	*Methanobacteriaceae*	*Raskin et al. 1994*
MB1174	Cy3	35	TACCGTCGTCCACTCCTTCCTC	*Methanobacteriaceae*	
MC1109	Cy3	35	GCAACATAGGGCACGGGTCT	*Methanococcaceae*	
MG1200	Cy3	35	CGGATAATTCGGGGCATGCTG	*Methanomicrobiales*	
SRB385	Cy3	35	CGGCGTCGCTGCGTCAGG	*Sulfate Reducing Bacteria*	*Amann et al. 1990*
Alf1b	Cy3	35	CGTTCGCTTGAGCCAG	*Alfa proteobacteria*	
Bet42a	Cy3	35	GCCTTCCCACTTCGTTT	*Betaproteobacteria*	*Manz et al. 1992*
Gam42a	Fluos	35	GCCTTCCCACATCGTTT	*Gamaproteobacteria*	
LGC354A LGC354B LGC354C	Cy3 Cy3 Cy3	35 35 35	TGGAAGATTCCCTACTGC CGGAAGATTCCCTACTGC CCGAAGATTCCCTACTGC	*Low GC gram positive bacteria (Firmicutes)*	*Meier et al. 1999*
CF319a	Cy3	35	TGGTCCGTGTCTCAGTAC	*Cytophaga-Flavobacterium*	*Manz et al. 1996*
BAC303	Cy3	0	CCAATGTGGGGGACCTT	*Bacterioidales*	
EUB338III	Cy3	35	GCTGCCACCCGTAGGTGT	*Verrucomicrobiales*	*Daims et al. 1999*
MA621	Cy3	35	TCAAAGGCAGTTCCGAGGTT	*Type II Methanotrophes*	*Bourne et al. 2000*
MG64	Cy3	35	CCGAAGGCCTRTTACCGTTC	*Type I Methanotrophes*	

III.3.6. Cloning and Sequencing

For identification of archaeal and bacterial species, which are present in the leachate, samples were cloned and sequenced. It is necessary to purify the PCR products before cloning.

III.3.6.1 PCR purification

QIAquick PCR Purification Kit was used for PCR purification. The protocol is below:

- 5 times volume of PB buffer is added to one time volume of PCR products,
- The mixture is put on the filter column and after centrifugation the liquid is discarded,
- The filter is washed with PE buffer,
- The DNA molecules which are bound on the filter, are dissolved with EB buffer.

III.3.6.2 Ligation

DNA molecules are ligated in cloning vector pGEM-T (Promega). The Ligation premix consists per sample of 2x Rapid Ligation buffer, T4 DNA Ligase, pGEM-T vector and the cleaned PCR product. The ligation reaction is performed at 4 ^{o}C overnight.

III.3.6.3 Transformation

Prior to transformation procedure, the plates containing LB (Luria-Bertani) medium, ampicilline, IPTG and X-Gal are prepared. The ligation reactions and the high effeciency competent *E. coli* JM 109 cells are transferred into sterile 1.5 mL microcentrifuge tubes. The transformation reactions are placed on ice for 20 min and after that the cells are heat shocked at 42 ^{o}C. After the heat shock the tubes are placed back on ice for 2 min. SOC medium at room temperature is added to the cells and incubated for 1.5 hours at 37 ^{o}C with shaking. Each transformation culture is placed onto antibiotic plates with LB medium. From each transformation two plates are cultivated: non-concentrated and concentrated. The plates are incubated overnight at 37 ^{o}C.

III.3.6.4 Screening

After incubation cells of the white colonies are picked up with sterile toothsticks and dissolved in TE buffer. With the same toothstick the clones are seeded in a LB plate with numbers. The tubes with TE buffer and cells are heated 10 min at 94 ^{o}C. After heating, the TE buffer with damaged and opened cloning cells is used as target DNA for PCR. The PCR products are treated with 10x buffer and restriction enzyme MspI and incubated for 1.5 hours at 37 ^{o}C. The restriction reaction is checked by electrophoresis at 150 V with 1x TBE (Tris/ Boric acid/ EDTA) buffer on 2% agarose gel. Then, depending on the RFLP analysis the plasmids thought to be resembling distinct species are checked for mobility comparison on DGGE. The selected plasmids are seeded in liquid LB medium containing ampicilline and incubated at 37 ^{o}C overnight. Overnight cultures are purified using the Wizard Plus SV miniprep DNA purification kit (Promega).

III.3.6.5 Plasmid isolation

For sequencing it is necessary to isolate the plasmids from clones. Clones are amplified in PCR using the primer pair of T7 for and Sp6 rev. QIAquick PCR purification kit (QIAGEN) is used for purification of the PCR products. Afterwards OD (optical density) analysis is performed to ensure that sufficient amount of DNA is present in the samples which would be sequenced.

III.3.6.6 Sequencing

The clones used in this study were prepared in the way expressed above. The samples were sent to a private company (SeqLab Sequence Laboratuaries, Göttingen, Germany) in Europe.

16S rDNA and mcrA sequences were aligned by using the multiple alignment Clustal W program (Thompson et al. 1994). Neighbor-joining phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis package (MEGA version 2.1) (Kumar et al., 2001) with the Jukes-Cantor algorithm and the robustness of the phylogeny was tested by bootstrap analysis with 1000 iterations.

CHAPTER IV

RESULTS

IV.1. CHEMICAL CHARACTERIZATION OF LEACHATE SAMPLES

The first part of the study includes chemical characterization of Kemerburgaz Sanitary Landfill leachate samples. That is because chemical characteristics of leachate is governed by microbial processes going on within the landfill. Since the project's aim is to determine the prevailent microorganisms in MSW leachate this part is necessary.

Applied parameters and characterization data of leachate samples were listed in Table IV.1. In addition to this parameters, BOD_5: COD and TKN: NH_4-N ratios and VSS amounts of the samples, which can be used as indicators to determine the landfill phases, were examined for the leachate samples. No obvious differences other than the parameters above were observed between leachate samples.

Tablo IV.1 Chemical Anaylyses Results of Istanbul Kemerburgaz Landfill Leachate

Samples	COD (mg/L)	BOD_5 (mg/L)	NH_3-N (mg/L)	TKN (mg/L)	SS (mg/L)	VSS (mg/L)	Alkalinity (mg $CaCO_3$/L)	Cl^- (mg/L)	pH	Color (PtCo)	Conductivity (mS/cm)
K1	3152	126	2980	3150	126	105	17,400	5600	8.0	4800	28,600
K2	3520	191	2240	3150	223	177	11,800	5600	7.9	5200	22,830
K3	10,475	5246	1560	2325	907	773	12,200	5550	7.8	5500	25,600
K4	9892	4684	1610	2225	724	648	10,400	5100	7.8	5400	24,210
YK1	7360	4500	1730	1875	1940	1580	9250	3125	7.5	5400	20,900
YK2	2980	183	3150	3225	344	240	15,250	4275	7.8	7850	28,260
YK3	3522	372	3440	3600	273	203	16,500	4225	7.8	6050	29,860
YK4	2212	89	1960	2025	277	197	10,250	2975	8.1	4850	21,600
YK5	3143	169	2570	2700	280	157	12,000	2925	7.8	6700	22,840
YYK1	11,900	7200	2320	2550	1235	812	13,000	3230	7.6	11,900	24,780
YYK2	3290	210	3260	3300	152	98	15,000	3170	8.0	5950	26,860
YYK3	4420	690	3575	3900	170	100	17,000	3170	8.0	5400	29,160
YYK4	3010	220	2120	2250	151	89	10,500	2670	8.2	4600	21,290
YYK5	2890	200	2380	2550	140	97	11,000	2030	8.0	5800	20,340

IV.1.1. BOD_5: COD Ratio

A parameter that can be used to evaluate the stability of the waste is the BOD_5: COD ratio in the leachate. According to Heyer and Stegmann (1998), BOD_5: COD ratio of 0.4 and above indicate acid formation phase. On the other hand, the ratio below 0.2 shows methanogenic phase. BOD_5: COD ratio indicating the stabilization phases of landfill leachate samples are given in Figure IV.1.

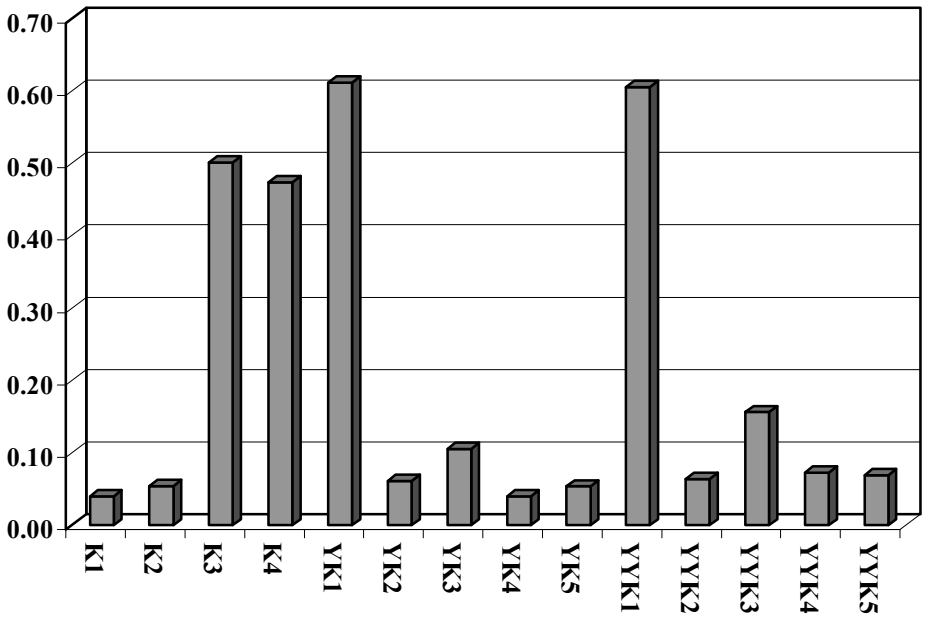


Figure.IV.1 BOD_5/COD Ratios of Kemerburgaz Lanfill Leachate Samples

Depending on the graph above one could say that leachate samples K3, K4, YK1 and YYK1 with quite high BOD_5: COD ratios point out the landfill sections, where they belonged to, are in acid formation phase. However, the rest of the samples have very low values indicating methanogenesis phase.

K3, YK3, YYK3, and K4, YK5, YYK5 are the samples coming from the same pipes only they were gathered at different times. K3 and K4 were collected a year earlier than YK3, YK5, YYK3 and YYK5. The landfill section which they belong to, was in acidogenic phase that time. A year later, chemical characterization data shows a shift through methanogenic phase with fairly low ratios.

IV.1.2. TKN: NH_3-N Ratio

TKN: NH_3-N ratio is another parameter, which indicates the biodegradation state of landfill sections that leachate samples come from. A ratio of higher than 1.1

demonstrates that hydrolysis reaction takes place in the landfill section, in other words acidogenesis phase prevails. TKN: NH_3-N ratios of leachate samples were given in Figure IV.2.

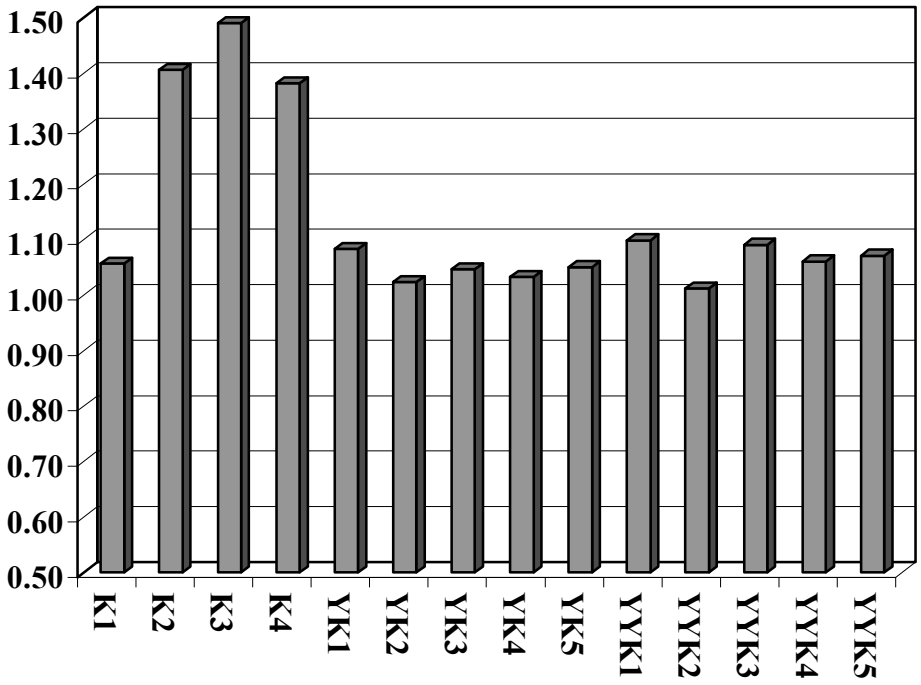


Figure IV.2 TKN/NH_3-N Ratios of Kemerburgaz Landfill Leachate Samples

While a landfill is being stabilized, NH_3 content increases and TKN: NH_3-N ratio decreases parallel with the landfill age. K2, K3, and K4 attract attention being the only ones with high ratios well above 1.1. K2 was sorted out as belonging to the section, which was in methanogenic phase. However, TKN: NH_3 ratio of that sample contradicts with that conclusion. Similar contradictions are valid for the samples YK1 and YYK1. They were thought to be arising from an acidogenic section of the landfill depending on the BOD_5: COD. But TKN: NH_3 ratios point out the methanogenic phase. The rest of the leachate samples have consistent values and in turn consistent conclusions.

IV.1.3. Volatile Suspended Solids (VSS)

In landfills where acidogenic phase prevails VSS amount rises due to the formation of VFAs. Thus, VSS values can also be an indicator. In fig.IV.3, where VSS values of the leachate samples were sketched, YK1 and YYK1 attract attention at first sight having very high values. That is consistent with the BOD_5: COD ratio.

K3 and K4 haven't got VSS amounts as high as YK1 and YYK1, but still these amounts are considerably high compared to the rest of the samples.

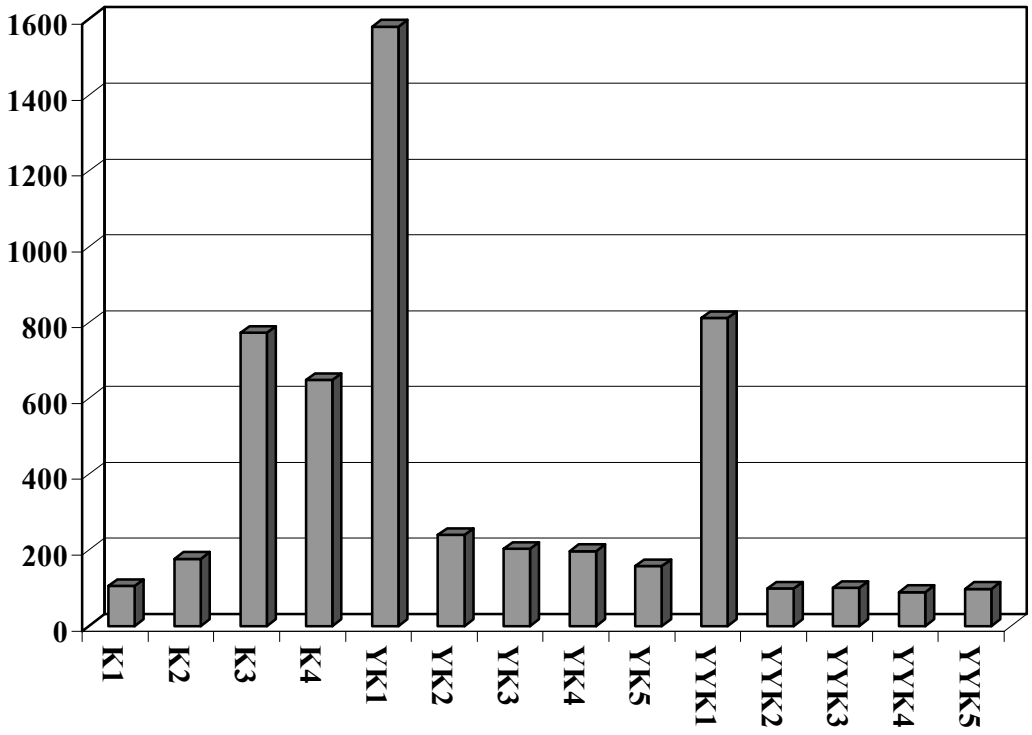


Figure IV.3 VSS Values of Kemerburgaz Landfill Leachate Samples

IV.2. MICROBIAL COMMUNITIES IN LANDFILL LEACHATES

To investigate both archaeal and bacterial diversities within a landfill leachate samples coming from the different sections of Kemerburgaz Lanfill were studied using molecular tools. These tools include PCR-DGGE method, cloning and sequencing, and fluorescent in situ hybridization technique. First, PCR-DGGE method was optimized with primers amplifiying total or sections of archaeal / bacterial 16S rDNA gene. Then these optimum conditions were utilized in cloning and sequencing. On the other hand, FISH allowed to see the microbial intensity and diversity of the samples visually.

IV.2.1. Identification of Microorganisms by PCR-DGGE

IV.2.1.1 Archaeal DGGE

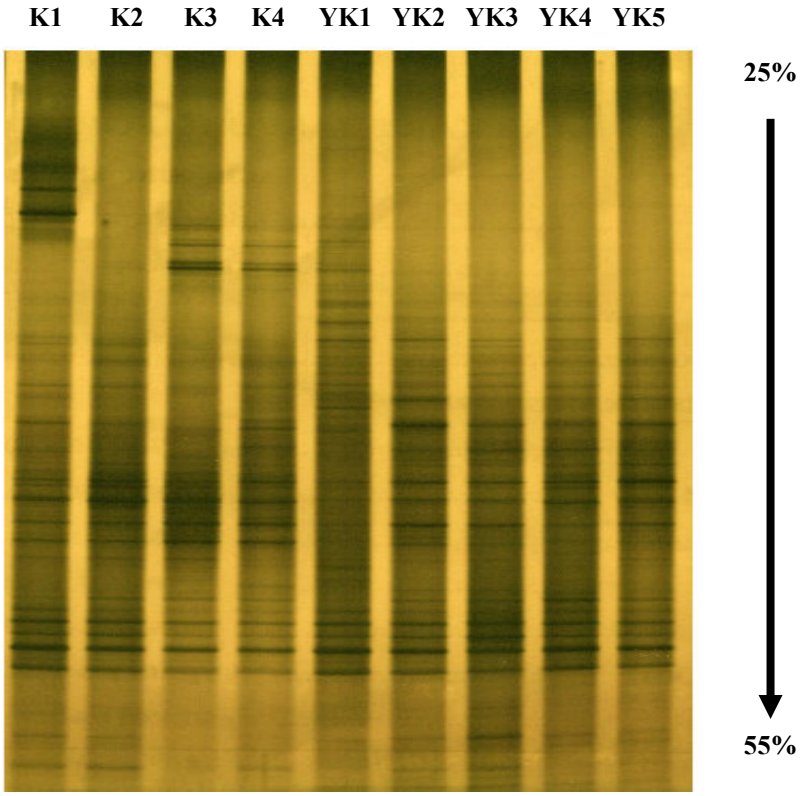


Figure.IV.4 Archaeal DGGE with Primers A109for – GC515rev

After PCR optimization, optimum conditions for DGGE were tried to get the best separation in banding patterns. DGGE gel banding pattern shown in Fig.IV.4 is the result of Nested PCR application to the samples. In Nested PCR, the complete 16S rDNA gene was amplified according to the premix and program shown in Table.III.3 using primers A109for and 1510rev . Then these PCR products used as template for PCR with A109for and GC515rev primers (program & premix in Table.III.4). In each lane excessive amounts of bands attract the attention. The reason of excessive bands could be explained by PCR bias or heteroduplexity phenomena. Mismatches in the two similar but nonidentical strands result in a lower denaturing temperature than that of homoduplex molecules; hence, the heteroduplexes melt at lower denaturant concentrations and appear as upper bands in parallel gradient DGGE analysis (Wise *et al.*, 1999).

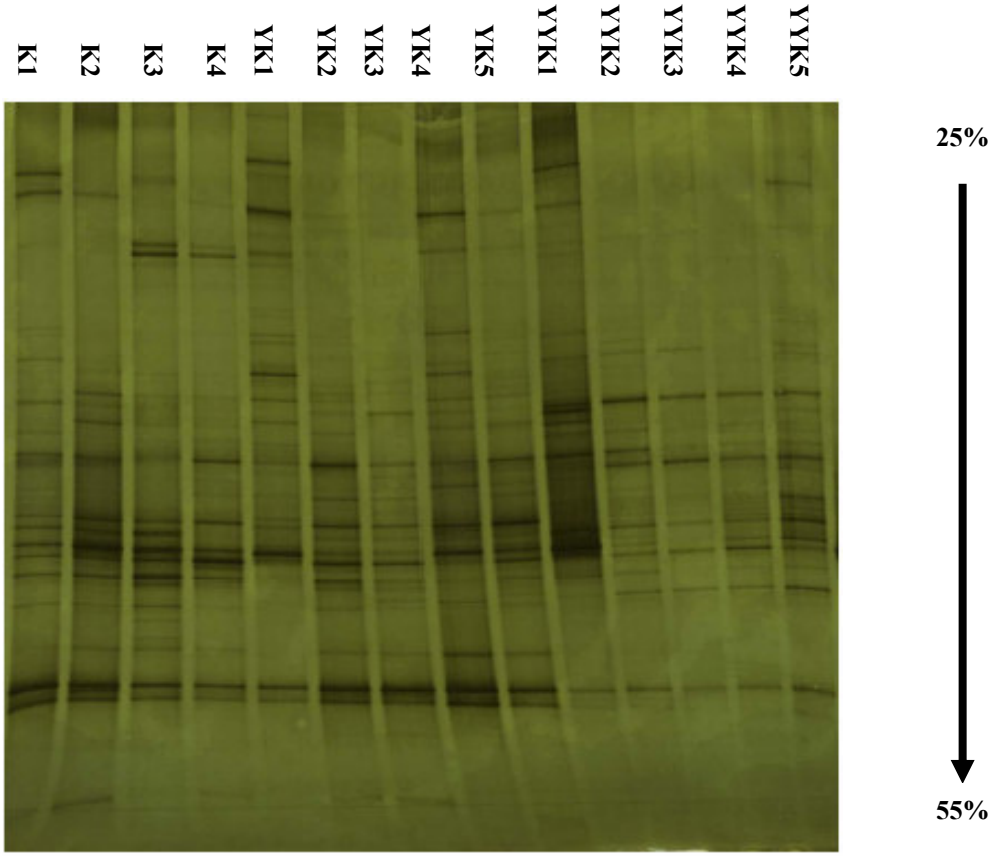


Figure.IV.5 Archaeal DGGE with Primers A109for – GC515rev

Archaeal DGGE PCR was directly applied to the DNA extracts of leachate samples to see the difference if any would be created against nested PCR. The result was more satisfying compared to the first trial (Fig.IV.5). The number of bands per sample decreased however, they were still excessive. Samples had some bands in common. The ones near to the bottom existed in all samples. No obvious increase in the number of bands was detected in young age leachate samples like K3, YK1 and YYK1. Although those samples shared some bands in common they had quite different DGGE profiles.

IV.2.1.2 Bacterial DGGE

PCR products of the leachate samples with the primer pair GC968 – 1401rev were used for DGGE analysis. The major difference between the DGGE gels shown in Fig.IV.6 and IV.7 is primer concentrations of the samples. The PCR products loaded on the first gel has a primer concentration of 0.1 µM while the other has that of 0.2 µM. Excluding that difference PCR reactions for samples were carried out according to the program given in Table.III.7. It is clearly seen that higher primer

concentration removed the low intensity bands resulting in less bands and changed the lower part banding pattern.

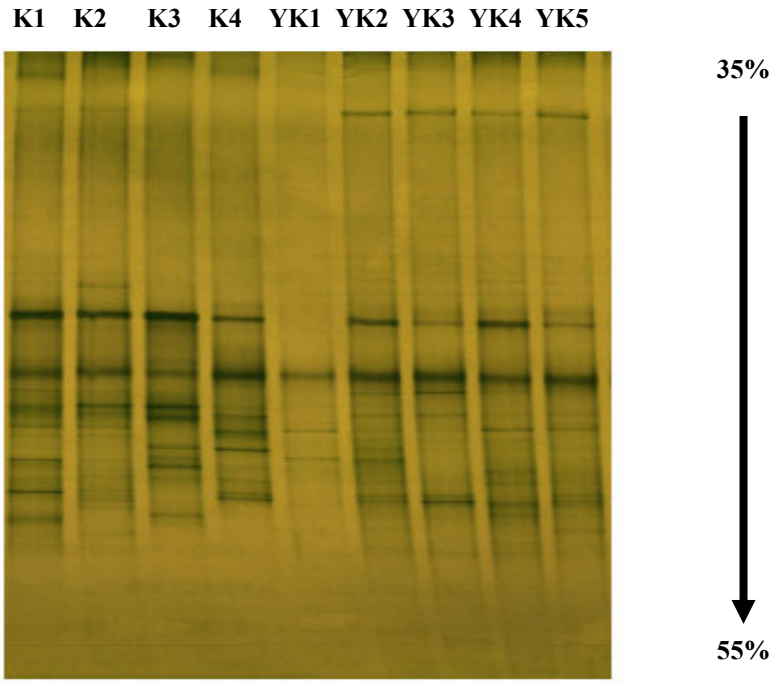


Figure.IV.6 Bacterial DGGE with Primers GC968 – 1401rev

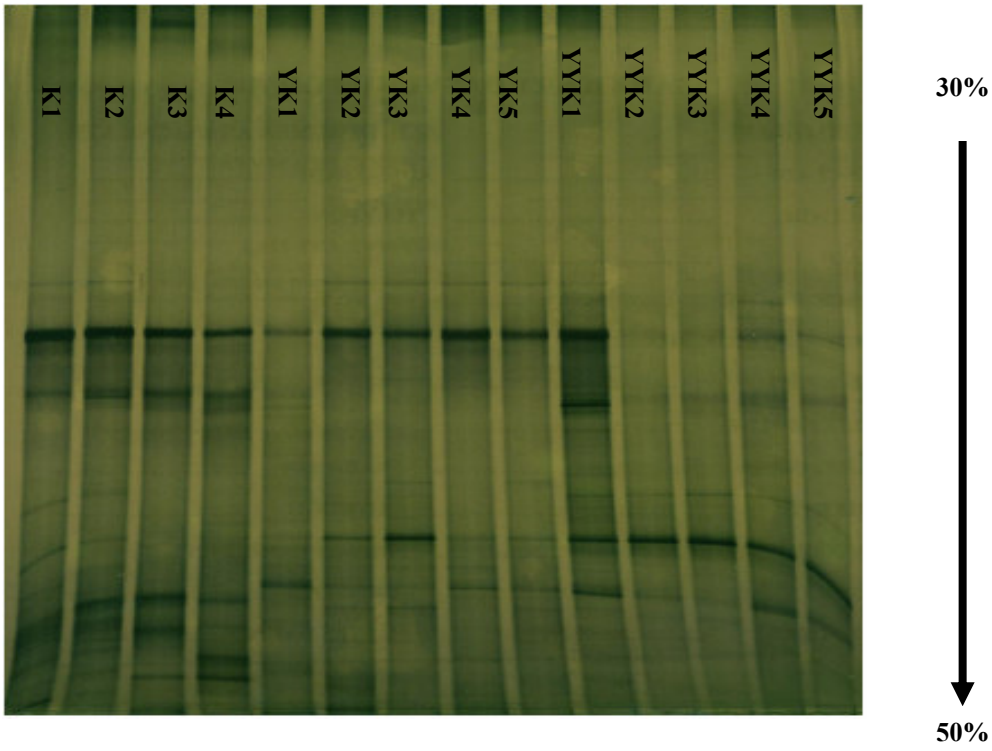


Figure.IV.7 Bacterial DGGE with Primers GC968 – 1401rev

The gel in Fig.IV.8 is another DGGE trial with bacterial DGGE primers. The different element from the other bacterial DGGEs is primers again. The primer pair used before was about to expire. Thus, a new set was ordered and that new primer pair was utilized in this DGGE PCR. As a result, the number of intensive bands increased and the lower part bands, which were ran through the gel in the previous analyses, were kept within. Generally, the banding pattern of the samples was similar to the previous bacterial DGGE analysis' excluding the additional bands at the bottom. Each group of leachate (Ks, YKs, and YYKs) shared similar bands. They also had many bands in common individually.

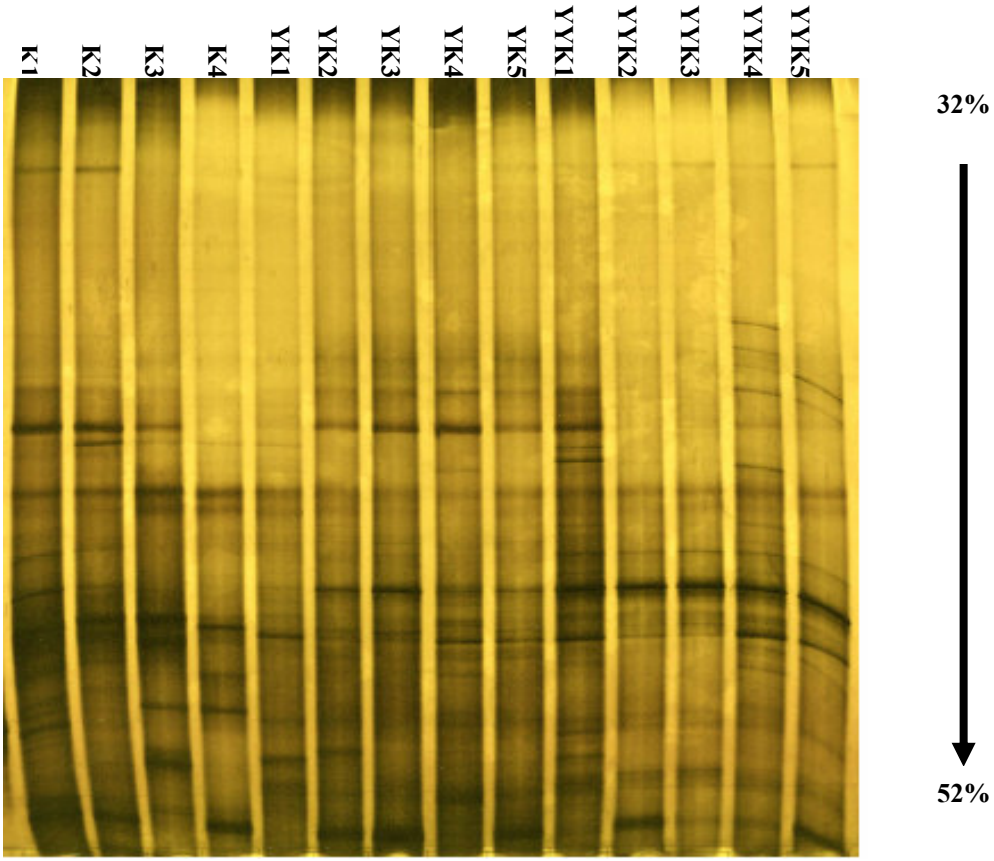


Figure.IV.8 Bacterial DGGE with Primers GC968 – 1401rev

IV.2.1.3 DGGE with plasmids

Archaeal DGGE analysis was conducted using both the samples and archaeal plasmids obtained from those samples by cloning (Fig.IV). The aim was to see the bands resembling the plasmids in the banding pattern of the samples. Except plasmid 39 (P39) all plasmids corresponded to the bands peculiar to themselves. When plasmids are in consideration one thing draw attention which is the double band configuration. That is especially obvious for the plasmid 1 (P1) and the

corresponding bands in the samples K1, K3 and YK2. This is called artifactual double banding and many researchers have been reported on that (Janse *et al*., 2004; Wise *et al*., 1999). These double bands should actually yield one band by the definition of DGGE analysis. No straightforward explanations could account for their formation as they were generated using non-degenerate primers, and after amplification of DNA from pure cultures as well as from plasmids. Artifactual bands hamper interpretation and analysis of DGGE gels since they lead to an overestimation of sequence diversity.

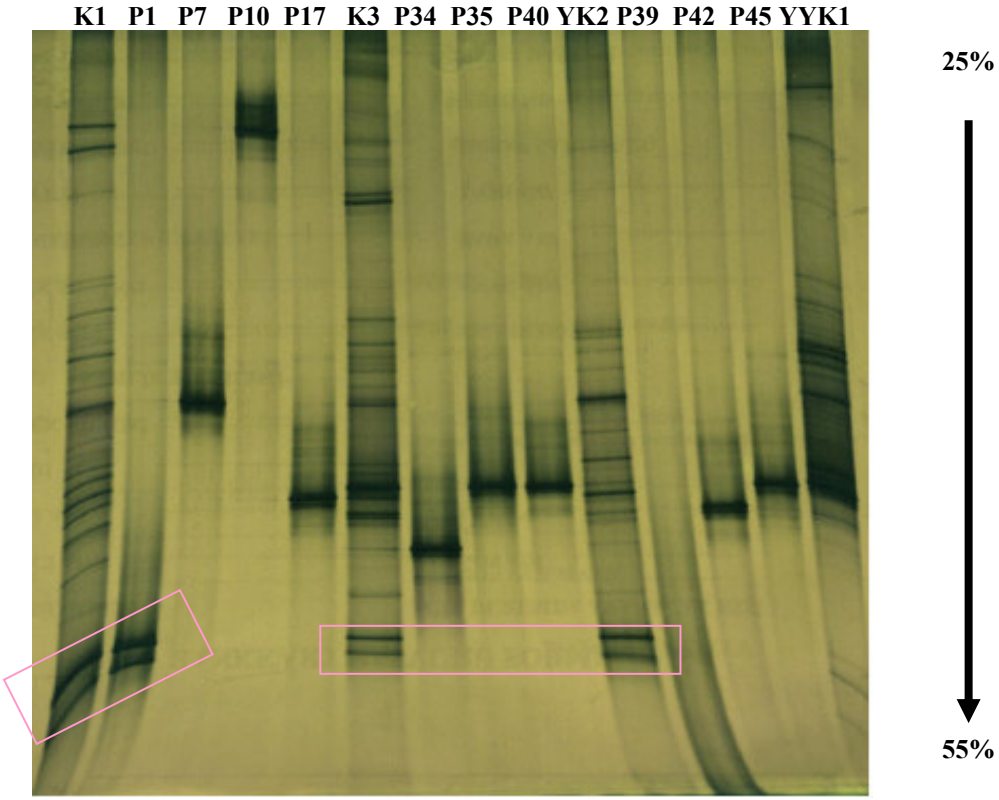


Figure.IV.9 Archaeal DGGE with Primers A109for – GC515rev

*Mcr*A gene plasmids obtained in cloning were amplified together with the samples using the primer pair ML for – GC ML rev according to premix and program shown in Table.III.9. Then the PCR products were loaded in DGGE gel to see the bands corresponding to the plasmids in the pattern created by samples (Fig.IV.10). However, plasmids didn't form one band as it is supposed to be. Also the banding pattern of the samples did have excessive numbers of bands indicating that DGGE for *mcr*A gene needed optimization.

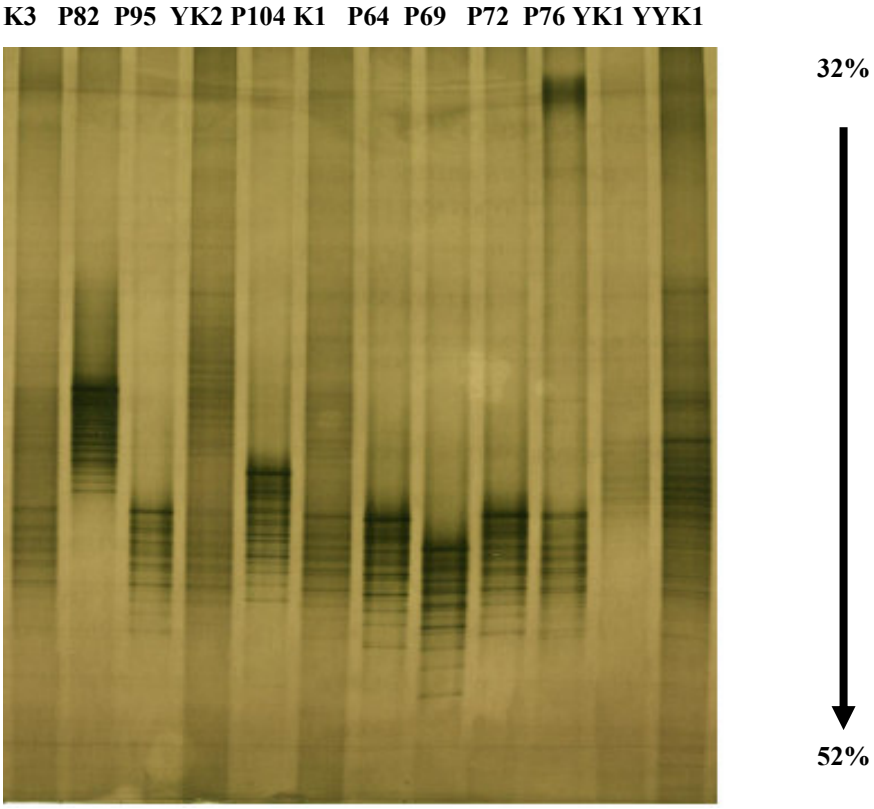


Figure.IV.10 *McrA* Gene DGGE with Primers ML for – GC ML rev

IV.2.2. Identification of Microorganisms by FISH

Whole cell in-situ hybridization with 16S and 23S rRNA targeted oligonucleotide probes was applied to the leachate samples. For every sample first thing to do was using the probes EUB338 and ARCH915 to have a general idea on the distribution of archaea and bacteria within. Afterwards, species-specific probes were hybridized with samples. The hybridization with EUB338 and ARCH915 probes revealed that in all samples bacteria amount is many times higher than archaea amount. Another fact, that was observed, is older the leachate sample less the amount of bacteria and archaea within (Fig.IV.11).

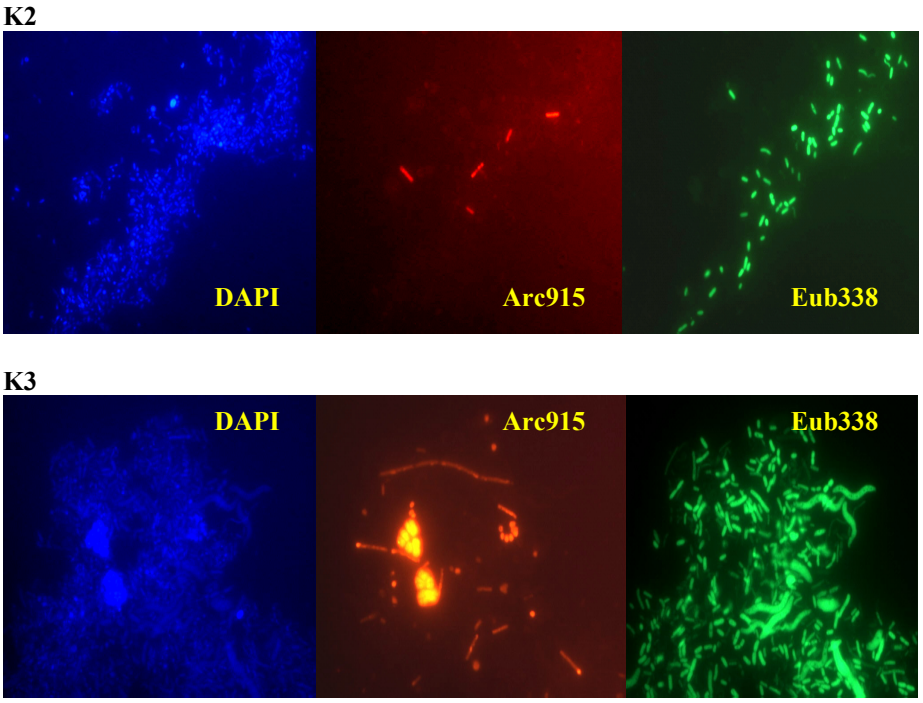


Fig.IV.11 Archaea and Bacteria Distribution of Leachate Samples K2 (old) and K3 (young)

IV.2.2.1. Archaea in Leachate Samples

Hybridizations with species-specific probes were performed to determine the distributions of acetoclastic and hydrogenotrophic methanogens in the leachate samples. First of all acetoclastic methanogens were investigated by using MsMx860 probe for *Methanosaeta*.

K1 and K2 leachate samples were dominated by *Methanosaeta*. M*ethanosarcina* was also present in both samples, only small in quantity and size. However, K3 leachate sample was dominated by acetoclastic Methnosarcina, which is an indication of high acetate concentration. Fig.IV.12 below shows the result of these samples hybridized with MsMx860 probe.

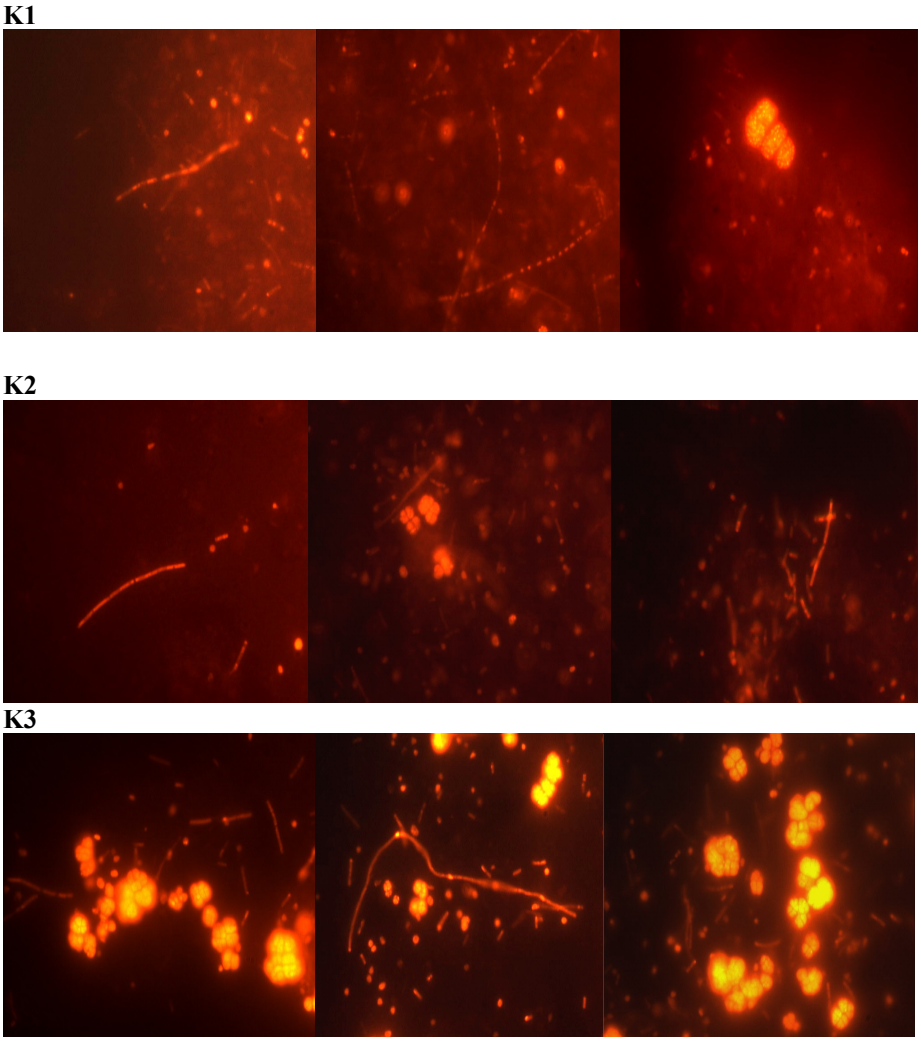


Figure.IV.12 *Methanosarcina* and *Methanosaeta* Distribution in Leachate Samples K1, K2, K3

When MsMx860 probe was applied to the leachate samples, which were collected in December, 2004, no signal was detected except YK1 sample. That sample contained very little amount of *Methanosarcina* (Fig.IV.13).

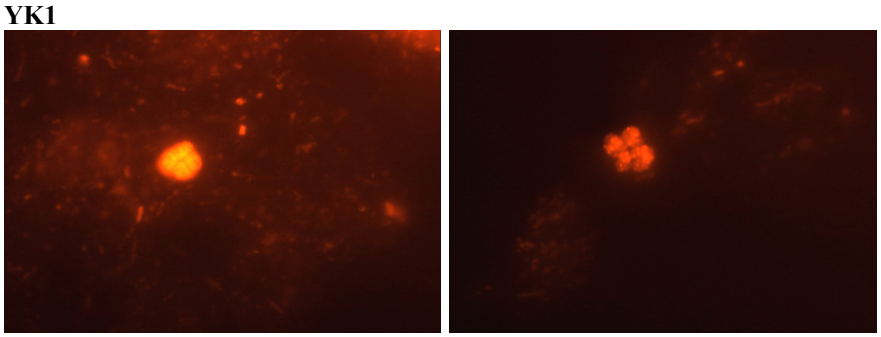


Figure.IV.13 *Methanosarcina* and *Methanosaeta* Distribution in Leachate Sample YK1

Samples collected in March, 2005 had significant amounts of *Methanosaeta* as shown in Fig.IV.14. However, hardly any *Methanosarcina* was observed even in the youngest leachate sample YYK1.

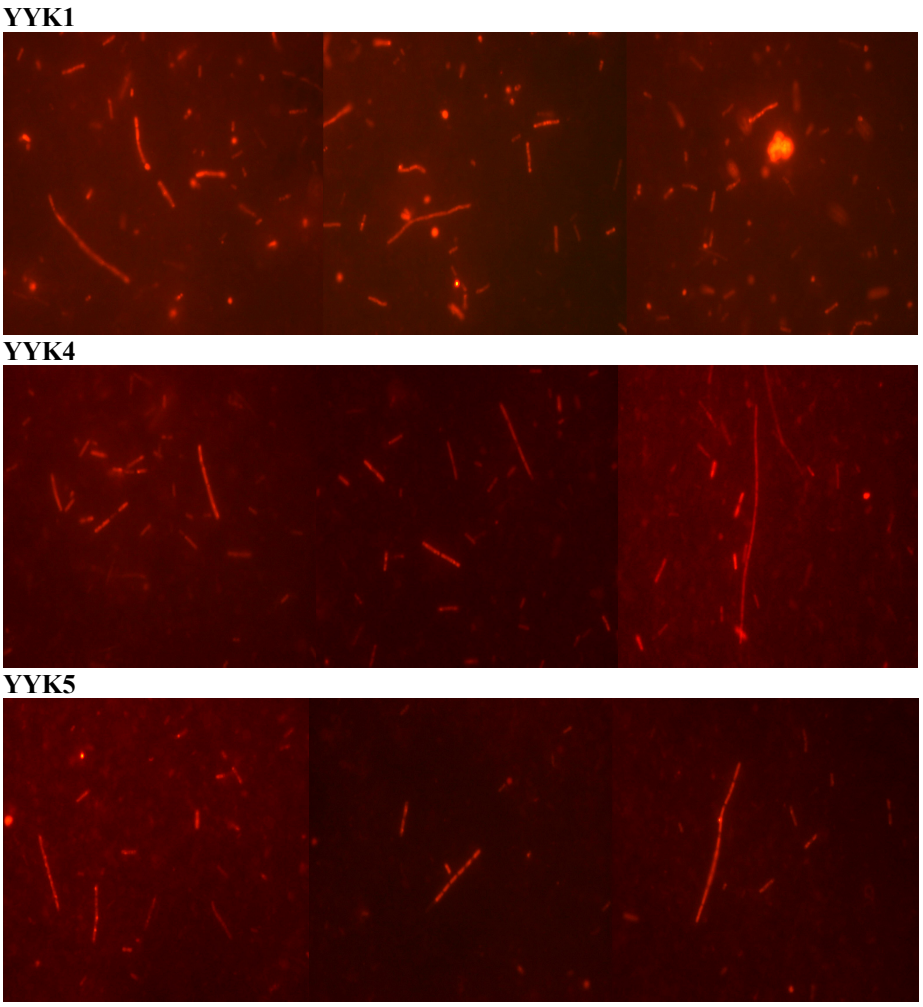


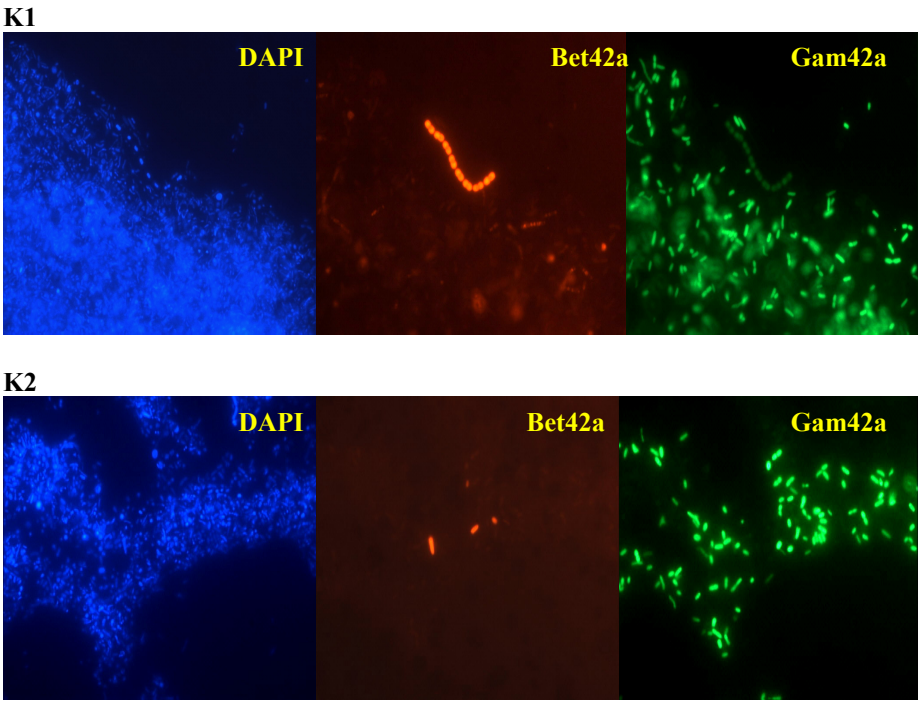
Figure.IV.14 *Methanosarcina* and *Methanosaeta* Distribution in Leachate Samples YYK1, YYK4, and YYK5

Unfortunately, the analyses with the probes of MB310 and MB1174 for *Methanobacterium* species, MC1109 for *Methanococcus*, and MG1200 for *Methanomicrobiales,* which are H_2-utilizing methanogens, identified very low in all leachate samples.

IV.2.2.2. Bacteria in Leachate Samples

To see the bacterial diversity in the leachate samples several probes besides EUB338 probe were used. They were Bet42a and Gam42a for beta and gamma subclasses of *Proteobacteria*; LGC354a, b, c for *low G + C gram positive bacteria* which also belong to *Proteobacteria* group; CF319a for *Cytophaga Flavobacterium* group of *Bacteroidetes*; BAC303 for *Bacteroidales* group of *Bacteroidetes*; SRB385 for sulfate reducing bacteria; MG64 for type-I methanotrophes; MA621 for type-II methanotrophes.

Gam42a probe hybridization revealed that the most abundant bacterial group in all leachate samples was gamma proteobacteria. Since Gam42a and Bet42a probes are only different from each other by one base it was necessary to hybridize the samples with these probes at the same time. Morphologically, basilles were the dominating group in both gammas and betas. There were also short and/or long chains of bacteria and short and thick or long and thin rods (Fig.IV.15).



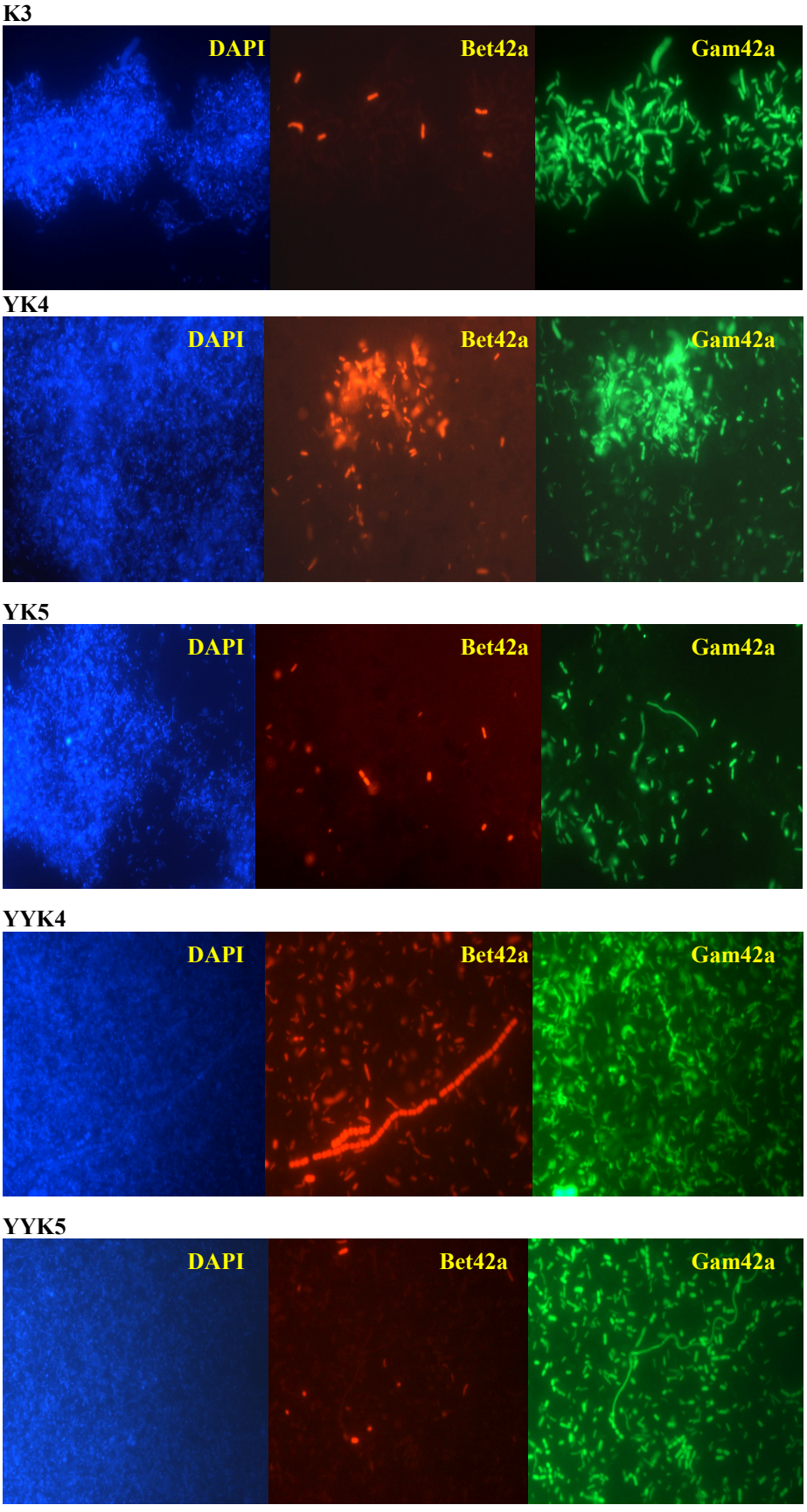


Figure: IV.15 *Beta* and *Gamma Proteobacteria* Distribution of Leachate Samples

LGC354a, b, and c probes were applied to the samples as a mixture which had a concentration of 50 ng/µL. The results were in contradiction with the studies of Huang *et al*. (2004 and 2005). They found low G + C gram positive bacteria being the most abundant organism in the leachate. However, we could detect hardly any of them. Only K3 and K4 leachate samples had some in the shape of thick rods (Fig.IV.16).

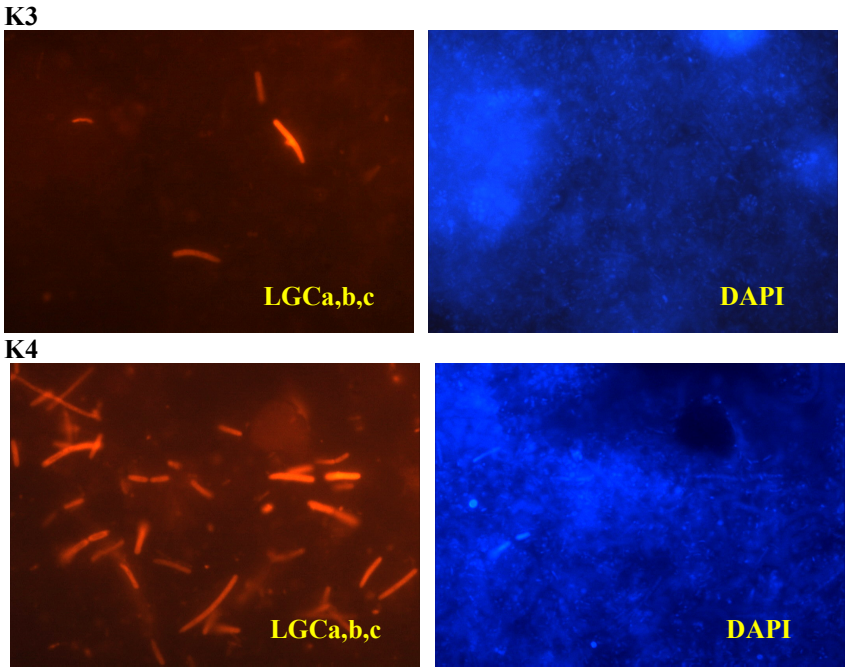


Figure: IV.16 *LGC Gram* + *Bacteria* Distribution of Leachate Samples

CF319a and BAC303 probes were utilized for the detection of Bacteroidetes. However, we could only detect some Cytophaga-Flexibacter group with the CF319a probe in the leachate samples K1, YK1 and YK4 (Fig.IV.17). Neither type-I nor type-II methanotrophes were visualized with the probes MG64 and MA621 in any samples. Virtually no sulfate reducing bacteria (SRB) were detected in the leachate samples.

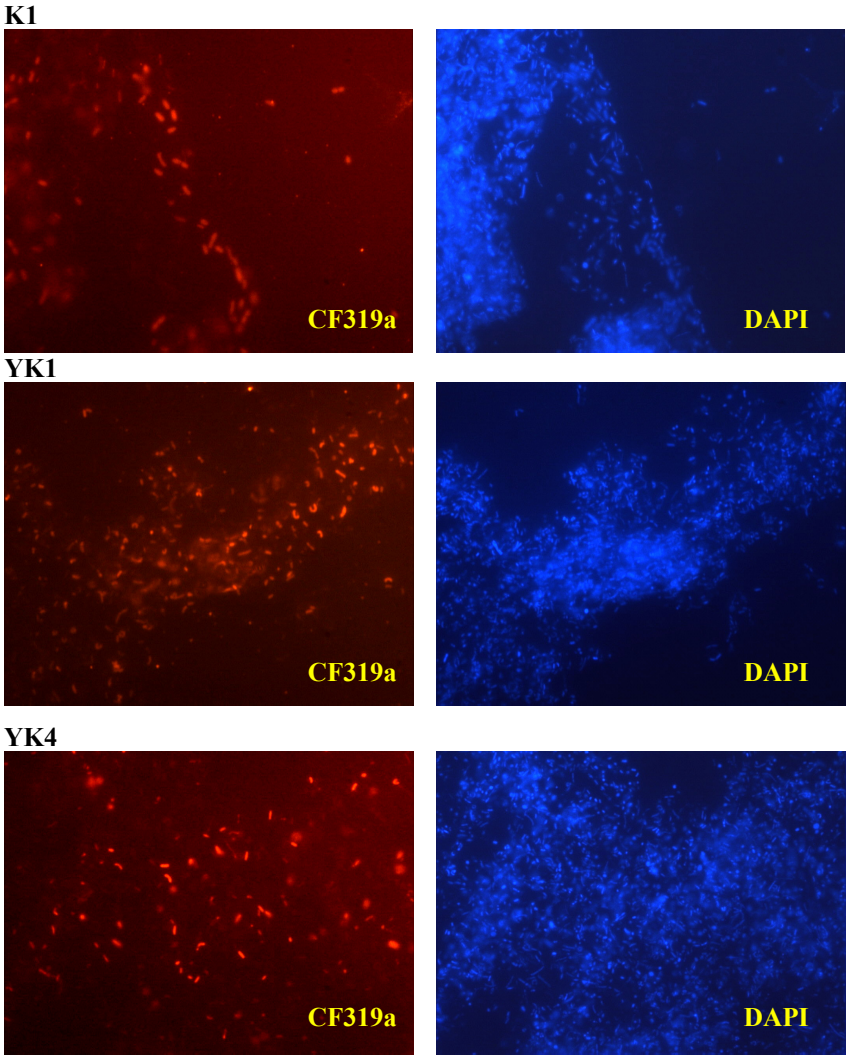


Figure: IV.17 *Bacteroidetes* Distribution of Leachate Samples

IV.2.3. Identification of Microorganisms by Cloning & Sequencing

For identification of dominant archaeal bands on DGGE gel, cloning analysis were applied to samples K1 and K3 and clones with frequencies of appearance higher than two were sequenced. Subsequently, amplicons of the cloned inserts were compared with the DGGE profiles of the samples by running them together on the same DGGE gel to identify the matching bands. After DNA sequencing and phylogenetic analysis of archaeal 16S rRNA clones, it was revealed that although some H_2-utilizing *Methanobacteriales* and *Methanomicrobiales* were present, acetate-utilizing *Methanosarcina* and *Methanosaeta* species were the major methanogenic archaea in acidogenic leachate sample K3. Clone group KA1 and KA42 showed 96.2% and 97.5% similarities to *Methanosaeta sp.*, respectively and

KA34 found 97.3% similar to *Methnosarcina siciliae*. While in all leachate samples only very small amounts of H_2-utilizing methanogens were detected with FISH analysis, some clones isolated from sample K1 clustered within *Methanomicrobiales* and *Methanobacteriales.* Clone group KA7 comprising 2 clones isolated from sample K1 showed 96.2% similarity to *Methanospirillum hungatei*, KA17 with 2 clones from K1 and 4 from K3 showed 97.1% similarity to *Methanofollis formosanus* and clone group KA64 consisted of 2 clones from sample K1 and K3 represented 99.3% similarity to *Methanothermobacter wolfeii* (Fig.IV.18). In the figure.IV.18 the significance of each branch is indicated by bootstrap values. The scale bar represents 0.02 inferred substitutions per nucleotide position. Sequences are submitted to the GenBank (DQ085318-DQ085324) and the accession numbers are shown in brackets. Sample source and numbers of identical clones among 73 16S rRNA clones are given in parentheses.

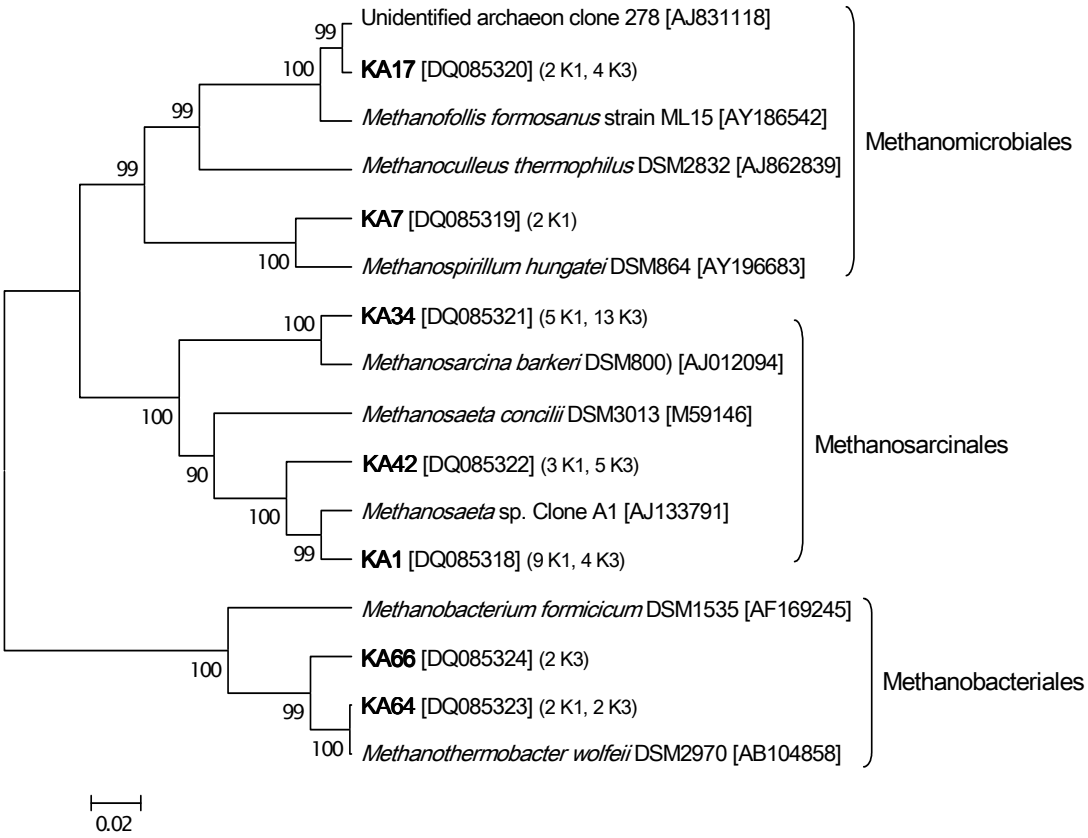


Fig.IV. 18 Neighbor-joining Tree of 16Sr RNA Clones from Leachate Samples K1 and K3.

The abundance of H_2-utilizing *Methanomicrobiales* especially in stabilized leachate samples was also verified with phylogenetic analysis of *mcrA* clones (Fig.IV.). While clone groups KM76, KM72 and KM82 showed similarities above 90%, group KM64 was 98.6% similar to *Methanoculleus bourgensis*. Group KM69 consisted of 2 clones isolated from sample K1 showed 87.0% similarity to an unidentified methanogen isolated from an anaerobic digestor. KM95 with 4 clones from acidogenic leachate sample K3 was found 91.9% similar to *Methanosarcina mazeii*. Another study based on the *mcrA* gene as an alternative to 16S rRNA in the phylogenetic analysis of methanogens in landfills revealed a greater diversity in the methanogenic population within the landfill and similarly reported high numbers of H_2-utilizing methanogens as compared with acetate-utilizing species in both of the young and mature landfill samples (Luton *et al*., 2002).

The significance of each branch in Fig.IV is indicated by bootstrap values. The scale bar represents 0.05 inferred substitutions per nucleotide position. Sequences are submitted to the GenBank (DQ085325-DQ085330) and the accession numbers are shown in brackets. Sample source and number of identical clones among 46 *mcrA* clones are given in parentheses.

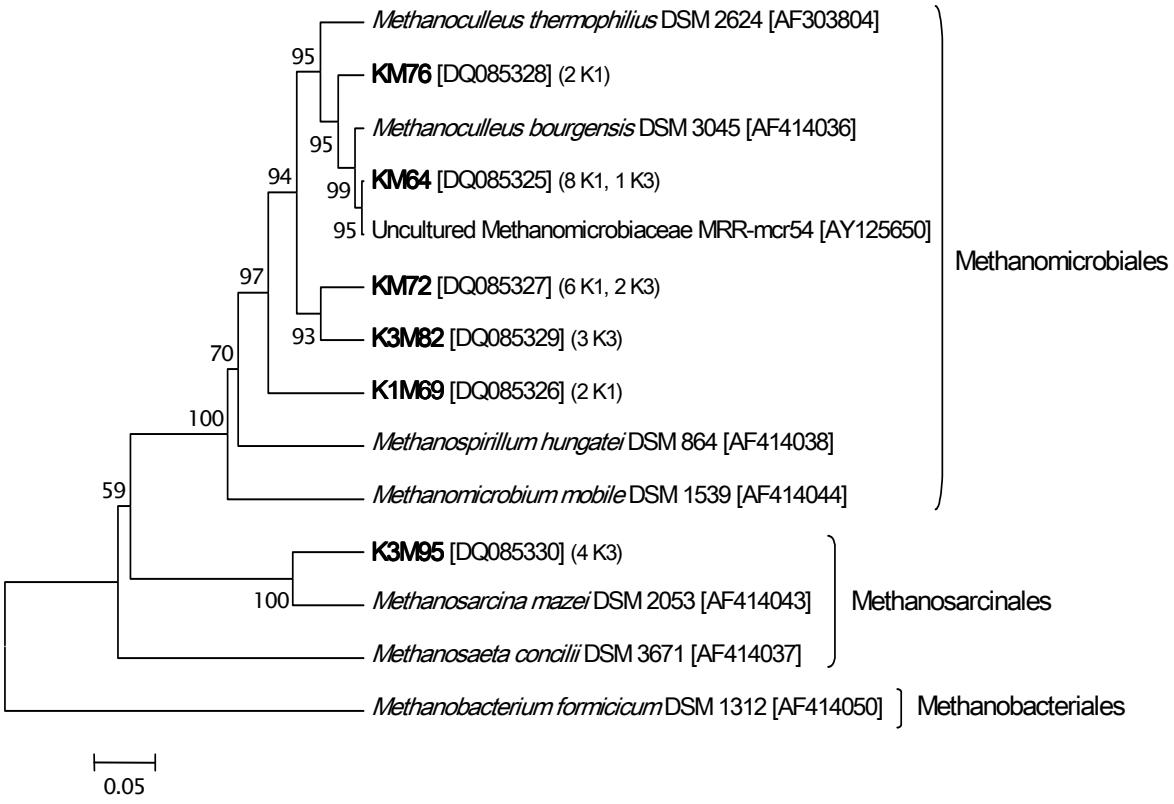


Fig.IV. 19 Neighbor-joining Tree of *McrA* Clones from Leachate Samples K1 and K3

CHAPTER V

DISCUSSIONS AND EVALUATIONS

In this study, a total of fourteen leachate samples originated from the different sections of Istanbul Kemerburgaz Sanitary Landfill were used for investigation of the prevalent microorganisms in MSW leachate. The study revealed that there are significant dissimilarities in microbial diversity between different sections of a landfill at different ages. Leachate samples were collected in three separate times. The sample group denoted by K (four samples) was collected in December 2003. The other groups denoted by YK (five samples) and YYK (five samples) were collected in December 2004 and March 2005, respectively.

The relations between chemical compositions and microbial constituents of leachate samples were investigated and strong correlations were found between the concentrations of organic pollutants, volatile suspended solids and the amount of microorganisms in leachate samples. The ratios like BOD_5: COD and TKN: NH_3-N aided in the phase determination of the lanfill sections where leachate samples originated from (Copa et al., 1995; Westlake, 1995). As biodegradation proceeds BOD_5: COD and TKN: NH_3-N ratios decreases gradually in time. On this respect, K3, K4, YK1, and YYK1 draw attention with their high BOD_5: COD ratios indicating the acidogenic phase. TKN: NH_3-N ratios of K3 and K4 leachates support that claim, however, that of YK1 and YYK1 contradicts with the BOD_5: COD ratio being very close to 1.1. The rest of the samples can be characterized as methanogenic since they have very low ratios and VSS values.

PCR-DGGE analyses of the leachate samples were quite challenging. The optimum conditions for PCR were found after many trials by changing the key elements of the procedure like concentrations of primers, Taq enzyme or the program used. The reason could be the inhibitory substances within the samples due to their heterogenous content. DGGE analyses of the leachate samples revealed that young age leachates (e.g. K3 - K4) shared similar archaeal and bacterial profiles as well as mature leachate samples did (e.g. YYK2 - YYK3). When *mcr*A gene was used as marker for archaeal communities excessive amount of bands were obtained for each sample as an indication of the need for optimization.

FISH experiments with fluorescently labelled oligonucleotide probes exposed the microorganisms inhabiting the leachate samples in a visual way. As the starting point, domain specific probes for archaea (ARCH 915) and bacteria (EUB 338) were utilized to get a general idea about the microbial composition of the samples. Then, group specific probes were used for each domain. Generally, for all samples bacteria were many times more than the amount of archaea free from the condition of the sample. That result is consistent with the findings of Mori et al. (2003) and Huang et al. (2003). They found that methanogens in the leachate constituted only a very small fraction (1% - 3%) of the total community.

Archaeal communities were investigated using the probes specific for acetoclastic (*Methanosarcina*, *Methanosaeta*) and hydrogentrophic (*Methanobacteria, Methanococcus*, and *Methanomicrobia*) methanogens. Very low signals of H_2-utilizing methanogens were detected in both young and mature samples. On the other hand, acetate-utilizing *Methanosarcina* was predominant in young age leachates as suggested by Huang *et al*. (2003). In K3 sample they were in the configuration of big clusters, but quite small and less in number in the samples YK1 and YYK1. M*ethanosaeta*, which is favored by low acetate concentration, existed in both YK1 and YYK1 more than the number of *Methanosarcina*. Other leachate samples either contained low amounts of *Methanosaeta* in the shape of long or medium-size thin rods or didn't contain any.

The bacteria amount was very high in young leachates (K3, K4, YK1, YYK1). It decreased significantly in old leachate samples. Without exception in all samples the most abundant bacteria group was gamma *Proteobacteria*. Beta-*Proteobacteria* followed it with relatively low intensities. However, low G+C gram-positive

bacteria and *Cytophaga-Flexibacter-Bacteroides* group were detected in inconsiderable amounts contradicting with the findings of Huang *et al*. (2004; 2005) and Burrell *et al*. (2004). Also neither type-I nor type-II methanotrophs were detected by FISH anaysis.

After DNA sequencing and phylogenetic analysis of archaeal 16S rDNA clones, it was revealed that although some H_2-utilizing *Methanobacteriales* and *Methanomicrobiales* were present, acetate-utilizing *Methanosarcina* and *Methanosaeta* species were the major methanogenic archaea in acidogenic leachate sample K3. On the contrary, most of the clones isolated from mature leachate samples were found similar to H_2-utilizing *Methanobacteriales* and *Methanomicrobiales* according to cloning analysis. The abundance of H_2-utilizing *Methanomicrobiales* in mature leachate samples was also confirmed with the results of the phylogenetic analysis of *mcrA* clones.

The results of cloning and sequencing are in agreement with the findings of Chen et al. (2003b) and Huang et al. (2003) which suggested that members of the genus *Methanosarcina* were dominant in young age leachate samples. However, Mori et al. (2003) detected the *Methanosaeta* species rather than *Methanosarcina* species in the leachate samples arising from a sea-based solid waste disposal site by cloning analyses. Another study by Luton et al (2002) includes the investigation of methanogen species in leachate by using mcrA gene as the target. This study also found that only one species of *Methanosatea* and a large number of species of *Methanosarcina* present in leachate samples.

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