T.C.
MARMARA UNIVERSITY
INSTITUTE FOR GRADUATE STUDIES IN
PURE AND APPLIED SCIENCES

INVESTIGATION OF MICROBIAL COMMUNITIES IN SANITARY AND BIOREACTOR LANDFILLS

Bülent MERTOĞLU

(Environmental Engineering, MSc)

THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

IN

ENVIRONMENTAL ENGINEERING PROGRAMME

SUPERVISOR
Prof. Dr. Ömer AKGİRAY

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ACCEPTANCE AND APPROVAL DOCUMENT

INVESTIGATION OF MICROBIAL COMMUNITIES IN SANITARY AND BIOREACTOR LANDFILLS

Established committee listed below, on …… and …… by the *INSTITUTE FOR GRADUATE STUDIES IN PURE AND APPLIED SCIENCES*' Executive Committee, have accepted Mr. Bülent MERTOĞLU's Doctor of Philosophy thesis, titled as "INVESTIGATION OF MICROBIAL COMMUNITIES IN SANITARY AND BIOREACTOR LANDFILLS" in Environmental Engineering.

COMMITTEE

Advisor :

Member :

Member :

Member :

Member :

Date of thesis' / dissertation's defense before the committee : ……

APPROVAL

Mr. Bülent MERTOĞLU has satisfactorily completed the requirements for the degree of Doctor of Philosophy in Environmental Engineering at Marmara University.
Mr. Bülent MERTOĞLU is eligible to have the degree awarded at our convocation on …… Diploma and transcripts so noted will be available after that date.

Istanbul **DIRECTOR**

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November, 2005 **Bülent MERTOĞLU**

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ABSTRACT

Investigation of Microbial Communities in Sanitary and Bioreactor Landfills

Main degradation processes that involved in landfills are not well understood and the microorganisms responsible for these processes are not very well known. In order to fully understand and characterize, microbial communities and activities, knowledge of their structure, diversity and function is necessary. In this study, microbial population diversities in sanitary and bioreactor landfills was monitored and analyzed by using molecular methods during stabilization periods of landfills.

Conventional landfill studies showed that methanogenic population diversity may be quite dissimilar in different landfill sites as well as in different stabilization phases. Acetate utilizing methanogens especially members of genus *Methanosarcina* were dominant in all young landfill leachate samples. On the other hand, only very few, long rod and filamentous methanogenic A*rchaea* belong to *Methanosaeta* genus were present in mature leachate samples.

In an aerated landfill bioreactor results showed that rapid bio-stabilization of landfilled MSW incineration bottom ash and shredded incombustible wastes are possible. Results also suggest that nitrification and denitrification can occur simultaneously in an aerated landfill bioreactor. In situ hybridization results have indicated that archaeal and bacterial activities increases with the acceleration of degradation process. It was revealed that *Methanobacteriales* and *Methanomicrobiales* were dominant methanogenic archaeal species at the beginning of bioreactor while *Methanosarcina* species were considerably dominant at the end of the one year operational period. *Nitrosomonas-like* ammonia oxidizing and *Nitrospira* related nitrite oxidizing bacteria were responsible for nitrification and intensively present nitrifiers in an aerated landfill bioreactor during one year operational periods.

November, 2005 **Bülent MERTOĞLU**

ÖZET

Düzenli ve Biyoreaktör Depo Sahalarındaki Mikrobiyal Toplulukların İncelenmesi

Günümüzde düzenli depo sahası ayrıştırma prosesleri ve ayrıştırmayı gerçekleştiren mikroorganizmalar tam olarak anlaşılamamaktadır. Mikrobiyal toplulukları ve aktivitelerini tam olarak anlamak ve karakterize etmek için mikroorganizmaların yapılarını, dağılımlarını ve görevlerini iyi bilmemiz gerekmektedir. Bu çalışma ile düzenli depo sahası ve havalandırmalı biyoreaktördeki mikrobiyal dağılım, stabilizasyon süreci boyunca, moleküler metotlar kullanılarak izlenmiş ve analiz edilmiştir.

Geleneksel depo sahalarında yapılan çalışmalarda farklı stabilizasyon fazlarında birbirinden çok farklı metanojenik popülasyon çeşitliliği tespit edilmiştir. Asetatı sübstrat olarak kullanan *Methanosarcina* türleri genç depo sahalarında baskın olarak görülmüştür. Diğer taraftan, uzun çubuk yapıdaki filament metanojen *Arke* türü, *Methanosaeta* olgunlaşmış depo sahalarında aktif olarak tespit edilmiştir.

Havalandırmalı depo sahası biyoreaktöründe, yakma tesisi taban küllerinin ve küçük parçalara ayrılmış yanmaya uygun olmayan atıkların stabilizasyonunun, hızlı bir şekilde gerçekleşebileceği gösterilmiştir. Ayrıca bu çalışmada, havalandırmalı biyoreaktör içerisinde nitrifikasyon ve denitrifikasyon eş zamanlı olarak gerçekleşmiştir. In-situ hibridizasyon sonuçları, *Arke* ve *Bakteri* aktivitelerinin, ayrışma proses hızıyla birlikte arttığını göstermektedir. *Arke* türleri içerisinde, ilk aylarda *Methanobacteriales* ve *Methanomicrobiales* türleri baskın olmakla birlikte zaman içerisinde bu türler azalmış ve yerini *Methanosarcina* türlerine bırakmıştır. Havalandırmalı depo sahasının bir yıllık işletme periyodunda, amonyağı nitrite oksitleyen bakterilerden *Nitrosomonas*, nitriti nitrata oksitleyen bakterilerden de *Nitrospira* türleri diğer nitrifikasyon bakterilerine göre baskın türler olarak görülmüştür.

Kasım, 2005 **Bülent MERTOĞLU**

CLAIM FOR ORIGINALITY

Investigation of Microbial Communities in Sanitary and Bioreactor Landfills

Main degradation processes that involved in landfills are not well understood and the microorganisms responsible for these processes are not very well known. In order to fully understand and characterize, microbial communities and activities, knowledge of their structure, diversity and function is necessary. In this study, microbial population diversities in sanitary landfills in Turkey and aerated bioreactor landfill in Japan was monitored and analyzed by using molecular methods.

Conventional landfill studies showed that methanogenic population diversity may be quite dissimilar in different landfill sites as well as in different stabilization phases. Acetate utilizing methanogens especially members of genus *Methanosarcina* were dominant in all young landfill leachate samples. On the other hand, only very few, long rod and filamentous methanogenic A*rchaea* belong to *Methanosaeta* genus were present in mature leachate samples.

In an aerated landfill bioreactor the results showed that rapid bio-stabilization of landfilled MSW incineration bottom ash and shredded incombustible wastes are possible. It was revealed that *Methanobacteriales* and *Methanomicrobiales* were dominant methanogenic archaeal species at the beginning of bioreactor while *Methanosarcina* species were considerably dominant at the end of the one year operational period. This is the first study that nitrification and denitrification was achieved simultaneously in one aerated bioreactor. *Nitrosomonas-like* ammonia oxidizing and *Nitrospira* related nitrite oxidizing bacteria were found responsible for nitrification in an aerated landfill bioreactor during one year operational periods.

November, 2005 **Prof. Dr. Ömer AKGİRAY** **Bülent MERTOĞLU**

LIST OF ABBREVIATIONS

MSW	**:** Municipal Solid Waste
WTE	**:** Waste to Energy
LFG	**:** Landfill Gas
BOD	**:** Biochemical Oxygen Demand
COD	**:** Chemical Oxygen Demand
VOA	**:** Volatile Organic Acids
MPN	**:** Most Probable Number
rRNA/DNA	**:** Ribosomal ribonucleic/deoxyribonucleic acids
FISH	**:** Fluorescence in Situ Hybridization
PCR	**:** Polymerase Chain Reaction
DGGE	**:** Denaturing Gradient Gel Electrophoresis
SRB	**:** Sulfate-Reducing Bacteria

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Solid wastes have serious impacts on public health and the environment if they are poorly stored, collected and disposed of. The most serious effects of poor solid waste management include air pollution, contamination of drinking water supplies and the spread of human diseases. It causes cities to become ugly and dirty, affects the health and morale of people, harms animals and plants, and hurts the economy and national pride. Integrated waste management techniques include source reduction, recycling, composting, incineration and landfilling. Landfilling is the most common method of solid waste disposal worldwide. Since it is the most prevalent management option, land disposal presents the greatest environmental challenges internationally. Preparation, management and control of the landfill must be the highest standard to minimize the risks to human health and the environment. Such preparation, management and control procedures should apply equally to the process of site selection, design and construction, operation and monitoring, closure and post-closure care.

A bioreactor landfill is a sanitary landfill that uses enhanced microbiological processes to transform and stabilize the readily and moderately decomposable organic waste constituents within 5 to 10 years of bioreactor process implementation. Bioreactor landfills can reduce the long-term pollution potential of the landfill by increasing the rate of waste settlement and stabilization, improving compaction densities, reducing the strength of leachate, and minimizing the long-term generation of landfill gas.

Reduction of stabilization time of landfills is an important task to minimize the post-closure care, maintenance, and risk, and to promote post-closure land utilization. However, it is difficult to accelerate the decomposition of refuse and stabilization of the landfills so as to shorten the regulated post-closure monitoring period. This is often too heavy a burden on landfill owners and regulators to achieve the long-term management of landfills and treatment of leachate after closure. Thus the cost effective accelerated landfill stabilization technology is required. The stabilization of landfills is judged from various aspects such as physical, chemical, and biological status, and biological processes such as organic compounds decomposition in the refuse and nitrogen removal in the leachate are essential. Therefore, establishment of the technologies and strategies for the best utilization of the microbial functions deeply related to the above mentioned processes is one of the important keys for the accelerated landfill stabilization. For this purpose, monitoring the functional microorganisms and the change of microbial community structure in landfills along with the measurement of physico-chemical indexes to elucidate the relationships between the biological status and stabilization process of the landfills is needed. The biological investigations in landfills will help to evaluate and optimize the previously proposed accelerated landfill stabilization technologies such as semi-aerobic landfills and leachate recirculation, and will lead to the establishment of biological indexes for the evaluation of the stabilization.

The main degradation process that is involved in a landfill bioreactor is not well understood, and the microorganisms responsible for these processes are not very well known. In order to fully understand and characterize the microbial communities and activities, knowledge of their structure, diversity and function is necessary. rRNA-based molecular methods have become the most important detection and identification methods in the determination of microbial diversity of complex microbial populations.

In this study, microbial population diversity in landfills was monitored and analyzed by using fluorescence in situ hybridization (FISH), slot-blot hybridization, polymerase chain reaction (PCR), and denaturing gradient gel electrophoresis (DGGE) techniques. These results were compared and evaluated with cloning and DNA sequencing data to understand the function of microbial diversities during stabilization periods of sanitary and bioreactor landfills.

CHAPTER II

GENERAL BACKGROUND

II.1. MUNICIPAL SOLID WASTE MANAGEMENT

Municipal solid waste (MSW), also called trash, garbage, refuse and rubbish, is the stuff we throw away everyday. The goal of sustainable solid waste management is the recovery of more valuable products from that waste with the use of less energy and a more positive environmental impact (McDougall *et al.,* 2001). The practice of the three R's (reduction, reuse, recycle) fits very well within the sustainable development concept. Rather than relying on a waste reduction hierarchy (Figure II.1), integrated solid waste management suggests optimization of the system. Per capita generation of municipal solid waste in the developed countries has increased threefold over the last two decades. It is predicted that waste generation in the developing countries will be doubling in the coming decade and global waste will be increased fivefold by 2025 (Brandsma, 1997). Indeed, how to resolve waste problem has become of enormous pressure for government policymakers.

Improper solid waste management leads to substantial negative environmental impacts (for example, pollution of air, soil and water, and generation of greenhouse gases from landfills), and health and safety problems (such as diseases spread by insects and rodents attracted by garbage heaps, and diseases associated with different forms of pollution). Municipal (or local) authorities charged with responsibility of providing municipal solid waste management services (together with other municipal services) have found it increasingly difficult to play this role. The difficulty has been aggravated by lack of effective legislation, inadequate funds and services, and

inability of municipal authorities to provide the services cost-efficiently. Changing lifestyles such as use of canned soft drinks, mobile phones, and disposable diapers (movement towards a "consumer society" in general), moreover, will pose special waste management challenges, as waste management systems in developing countries are incapable of frequent adjustment to match these lifestyle changes.

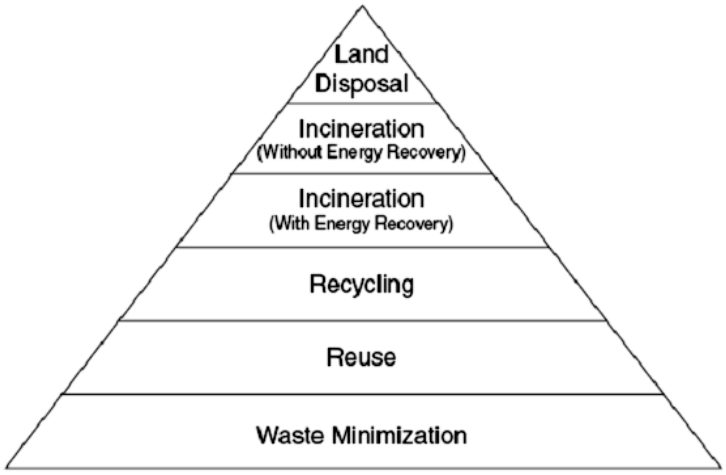


Figure II.1 Waste management hierarchy.

The composition of solid waste is a crucial element for defining waste management strategies. Moreover, 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. In Table II.1, different MSW compositions in different countries are given (Girgin, 2004).

Table II.1 Municipal solid waste composition in different countries (All data are in % by weight).

Material Groups	England	Germany	Greece	Syrian	China	USA	Japan
Organic Matter	19.8	44	48.5	72.5	60	27.5	32
Paper	34.8	17.9	22	5.0	3.1	41.1	38
Plastic	11.3	5.4	10.5	5.1	4.5	7.5	11
Glass	9.1	9.2	3.5	0.6	0.8	8.0	7
Metal	7.3	3.2	4.2	0.8	0.3	9.4	6
Others	12.2	20.3	11.3	16.0	31.3	6.5	7

Even at the high levels of income characteristic of most OECD members, the entire population may not be served by municipal waste services, indicating market

opportunities for the extension of basic household and commercial service. Among middle-income OECD members, the share of the population not yet served by MSW services was 14.9 percent in Hungary (2000), 15.0 percent in Greece (1997), 16.5 percent in Mexico (2000), 27.0 percent in the Russian Federation (1992), and 28.1 percent in Turkey (1998).

Table II.2 Municipal solid waste disposal methods for OECD member countries (OECD Environmental Data Compendium, 2002).

Country	**Year**	**Total**	**Recycling**	**Composting**	**Incineration**	**Landfill**
		(1000 tons)	%	%	%	%
Austria	1999	3,096	34	15	15	29
Belgium	1999	5,473	40	16	27	32
Canada	1998	9,926	30	11	N/A	N/A
Denmark	2000	3,546	22	16	52	10
Finland	1999	2,400	N/A	N/A	8	60
France	1999	30,744	10	8	33	48
Germany	1998	44,094	34	7	21	37
Greece	1997	3,900	8	N/A	N/A	91
Hungary	2000	4,084	N/A	N/A	9	91
Iceland	2000	192	9	2	9	81
Ireland	2000	2,302	8	1	0	91
Italy	1997	27,425	7	9	6	78
Korea	2000	16,950	4	N/A	12	47
Japan	1999	51,446	9	N/A	78	21
Luxembourg	1999	227	N/A	15	59	26
Mexico	2000	30,733	2	N/A	N/A	98
Netherlands	2000	9,691	23	24	41	13
Norway	2000	2,755	22	9	15	55
Poland	2000	12,226	N/A	2	3	98
Portugal	2000	4,531 6	6	21	67	
Russian Federation	1992	26,000	N/A	1	4	95
Slovak Republic	2000	1,706	2	5	12	62
Spain	1999	18,377	5	18	6	72
Sweden	1998	4,000	25	8	35	32
Switzerland	2000	4,681	32	14	48	6
Turkey	1999	24,945	N/A	1	N/A	96
United Kingdom	1999	33,200	9	2	8	81
United States	1999	208,520	22	6	15	57

Table II.2 provides information on the share of MSW destined for different disposal methods across OECD member countries. The share of MSW destined for landfills ranged from 17 percent (Denmark) to a high of 100 percent (New Zealand). Countries with high landfill use (between 90-100 percent of MSW) include Hungary, Iceland, Ireland, Mexico, New Zealand, Poland, and Turkey.

II.1.1. Municipal Solid Waste Management in Japan

A top priority of federal and local government in Japan has been to minimize the generation of wastes and reduce landfilling, by means of recycling and combustion to generate electricity. Japan is now one of the leading countries in managing waste generation. The rate of MSW generation in Japan (principally residential and commercial wastes) in fiscal 1999 totaled 53.7 million tons. This corresponded to a per capita generation of 0.42 metric tons per year (Matsunaga and Themelis, 2005). In Japan, 74.5% of the total MSW was combusted and only 20.3 % was landfilled, including ash from incineration.

Waste management in Japan is mainly the responsibility of local government. There are several waste-reduction methods such as reusing shopping bags or requesting people to bring their carryon bags while shopping; minimization of packaging materials; extending the life of products; and design of products so as to reduce the use of materials (de-materialization).

Recycling is very successful in Japan with regard to some materials: In 1999, an estimated 55% of the paper, 78-83% of metal cans, and 22.8% of PET bottles were recycled. Air-conditioners, television sets, washing machines and refrigerators are required to be recycled by the user, at a certain fee. The target is to close the material loop. The local government (e.g., at Nagoya, population 2.18 million) has organized recycling “flea” markets where people can buy used products and materials.

Japan has tried to reduce waste generation and re-use or recycle as much as possible of the MSW generated. Due to land limitation, the first priority in Japan has been to reduce the amount of landfilled waste. Presently 74.5% of waste goes to combustion plants and only 6.3% of the MSW is directly landfilled (Matsunaga and Themelis, 2005). There are 1717 combustion plants in Japan of total capacity of 193,00 tons per day; of these, 1103 (64.2%) are Waste-To-Energy (WTE) plants and

215 plants (12.5%) generate a total of 1060 MW. In comparison, there are 102 WTE facilities in the U.S. and generate 2800 MW.

In Tokyo, as more wastes began to be processed by incineration than landfills from 1974, incineration rate sharply increased as seen in Figure II.2. As of 1998, the Bureau of Waste Management, TMG has 18 incineration plants and these facilities process 87.1% of post-recycling and 100% of the Bureau-collected combustible waste. Meanwhile, landfills receive less waste over time, and two landfills are currently in service to process about 13% of municipal waste from the ward area of Tokyo (Yoon and Jo, 2002).

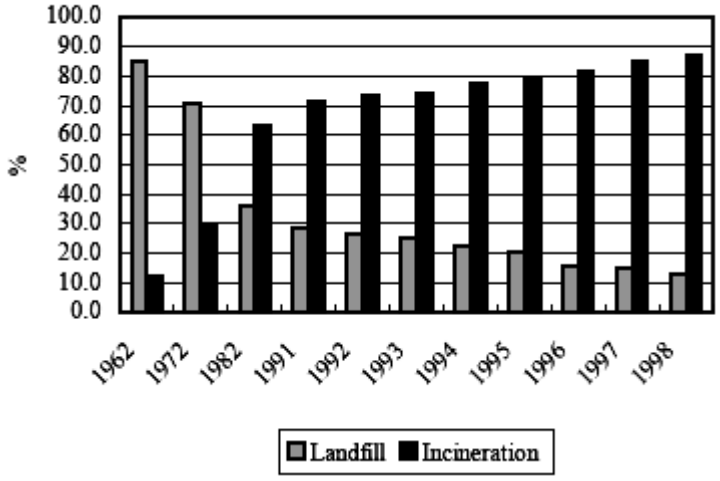


Figure II.2 Treatment of MSW in Tokyo.

However, since the landfilled material consists mostly of incombustible and incinerator ash, the demand for landfills as the final disposal method still remains. Although incineration rate is high, because of increasing pressures about environmentally sound waste disposal, rising disposal costs, and energy saving, recycling/resource recovery has become of the most attractive and desirable alternative for an eco-society with zero-emission that the Japanese government has pursued recent years. Indeed, recycling is not an ultimate purpose in itself. Rather, the most beauty that recycling can bring about is that it reduces the overall environmental load resulting from waste generation (Nakamura and Kondo, 2000). Recycled materials increased from less than 100,000 tons (recycling rate: 1.5%) in 1982 to 664,289 tons (recycling rate: about 13%) in 1998.

II.1.2. Municipal Solid Waste Management in Turkey

Waste is controlled by regulations on Control of Solid Wastes, Control of Medical Wastes and Hazardous Waste Control Management with the aim of assessing any adverse impacts. The responsible authorities for solid waste management in Turkey are the ministries of Environment, Industry and Trade, Interior Affairs, Public Works and Settlement; municipalities; the chambers of trade and industry; and the Turkish Standards Institute.

The majority of the household waste in Turkey is organic in nature (Table II.3). 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).

Table II.3 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. (Figure II.3-4).

State Institute of Statistics has been collecting data on the current status of waste services and waste disposal sites of all municipalities in Turkey within the scope of Environmental Statistics since 1994. According to the results of municipal solid waste statistics, wastes of 3018 municipalities out of 3215 were collected in 2003.

The amount of solid waste collected from municipalities receiving waste collection services was realized as 12.86 million tones in summer, 13.26 million

tones in winter and an annual average of 26.12 million tones. From these results daily amount of solid waste per capita is calculated as 1.32 kg/capita-day in summer, 1.34 kg/capita-day in winter and 1.34 kg/capita-day for yearly average.

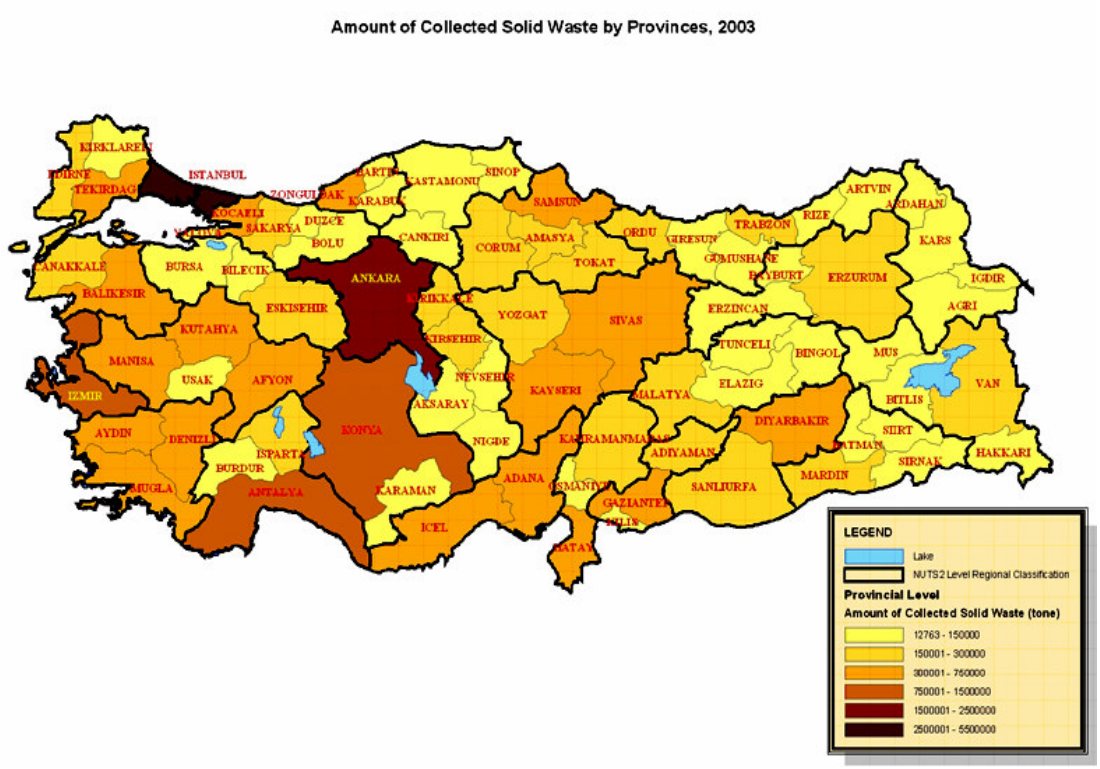


Figure II.3 Amount of collected solid waste by provinces (SIS, 2003).

Of the 26.12 million tones of solid waste collected municipalities in 2003, 45.3% was disposed of in municipality's dump, 28.5% was disposed of in controlled landfill, 15.2% was disposed of in metropolitan municipality's dump, 2.9% was disposed of in another municipality's dump, 2.3% was disposed of by burial, 1.2% was disposed of in composting plant, 1.0% was disposed of by burning in an open area, 0.9% was disposed of into lake and river (Figure II.4).

As it is seen in the Figure II.4, only a small fraction of the generated waste is composted or incinerated, and the unprocessed part is sent directly to waste disposal sites. While the numbers of sanitary landfills are increasing in numbers, especially with the new sites operating in certain large cities and in some touristy regions, the amount of sanitary landfilling is still less than 50%. Disposal of municipal solid waste into uncontrolled garbage dumps that are not properly regulated is the most important problem regarding solid waste management in Turkey. On the other hand,

inappropriate removal methods such as open burning or pouring into rivers that can pose risk to both environmental impact and public health are still being applied.

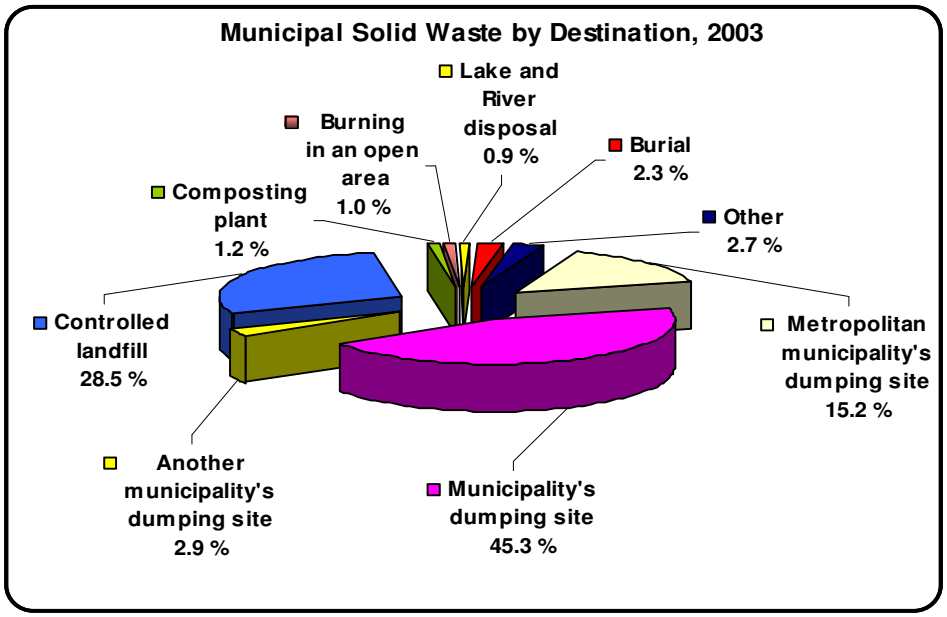


Figure II.4 Municipal solid waste by destination (SIS, 2003).

Percentages of employed disposal methods in different countries are represented in Table II.2. When disposal strategy in Turkey is compared with other countries, it is revealed that the application of appropriate methods like landfilling, incineration or composting is crucially insufficient in Turkey.

In Table II.4, data about capacities and numbers of plants using for treatment of solid waste is represented. Landfilling and composting are the major processes used for the treatment of municipal solid waste. Moreover, incineration is used for only removal of medical wastes.

Table II.4 General information about waste removal processes in Turkey (SIS, 2001).

Process for Removal	**Municipal Solid Waste**		**Medical Waste**
	Landfilling	Composting	Incinerating
Number of plant	12	3	3
Total Capacity	261,3 million tones	299 000 tones	44 000 tones
Total amount of removed waste	8,3 million tones	218 000 tones	11 000 tones

Industrial wastes for 1995 amounted to 17.5 million tones of which 7 to 8 million tones were hazardous waste. Mostly industrial solid waste is stored in waste disposal dumps and mixed with municipal waste in landfill sites. Concerning clinical

waste the existing data is incomplete; however, calculations based on hospitals' capacity estimate the annual amounts to be around 7.5 million tones.

Since clinical waste is not measured properly, the nature of its disposal is not quite clear. The principal management option adopted is incineration. The number of incineration plants is limited (six such facilities operating in Turkey) due to their high operational cost. However, it is believed that much medical wasted gets mixed with municipal refuse. There are 1,120 hospitals in Turkey and the total number of beds is 160,884.

There is only one hazardous waste disposal facility in Turkey, obviously inadequate for the current level of waste production. The lack of infrastructure results in large percentages of hazardous waste reported as sold (25 percent) or disposed of in uncontrolled ways (66 percent). The recycling of industrial waste could be improved if organizations such as the chambers of industry established waste exchange inventories and other schemes to facilitate the use of one enterprise's waste as an input to another (Okumuş, 2002).

II.1.3. Management of MSW Incinerator Residues

Biological treatment technologies (composting, anaerobic digestion, etc.) are now reemerging as commercially viable means to permanently remove the organic material fraction from the waste stream. Because the success of these technologies relies on securing a stable market for the treated product, countries are implementing regulatory measures to ensure that compost quality is commensurate with the intended application of the product. Typically, this has resulted in a move away from mixed solid waste processing to the processing of only the putrescible fraction of the waste stream (garden, kitchen and commercial food wastes).

The main objectives of MSW incineration are to sterilize the waste and reduce the volume of material requiring final disposal. The majority of new incineration facilities are also designed for energy recovery, either in the form of electricity or process steam for industry or district heating. Over the past decade, the concern over air emissions from these facilities has resulted in most countries adopting very stringent air emission control regulations which have increased the cost of constructing and operating incinerators. However, some countries are now implementing new measures to reduce the volumes of post-recycled waste destined

for landfill by limiting the organic content of the material to less than 5%, thus promoting the use of incineration systems within an integrated waste management strategy (Sakai *et al.,* 1996).

Although MSW incineration is capable of reducing the volume of waste by 90%, 20-30% of the original weight of the waste is left as ash which requires further management. There are two generic ash streams discharged from incinerators. Bottom ash is generally defined as the material collected off the incineration grates, whereas fly ash is a collective term for the finer material captured downstream of the furnace, i.e. in the heat recovery and air pollution control system.

In most countries, these two streams are classified and managed differently due to the significant differences in their physical, chemical and leaching characteristics. Although most countries have deemed bottom ash suitable for disposal in landfills or monofills, many European countries have also permitted extensive use of processed bottom ash in various construction applications.

In general, the classification of an ash stream, and determining how it needs to be managed, is based on the trace metal analytical results from regulatory leach tests compared against established regulatory limits. As indicated in Table II.5, these regulatory tests and the respective limits differ significantly within the seven countries.

One objective of landfilling of waste, including MSWI residues, is to remove from general circulation materials and products that are no longer useful in any respect. It is preferable to do this in a manner that ultimately returns the basic constituents of the waste to the ecological cycle, possibly after they have undergone chemical and/or physical reactions and transformations. A second and equally important objective of waste disposal is to ensure that the waste does not cause any unacceptable short- or long-term impact on the environment or on human health. Disposal methods must ensure that this is accomplished in a sustainable manner, i.e. without excessive and/or prolonged maintenance or operation requirements and without a prolonged need for aftercare (Sabbas *et al.,* 2003).

Table II.5 Energy recycling of MSW incineration.

Item	Canada	Denmark	Germany	Netherlands	Sweden	USA	Japan
Area	9,980,000 km^2	43,000 km^2	357,000 km^2	42,000 km^2	450,000 km^2	9,160,000 km^2	378,000 km^2
Population	29 x 10^6 (1995)	5.2 x 10^6 (1995)	82 x 10^6 (1995)	15 x 10^6 (1995)	8.9 x 10^6 (1995)	263 x 10^6 (1995)	125 x 10^6 (1994)
MSW generate	23.2 x 10^6 tons (1994)	2.6 x 10^6 tons (1993)	43.5 x 10^6 tons (1993)	12.8 x 10^6 tons (1993)	3.2 x 10^6 tons (1991)	207 x 10^6 tons (1993)	50.2 x 10^6 tons (1992)
MSW combusted	1.2 x 10^6 tons (1992)	1.5 x 10^6 tons (1994)	11.0 x 10^6 tons (1993)	2.8 x 10^6 tons (1993)	1.7 x 10^6 tons (1991)	32.9 x 10^6 tons (1993)	37.3 x 10^6 tons (1992)
Percentage combusted	5% (1992)	58% (1994)	25% (1993)	23% (1993)	55% (1991)	16% (1993)	74% (1992)
Bottom ash generated	0.3 x 10^6 tons (1993)	0.5 x 10^6 tons (1993)	3.0 x 10^6 tons (1993)	0.65 x 10^6 tons (1993)	0.4 x 10^6 tons (1990)	6.8 x 10^6 tons (1990)	5.0 x 10^6 tons (1991)
Bottom ash used	0% (1993)	90% (1993)	60% (1993)	90% (1993)	0% (1990)	0% (1990)	0% (1991)
APC residues generated	0.02 x 10^6 tons (1993)	0.05 x 10^6 tons (1993)	0.3 x 10^6 tons (1993)	0.09 x 10^6 tons (1993)	0.06 x 10^6 tons (1990)	0.91 x 10^6 tons (1990)	1.16 x 10^6 tons (1991)
Number of facilities	17	31	53	11	21	148	1841 (1991)

II.2. SOLID WASTE MANAGEMENT TECHNIQUES

Solid waste management is an integral part of public health and environmental control, being of particular importance in highly populated urban areas. Over the last twenty years, waste management has begun to emerge in developed countries as a scientific and engineering profession in its own right. Environmental standards of refuse incineration and landfilling have gradually improved, and new methods of refuse sorting and resource recovery have begun to emerge. With complex legislation and control systems, and networks of sophisticated treatment and disposal facilities, being developed in parallel. The political priority given to waste management has increased sharply, largely due to public concern over well publicised incidents.

Solid waste management techniques vary from country to country depending upon physical geography, demographics, and level of economic development. Most industrialized countries have regular solid waste collection and disposal services. Most waste disposal sites are required by law to have at least some environmental prevention and control technologies. In contrast, most developing countries provide formal waste collection and disposal services to only a portion of the population, the urban poor as well as residents of rural areas often have no formal collection services or designated dumping areas. Even when areas are designated as disposal sites, often they are open dumps that pose serious threats to public health and the surrounding environment.

Integrated waste management techniques include source reduction, recycling, composting, incineration and landfilling. Landfilling is the most common method of solid waste disposal worldwide. Since it is the most prevalent management option, land disposal presents the greatest environmental challenges internationally.

II.2.1. Composting

Composting is a natural process by which *Bacteria* and other microorganisms break down organic matter into simple nutrients in the presence or absence of air (oxygen). Where the composting takes place in the absence of air, the process is called anaerobic composting and where it takes place in the presence of air, it is called aerobic composting (Tchobanoglous *et al.,* 1993). Composting (wherein elements conducive for the process of breaking down of the organic matter like air,

moisture, micro fauna, etc. are introduced) has been identified as the most efficient way of converting municipal organic waste into manure, thereby recycling nature's resource to nutrients. Aerobic composting is the most widely accepted way of composting organic wastes. This can be carried out in several ways and stages. The aim of organic composting is two fold; that of breaking down the complex organic matter into a simpler and acceptable form for the plants to absorb, and that of improving the nutrient (NPK - Nitrogen, Potassium, Phosphorus) value of the organic matter to increase yield by the plants.

It is important to view compostable materials as usable, not as waste requiring disposal. When developing and promoting a composting program and when marketing the resulting compost, program planners and managers should stress that the composting process is an environmentally sound and beneficial means of recycling organic materials, not a means of waste disposal.

In the broadest sense, any organic material that can be biologically decomposed is "compostable". In fact, humans have used this naturally occurring process for centuries to stabilize and recycle agricultural and human wastes. Today, composting is a diverse practice that includes a variety of approaches, depending on the types of organic materials being composted and the desired properties of the final product.

II.2.2. Incineration

Incineration is an important component of the integral management of municipal solid wastes (MSWs) in several countries. The incineration process transforms organic materials in CO_2 and H_2O but yields inorganic residues, from ferrous and non-ferrous metals to silicates. These residues can be broadly classified as bottom and fly ashes. The former is the by-product of the combustion process, whereas fly ashes are the solid residues derived from the combustion chamber which are collected from the reactor and filters. Bottom ash, which is the residue produced in greatest amount (25% of the incinerating mass), is mainly composed of Si, Fe, Ca, Al, Na and K in the form of oxides, and thus, presents a similar composition to that of geological materials.

The chemical properties of ash exhibit a wide range of constituents. Essentially, the total quantity of metals in the ash will equal the quantity of metals in

the bio-solids being incinerated. Because incineration has the effect of reducing the total mass of material, the concentration of metals in the ash is greater.

Some metals will vaporize at the temperatures found in an incinerator and exit with the exhaust gas. Metals that are expected to partially vaporize during incineration are cadmium, lead, mercury and zinc.

Usually, excess air is supplied to the incinerator in order to ensure complete mixing and combustion. The combustion principle gas products include carbon dioxide, carbon monoxide, water, oxygen, and oxides of nitrogen. Excess air is also added to the incinerator to regulate operating temperature and control emissions. Excess air requirements will differ with waste moisture contents, heating values, and the type of combustion technology employed.

Many incinerators are designed to operate in the combustion zone at 1,800 °F to 2,000 °F. This temperature is selected to ensure good combustion, complete elimination of odors, and protection of the walls of the incinerator. A minimum of 1,500 °F is required to eliminate odor. As more excess air is supplied to the incinerator, the operating temperature is lowered.

Waste-to-energy systems are designed to maximize waste burn out and heat output while minimizing emissions by balancing the three -T- time, temperature, and turbulence - plus oxygen (air). The heterogeneous nature of municipal solid waste requires that waste-to-energy systems be carefully designed to operate efficiently over a wide range of waste input conditions.

Interest in slagging combustion processes for municipal bio-solids is strong in Europe and Japan, because high temperatures ensure volatile organic compound destruction and glassified ash stabilizes heavy metals so that they can not be leached. This technology is in the developing phase, and the available database is inadequate to define if whether there is metal fuming at elevated temperatures. The majority of ash generated by bio-solids incineration is disposed of in either Municipal Solid Waste (MSW) landfills or ash monofills. Due to the solids concentration within the ash, dust control is a significant issue.

II.2.3. Landfilling

Landfilling is defined as “an engineered method of disposing of solid wastes on land in a manner that protects the environment, by spreading the waste in thin layers,

compacting it to smallest practical volume, and covering it with compacted soil by the end of each working day or at more frequent intervals if necessary". Preparation, management and control of the landfill must be the highest standard to minimize the risks to human health and the environment. Such preparation, management and control procedures should apply equally to the process of site selection, design and construction, operation and monitoring, closure and post-closure care.

Construction and operation of landfills is generally straightforward, despite the complexity of the natural processes involved in waste decomposition. Before wastes are deposited in the ground, a site is often excavated to allow more waste to be deposited on a given plot of land. The lowest component of a landfill is the liner system, which includes drains and impermeable barriers designed to minimize the migration of leachate to groundwater. Liners usually consist of layers of compacted clay and geo-membrane material (Tchobanoglous *et al.,* 1993).

As solid waste is deposited in the landfill, waste is compacted and covered daily. The total amounts of waste a site can receive are determined by the area of the plot and by the maximum slope of the sides of the landfill that still ensure slope stability. Once waste has reached the final design level of the landfill, 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.

Prior to landfilling, waste may be shredded or baled. The purpose of shredding is to increase the compaction rate and thus the capacity or life expectancy of the landfill. Indeed, shredded waste can be compacted to a density approximately 27% greater than unshredded waste. Moreover, gas production and land setting occur over a shorter period of time in shredded waste landfills, thus reducing site maintenance. On the other hand, the shredding process entails additional costs.

The major types of MSW landfill are the area and the canyon landfills. The area landfill is generally used in a rolling terrain where cover soil can be obtained from an area adjacent to the landfill itself. Through proper coordination, the cover soil is brought in as necessary to provide the various forms of cover and to prepare the berms (Figure II.5).

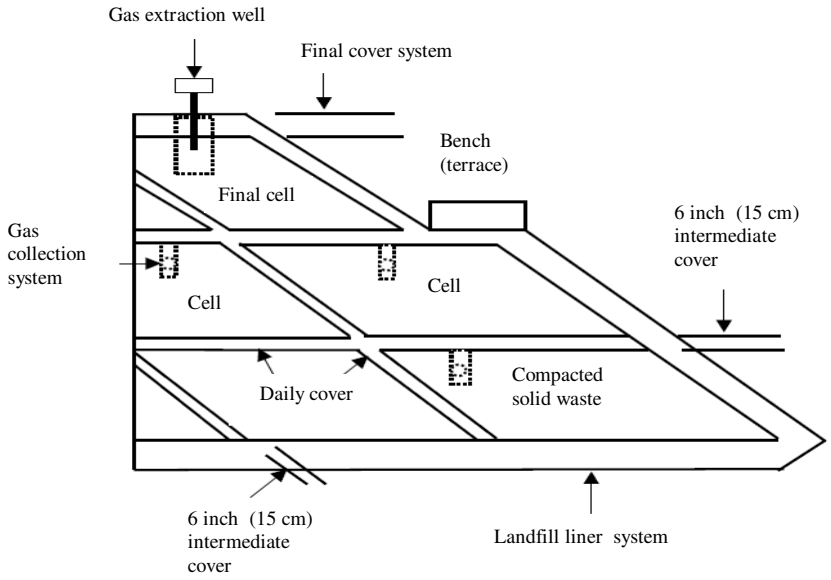


Figure II.5 Section view through a typical solid waste sanitary landfill.

After a load of solid waste is deposited in a landfill cell, the decay process that essentially constitutes the backbone of stabilization, involve both aerobic and anaerobic processes. The nature of microbial growth and the landfill environment will give rise to a succession of major types of microorganisms depending on the stages of stabilization.

As waste degrades, it compacts as mass is converted to methane and escapes. The capacity of a landfill is defined by the available volume is a function of plot area and maximum slope. Faster decomposition provides faster settling in turn yields more waste capacity in a given landfill resulting in less need for more landfills. Understanding the decomposition processes is therefore important to reducing the need for more landfills.

Within the landfill biological, chemical, and physical processes occur that promote the degradation of wastes and result in the production of contaminated leachate and gas. Thus, the landfill design and construction must include elements that permit control of landfill leachate and gas.

II.3. LANDFILL BIOREACTOR TECHNOLOGIES

A bioreactor landfill is a sanitary landfill that uses enhanced microbiological processes to transform and stabilize the readily and moderately decomposable organic waste constituents within 5 to 10 years of bioreactor process implementation. The bioreactor landfill significantly increases the extent of organic waste decomposition, conversion rates and process effectiveness over what would otherwise occur within the landfill. Stabilization means that the environmental performance measurement parameters (landfill gas composition and generation rate and leachate constituent concentrations) remain at steady levels, and should not increase in the event of any partial containment system failures beyond 5 to 10 years of bioreactor process implementation.

Bioreactor landfill technology is not a new idea. Its genesis springs from the systematic treatment of wastewater that began in the late 1800s and early 1900s (Tchobanoglous and Burton, 1991). Bioreactor landfills can be thought of as an extension of anaerobic and aerobic digestion at wastewater treatment plants. They accelerate the biodegradation rate of MSW by adding leachate/water and possibly air and some nutrients.

Landfills continue to generate leachate and gas for decades following waste placement, possibly beyond the current 30-year post-closure monitoring period. Research has shown that a significant portion of the biodegradable fraction of waste placed in conventional MSW landfills remains relatively unstabilized following decades of landfilling (Rathje, 1999). Environmental impacts related to gas generation, leachate contamination of groundwater, and the long-term structural integrity of the containment system require long-term monitoring.

Bioreactor landfills represent a fundamentally safer and better method of land disposal than the currently defined conventional landfills since waste is stabilized more rapidly. Bioreactor landfills can reduce the long-term pollution potential of the landfill by increasing the rate of waste settlement and stabilization, improving compaction densities, reducing the strength of leachate, and minimizing the long-term generation of landfill gas (Reinhart and Townsend, 1998).

The bioreactor landfill requires certain specific management activities and operational modifications to enhance microbial decomposition processes. The single

most important and cost-effective method is liquid addition and management. Other strategies, including waste shredding, pH adjustment, nutrient addition, waste pre-disposal and post-disposal conditioning, and temperature management, may also serve to optimize the bioreactor process. Successful implementation also requires the development and implementation of focused operational and development plans.

The bioreactor landfill is a waste treatment system. During landfill operations, it requires closer attention to system performance than the drier landfill. Successful operation of a bioreactor landfill depends upon control and monitoring of biological, chemical, and hydrologic processes occurring within the landfill. Operational and maintenance programs addressing settlement, landfill gas, and leachate may be reduced to a minimal level once the landfill is closed and the refuse is largely stabilized.

Bioreactor operations should be concentrated on waste segregated to maximize its organic content and shredded, flailed, or otherwise manipulated to increase its exposed surface area. Waste segregation could include separation of construction and demolition (C&D) wastes from MSW. Limited shredding can be obtained by spreading refuse in thin lifts and using landfill equipment to break open plastic bags and break down containers. Mechanical shredding can be efficient and effective in reducing particle size and opening bags; however it is an intensive, high maintenance and high cost activity, which may not be cost-effective. Moreover, shredded wastes may become exceedingly dense after placement, thereby limiting moisture penetration.

Leachate recirculation is the fundamental process used in bioreactor landfills to treat the contained waste (Reinhart and Townsend, 1998). The U.S. EPA reported that more than 200 landfills use leachate recirculation as a means of leachate management (Reinhart and Carson, 1993). Leachate is produced in landfills as a result of water entering and moving through solid waste and may contain dissolved or suspended material associated with the waste, as well as byproducts of biological and chemical reactions. Leachate may also contain hazardous and toxic constituents that are found in the waste (Reinhart and Townsend, 1998).

Bioreactor landfills can be categorized broadly as aerobic or anaerobic. However, there are also ongoing studies of aerobic/anaerobic and semi-aerobic bioreactors, which combine elements of both aerobic and anaerobic systems.

II.3.1. Aerobic Landfill Bioreactor

Aerobic bioreactors operate by the controlled injection of moisture and air into the waste mass through a network of horizontal and/or vertical pipes. Aerobic landfill processes are analogous to wet composting operations in which biodegradable materials are rapidly biodegraded using air, moisture, and increased temperatures created by biodegradation. Prior to air injection, liquid is pumped under pressure into the waste mass through injection wells in order to wet the waste mass to moisture content between 50% and 70% by weight. Once optimal moisture conditions have been reached, air injection commences (Figure II.6).

Blowers typically are used to force air into the waste mass through a network of perforated wells that have been installed in the landfill. The rates of injection of air and leachate into the landfill are similar to the air and moisture application rates used in many composting systems. The aerobic process continues until most of the easily and moderately degradable compounds have been degraded and the compost temperature gradually decreases during the final phase of "curing" or maturation of the remaining organic matter.

Optimum temperatures for waste degradation within an aerobic bioreactor landfill are between 140º and 160ºF. Due to the substantial amounts of heat generated, large quantities of leachate can be evaporated. Hudgins and Green (1999) reported leachate volume reductions of 86% and 50% at two aerobic bioreactor landfills. Waste temperatures are controlled by changing the rate of air and liquid injection. The potential for waste combustion typically is managed by ensuring that the waste mass is wetted adequately and air injection is uniform throughout the waste mass to minimize methane generation. Waste temperatures are maintained in the optimal range, and only enough air is injected into waste to support aerobic biodegradation.

Aerobic bioreactor landfills are much more operationally intense than anaerobic bioreactor landfills. Weathers *et al.* (2001) determined that the additional power required to inject air into an aerobic bioreactor was 12 times higher than the power required extracting landfill gas in an anaerobic bioreactor. However, post-closure costs should be reduced substantially due to reductions in landfill gas generation and cover settlement.

Because of higher reaction rates, aerobic biodegradation is a more rapid process than anaerobic biodegradation. Consequently, aerobic landfills offer the potential to achieve the same waste stabilization in two or four years that conventional landfills require decades or longer reaching. The rapid rate of waste stabilization in aerobic landfills offers the potential for mining of the landfill waste.

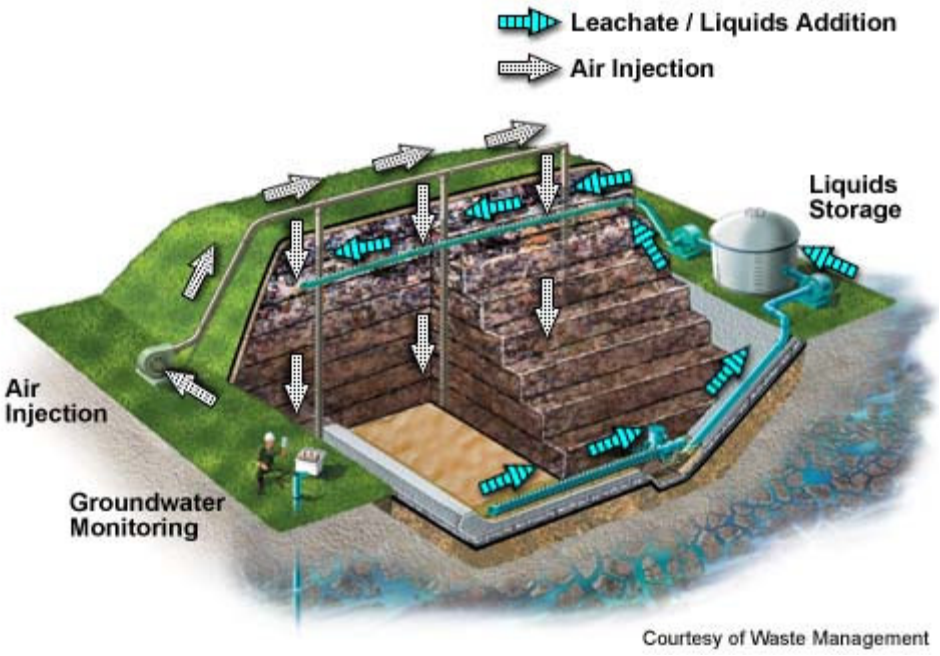


Figure II.6 Aerobic landfill bioreactor.

II.3.2. Anaerobic Landfill Bioreactor

Anaerobic bioreactor landfills seek to stabilize landfilled waste rapidly by the addition of moisture to uniformly wet the waste mass. Landfill degradation of MSW frequently is rate-limited by insufficient moisture. The maximum methane production in landfills occurred at moisture content of 60-80% wet weight. This suggests that most landfills are well below the optimum moisture content for methane production. Also, the liquid absorptive capacity is about 16-29% or 30-60 gal/yd^3 of waste which represents a large potential capacity for leachate storage.

In an anaerobic bioreactor landfill, moisture is added to the waste mass in the form of re-circulated leachate and other sources to obtain optimal moisture levels. Biodegradation occurs in the absence of oxygen and produces landfill gas. Landfill gas, primarily methane, can be captured to minimize greenhouse gas emissions and

for energy projects. Table II.6 compares conventional landfills with anaerobic and aerobic bioreactor landfills.

Liquid can be injected into the waste via horizontal trenches, vertical wells, surface infiltration ponds, spraying, and prewetting of waste (Figure II.7). Anaerobic bioreactor landfills initially should be carefully monitored. If the waste is wetted too rapidly, a buildup of volatile organic acids might lower the leachate pH, inhibiting the methane-producing *Bacteria* population and reducing the rate of biodegradation. Leachate parameters (such as pH, volatile organic acids, and alkalinity) and LFG (landfill gas) parameters (such as methane content) are direct indicators of an established methane-producing *Bacteria* population. Optimal conditions for methane-producing *Bacteria* are a pH of greater than 6.5. A high volatile organic acids-to-alkalinity ratio (>0.25) indicates that the leachate might have a low buffering capacity and conditions could soon inhibit methane generation.

Table II.6 Comparison of bioreactor landfills.

	Conventional Landfill	**Anaerobic Bioreactor**	**Aerobic Bioreactor**
Typical settlement after 2 years	2-5%	10-15%	20-25%
Typical settlement after 10 years	15%	20-25%	20-25%
Anticipated Waste-Stabilization Time Frame	30-100 years	10-15 years	2-4 years
Methane Generation Rate	Base case	2 x base case	10-50% base case
Liquid Storage Capacity Utilized in Waste Mass	None	30-60 gal./yd.^{3}	30-60 gal./yd.^{3}
Liquid Evaporation	Negligible	Negligible	50-80 %
Average Capital Cost	Low	Medium	High
Average O&M Cost	Low	Medium	High
Average Closure/ Post-closure Cost	High	Medium	Low

The gas content of anaerobic bioreactors is similar to that of conventional landfills, with methane and carbon dioxide each making up approximately 50% of the total LFG volume. When the methane content of the LFG exceeds approximately 40%, the methane-producing *Bacteria* population can be considered established. A decrease in the methane gas content below 40% is a possible indication that the waste is becoming too wet or dry. Once the methane-producing *Bacteria* population

has become established, the rate of leachate recirculation may be increased (Campman and Yates, 2002).

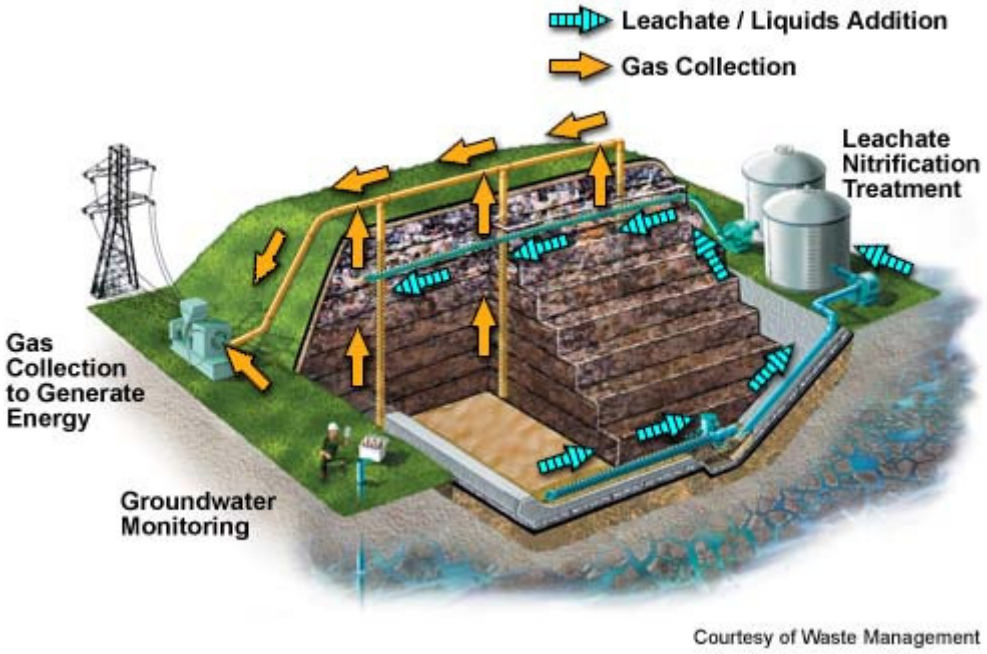


Figure II.7 Anaerobic landfill bioreactor.

II.3.3. Stabilization Phases of Landfill

A MSW landfill does not have a single waste age, but rather different ages associated with the various cells within the landfill and their respective stabilization stages (Pohland *et al.,* 1993). As a result, the different landfill stabilization phases often overlap. These phases are usually viewed collectively which tends to limit understanding of their progression. Operating a MSW landfill as a bioreactor has an effect only on the rates and not the sequence of the degradation phases (Pohland and Al-Yousfi, 1994; Reinhart and Townsend, 1998; Kim and Pohland, 2003). It is important for those responsible for landfill management to understand each of these events.

Figure II.8 illustrates the five sequential phases of landfill stabilization. Since landfills have various sections, a landfill is not experiencing a single phase of waste stabilization but rather many phases of stabilization are occurring simultaneously.

Initial adjustment phase is associated with initial placement of solid waste and accumulation of moisture within landfills. An acclimation period is observed

until sufficient moisture develops and supports with an active microbial community. Preliminary changes in environmental components occur in order to create favorable conditions for biochemical decomposition. During this first stage of decomposition, aerobic microorganisms degrade the organic materials to CO_2, H_2O, and partially degraded residual organics; producing considerable heat (McBean *et al*., 1995). 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). Any leachate produced during this initial phase is most likely a result of moisture squeezed out of the waste during compaction and cell construction (Lu *et al*., 1985). Leachate formed during this phase is characterized by the entrainment of particulate matter, dissolution of highly soluble salts initially present in the landfill, and the presence of relatively small amounts of organic species from aerobic degradation (Lu *et al*., 1985; McBean *et al*., 1995).

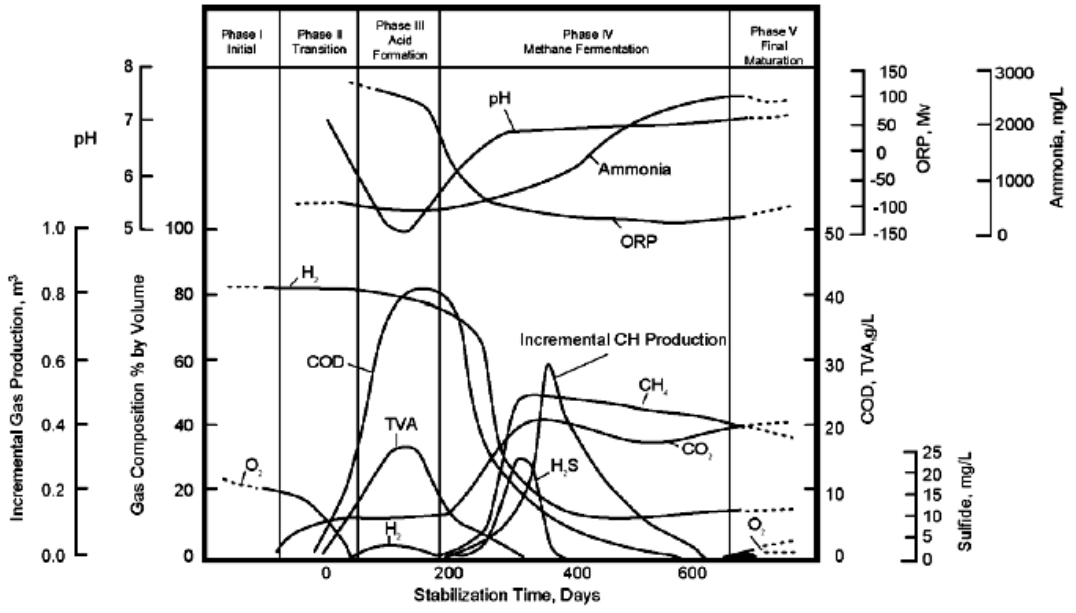


Figure II.8 Phases of anaerobic decomposition in MSW landfills (adopted from Pohland and Kim, 1999).

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.

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 **acid formation phase**. The process generally involves fermentation (combined oxidation/reduction of organics) of insoluble long chain sugars and thus referred to as solid-state fermentation (Blackall and Silvey, 1994). Products include propionate, butyrate, valerate, caproate and heptoate and longer carbon chains, as well as other more complex acids and alcohols.

In this 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 *Archaea* can grow. A decrease in pH values is often observed, and is accompanied by metal species mobilization resulting in a chemically aggressive leachate. Also, a decrease in the sorptive capacity of the refuse is seen during this phase (Lu *et al*., 1985). The highest concentrations of BOD (1000 to 57700 mg/L), COD (1500 to 71100 mg/L), and specific conductance (1600 to 17100 μmhos/cm) 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. This phase is also characterized by increasing CO_2 production, the appearance of H_2 and a reduction in pH due to the formation and dissolution of organic acids.

During **methane fermentation phase**, 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. These lignin-type compounds are important factors in adsorption and complexation mechanisms (Lu *et al*., 1985). The pH level is

elevated, being controlled by the bicarbonate buffering system, and 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. Phase IV also provides substrate for methanotrophic *Bacteria* which convert methane to CO_2 and water in the presence of oxygen. These *Bacteria* can significantly reduce methane emissions.

During the final stage of landfill stabilization, **maturation phase**, nutrients and available substrate become limiting and the biological activity shifts to relative dormancy. Gas production dramatically drops and leachate strength remains steady at much lower concentrations. Oxygen and oxidized species may slowly reappear. However, the slow degradation of resistant organic fractions may continue with the production of humic-like substances.

II.3.4. Factors Affecting Landfill Stabilization

The factors that affect the biochemical reactions occurred within the landfill are discussed in the following:

II.3.4.1. Oxygen

Methanogens require the absence of O_2 with a redox potential of below –330 mV (Christensen and Kjeldsen, 1989). The type and size of any gas extraction system will affect the presence of oxygen, particularly if an active system is employed (air is drawn into the structure). Generally there exists an aerobic zone at the top of the waste, which is generally up to 1 m.

II.3.4.2. pH

Methanogenic *Archaea* are particular sensitive to pH and prefer the range 6 and 8 (Christensen and Kjeldsen, 1989). In laboratory studies it has been shown that methanogens cannot survive under lower pH conditions. However, in landfills this has been found not to be the case and methanogens can still survive though the rate of growth is severely limited. Sulfate reducing bacteria are slightly more tolerant of low pH than methanogens which can also inhibit methanogenesis.

II.3.4.3. Alkalinity

Alkalinity is generally expressed as a concentration of calcium carbonate. It acts as an effective pH buffer, which may significantly improve the efficiency of the degradation by maintaining a close to neutral pH range in the landfill ecosystem. Its major source would generally come from soil and demolition waste. It has been reported that an acetic acid to alkalinity ratio less than 0.8 is essential to start methane production. Alkalinity in excess of 2000 mg per liter and a concentration of volatile acids less than 3000 mg per liter is required for good methane production.

II.3.4.4. Sulfate

The presence of sulfate can have a significant impact upon methanogenesis. Sulfate reducers can metabolize both acetate and hydrogen. Acetate and hydrogen are, of course, the major substrates of methanogens. Sulfate will compete methanogens for these substrates due to the more favorable thermodynamics of sulfate reduction and a greater resistance to low pH. Although inhibited, methanogenesis continues alternate pathways such as alcohols, which are produced in small quantities during acidogenesis. Sulfate reduction can also have a beneficial impact upon methanogens. The reduction sulfate creates sulfide, which readily combines with metals such as Cu, Zn and Fe that precipitate out of solution. This can have the effect of reducing potentially toxic metal concentrations that could further inhibit methanogenesis (Mori *et al.,* 2000).

II.3.4.5. Nutrients

The suggested optimum ratio for C:N:P is 100:0.44:0.08 (Christensen and Kjeldsen, 1989). Landfills are generally around optimum or better but the heterogeneous nature of the waste may result in zones of nutrient deficiency. The most likely deficient nutrient is phosphorus.

II.3.4.6. Temperature

There are three groups of methanogens operating at different temperature ranges, namely psychrophilic (<20ºC), thermophilic (>44ºC), and mesophilic (20 to 44ºC). It is the mesophilic group that is relevant to landfill methanogenesis. Laboratory studies have reported that the production of methane increased

significantly (up to 100 times) with temperature raised from 20 to 40ºC (Christensen and Kjeldsen, 1989). The optimum temperature for methane production was between 34 to 38ºC. The amount of heat energy generated by anaerobic decomposition processes is small compared to aerobic degradation. However, because landfill wastes and earth capping are good insulation materials, the heat loss to the external environment is generally minimal. Thus, the heat generated by the anaerobic processes is often enough to maintain an elevated temperature within the landfill mass. In a temperate climate, landfill temperature between 30 and 45ºC has been reported.

II.3.4.7. Moisture

Moisture is a major contributor to successfully methanogenesis by providing; more efficient hydrolysis, improved mixing of substrates and nutrients, dilution of inhibitors such as organic acids, H_2, O_2 etc. and a biofilms on the waste surface for microorganisms to spread and colonize. Many laboratory studies report that optimal methanogenesis is achieved at moisture contents of above 60% moisture content. As landfill waste typically has field capacities of the order of 35% moisture is often at sub-optimal quantities.

II.3.4.8. Inhibitors

While oxygen, pH, and sulfate all have inhibitory effects on the methanogenic *Archaea* as discussed above, the inhibitors here referred to are cation concentrations, heavy metals and organic compounds. The cations such as sodium, potassium, calcium, magnesium and ammonium, in low concentrations, are essential as micronutrient. But in high concentrations, they significantly inhibit methane production. For heavy metals, their concentrations commonly present in landfill wastes are not high enough to influence significantly the sensitive methanogenic *Archaea*. The toxic effects caused by various organic compounds have been studied by several researchers and are summarized by Christensen and Kjeldsen (1989). They concluded that fairly high concentrations of these toxic organic compounds are required to impose a significant inhibitory effect on a methanogenic system. In MSW landfills, their concentrations would generally be too low to have any inhibitory effect.

II.4. LANDFILL MICROBIOLOGY

Reduction of stabilization time of landfills is an important task to minimize the post-closure care, maintenance, and risk, and to promote post-closure land utilization. However, it is difficult to accelerate the decomposition of refuse and stabilization of the landfills so as to shorten the regulated post-closure monitoring period. This is often too heavy a burden on landfill owners and regulators to achieve the long-term management of landfills and treatment of leachate after closure. Thus the cost effective accelerated landfill stabilization technology is required. The stabilization of landfills is judged from various aspects such as physical, chemical, and biological status, and biological processes such as organic compounds decomposition in the refuse and nitrogen removal in the leachate are essential. Therefore, establishment of the technologies and strategies for the best utilization of the microbial functions deeply related to the above mentioned processes is one of the important keys for the accelerated landfill stabilization. For this purpose, monitoring the functional microorganisms and the change of microbial community structure in landfills along with the measurement of physico-chemical indexes to elucidate the relationships between the biological status and stabilization process of the landfills is needed. The biological investigations in landfills will help to evaluate and optimize the previously proposed accelerated landfill stabilization technologies such as semi-aerobic landfills and leachate recirculation, and will lead to the establishment of biological indexes for the evaluation of the stabilization.

Stabilization of organic solids in a landfill is a dynamic, complex, biologically mediated process, which is primarily influenced by waste characteristics, availability of moisture and nutrients, and prevailing operation circumstances. A large volume of methane is formed during the period of the stabilization process which indicates that methanogenic degradation of organic compounds plays significant roles. Since methane production in landfills is important with respect to the economic and environmental issues, optimizing the microbial activities in landfills is one of the major issues in the management of such sites. These features are tightly associated with microorganisms occurring in the processes, much attention has to be paid to the fundamental knowledge on the microbial ecology in landfills to further establish

more efficient municipal solid waste (MSW) stabilization processes (Chen *et al*., 2003)

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. 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). Microorganisms degrade these organics into mineral form, principally CO_2, by facilitated chemical oxidation.

Most of the microorganisms in the landfill use organic compounds and chemical pathways to generate energy are termed chemoorganotrophs (Blackall and Silvey, 1994). As redox reactions tend to produce more energy than other types of reactions, microorganisms, which utilize these reactions, flourish within the landfill. During such reactions microorganisms use enzymes and other mechanisms to enhance the rate of such reactions and to some extent influence the point of equilibrium. 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).

Over 50% of municipal solid wastes are potentially biodegradable. Within the landfill environment consortia of anaerobic microorganisms mediate the decomposition process through polymer hydrolysis, fermentation to organic intermediates, and mineralization by methanogenesis. This final step in the decomposition process has received considerable attention (Barlaz *et al*., 1987) due the potential of methane as a fuel source, and also because of the environmental impact of landfill gas (which typically has a CO_2: methane content of 60:40). In addition to landfill gas, leachate is also generated through microbial degradation processes and can also associate with potential pollution problems (Rugge *et al*., 1995; El-Fadel *et al*., 1997). The microorganisms in the anaerobic landfill ecosystem can be divided into two main groups; *Bacteria* and *Archaea*.

II.4.1. *Bacteria* in Landfill Ecosystem

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.

In literature, there are only a few studies about the investigation of *Bacteria* population in landfill by using molecular tools. Kim (2003) has investigated the landfill microbial communities using landfill gas. The results stated that *Bacteria* are dominant group in the landfill environment.

Qian and Barlaz (1996) measured hydrolytic, acetogenic and methanogenic *Bacteria* along with total anaerobes from different kinds of landfill wastes (grass, leaves, food wastes) by the most probable number (MPN) technique. Their results showed that methanogens were only a small portion (less than 0.2%) of total anaerobes. Hemicellulolytic organisms accounted for the major portion of total anaerobes. Also, in the natural anaerobic ecosystems studied by others, methanogens were detected in only limited amounts.

Boon *et al.* (2000) has investigated the bacterial community in landfill by using DGGE technique and only some differences in a few bands of the bacterial community structure were observed between soil samples taken from different depths. These minor differences, however, resulted in two clusters of samples according to sampling depth. These results confirm the findings of Felske and Akkermans (1998) namely that some *Bacteria* can be suppressed or stimulated as a function of depth.

In the studies of Daly *et al.* (2000) and Van Dyke and McCarthy (2002), the authors designed the PCR primers for phylogenetic subgroups of sulfate-reducing bacteria, and cellulose-degrading bacteria respectively. In particular, Daly *et al.* (2000) mentioned that, due to the difficulty of sampling of landfill wastes, leachate was used as the source for samples in their study. Daly *et al.* (2000) found an unexpected high level of diversity among sulfate-reducing bacteria (SRB) in landfill leachate. SRB can compete with methanogens for electron donors such as acetate and H_2, and have the potential to inhibit methanogenesis. Daly *et al.* (2000) believed that leachate conditions favor to fermentative microorganisms for producing various

volatile fatty acids that serve as substrates for SRB. Then they suggested that the scale of landfill sites and their extreme heterogeneity would promote microbial diversity. In addition, because leachate results from the percolation of water through the site, this may explain the high diversity of SRB in leachate.

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 *Clostridium thermocellum* and *C. leptum* species.

Pourcher *et al.* (2001) found that the predominant cellulolytic groups could be assigned to the family of *Bacillaceae* and to the genera *Cellulomonas*, *Microbacterium* and *Lactobacillus*. Furthermore, chemical parameters such as pH, carbohydrates and volatile solid contents influenced the composition of the cellulolytic bacterial groups which were reduced essentially to the family of *Bacillaceae* in the oldest refuse samples.

Boothe *et al.* (2001) has investigated aerobic microbial populations in landfill leachate and bulk material during an engineered aerobic bioreduction process in a test cell of a municipal landfill. In the study, two gram positive and six gram negative species were isolated from both leachate and bulk material, and none of the yeast *Candida sp.* or *Cryptococcus sp*. isolated from solid samples was found in leachate.

In another study, Wise *et al.* (1999) investigated the diversity of the methanotrophic community in mildly acidic landfill cover soil by using three methods: two culture-independent molecular approaches and a traditional culture-based approach. Methanotrophic *Bacteria* are a physiologically unique group of microorganisms distinguished by their ability to use methane as sole source of carbon and energy. High rates of CH_4 oxidation and large methanotrophic populations have been reported in the oxic portion of landfill cover soils. Phylogenetic analysis suggested the presence of a new phylotype related to the *Methylobacter-Methylomicrobium* group and the existence of a novel group of related species distinct from the validly published *Methylosinus* and *Methylocystis* genera. Finally, not all of the bands separated by DGGE could be accounted for by the clones and isolates. This polyphasic assessment of community structure demonstrates that much diversity among the obligate methane oxidizers has yet to be formally described.

II.4.2. *Archaea* in Landfill Ecosystem

Methanogens are a very diverse group of the *Archaea*, and are oxygen-sensitive, fastidious anaerobes. Despite the enormous phylogenetic diversity, as a group methanogens can only use a small number of simple compounds, most of which contain one carbon (Zinder, 1993). Most methanogens can grow on molecular hydrogen and CO_2 as sole energy sources, except for a few obligate methylotrophic and acetotrophic species (Müller *et al*., 1993). However, most of the methane produced in nature originates from acetate. Acetotrophs grow more slowly than CO_2-reducers, therefore, methane from acetate is not likely to predominate where the residence time for organic matter is short (Ferry, 1993). Other substrates include formate, methanol, methylated amines, and methylated sulfides. Because many methanogens use only one or two substrates, methanogens are dependent on other organisms for their substrates.

Methanogens can be separated into three main nutritional categories. (i) Hydrogenotrophs (38 species) oxidize H_2 and reduce CO_2 to form methane, and among those some are able to oxidize formate for methane formation. (ii) The second nutritional group includes methylotrophs (20 species), which utilize methyl compounds as methanol, methylamines, or dimethylsulfides to produce methane. H_2 is also here used as an external electron donor. Thirteen species are obligate methylotrophs. (iii) The last category, acetoclastic (or acetotrophic) methanogens (9 species), utilizes the methyl group of acetate to produce CH_4; only two species are obligate acetotrophs. Some species share nutritional characteristics and cannot be classified in a single group (Garcia *et al.,* 2000).

A number of additional standards have been used to classify methanogens. They include morphology, motility, electron microscopy images, colony morphology, nutritional spectrum, growth rates, growth conditions, metabolic end-products, gram staining, susceptibility to lysis, antigenic fingerprinting, lipid analysis, distribution of polyamines, nucleic acid hybridization, G + C content of the DNA, 16S rRNA sequencing and sequence analysis (Boone and Whitman, 1988). According to those criteria, five orders, ten families, 26 genera, and 74 valid species have been defined (Boone *et al.,* 1993).

Hydrogenophilic methanogens include members of the order *Methanomicrobiales* produce slightly more energy thermodynamically and are

therefore more favorable when hydrogen is available. Consequently measured hydrogen concentrations within landfills are generally small and disappear once acidogenesis is complete.

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 digester (Zinder *et al.,* 1984).

Methanogens have very slow growth rates and also the greater part of the digestible organic matter is eliminated during the methane-formation; as a result methane-formation is assumed to be the rate-limiting step of the digestion process; the metabolism rate of the methanogens may greatly influence the process efficiency. Moreover, the methane-formation rate also can affect the process stability which can be said that the methanogens are the most sensitive of all microorganisms involved in anaerobic digestion for environmental stress, and inhibition of methanogens may lead to an unbalance between acid production and acid consumption.

Methanogens are sensitive to both pH and hydrogen concentrations, yet are dependent upon both. In this manner methanogens are involved in a symbiotic relationship with acetogens. Generally the rate of acid and hydrogen formation is matched by their conversion to methane. In this manner they play a very important role in the landfill ecosystem (Barlaz *et al*., 1989).

As it is seen in the anaerobic pathway process, a consortium of anaerobic organisms to work together to bring about the conversion of organics is the main idea. Methanogenic microorganisms play an important role in this degradation (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.,* 1998). 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. Several studies using 16S rDNA sequence analysis on microbial communities in anaerobic treatment systems have been reported. They have provided unprecedentedly detailed insights into the microbial communities in these poorly characterized ecosystems and have recovered a number of previously unknown 16S rDNA sequences (Huang *et al.,* 2002).

Chen *et al*. (2003) investigated the archaeal community within the old and young age landfill samples respectively. Chen *et al.* (2003) 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.

In addition, studies on archaeal community compositions in leachate from a landfill (Huang *et al.,* 2002) and in a landfill site (Mori *et al.,* 2003) indicated that members of *Methanomicrobiales* and *Methanosarcinales* could be significant archaeal populations in such environments. Mori *et al.* (2003) suggested that the majority of methane emitted from the landfill site originated from the acetate utilizing *Methanosaeta*. However Huang *et al.* (2003) showed that methanogens in the leachate accounted for only a very small fraction of the total community (approximately 2%) and that *Methanomicrobiales* and *Methanosarcinales* constituted the majority of the total methanogenic population.

Luton *et al.* (2002) stated that of the four *Methanomicrobiales* group sequences; two have been detected in landfill, *Methanoculleus bourgensis* and *Methanospirillum hungatei*, using species-specific probes in previous studies.

II.5. IDENTIFICATION OF LANDFILL MICROORGANISMS

One of the most fundamental discoveries made by microbiologists during the last decade has been the realization that the vast majority of *Bacteria* in the environment have not yet been cultured (Amann *et al.,* 1995). Using molecular biological tools and employing the 16S rRNA gene as a marker, microbiologists have been able to identify the presence of novel, uncultured organisms in situ. These 16S rRNA-based approaches have led to estimates of prokaryotic biodiversity and surveys of the microbial community structure of many environments (Britschgi and Giovannoni, 1991; Ward *et al.,* 1992; Barns *et al.,* 1994; Wise *et al.,* 1997). At present, one of the most burning questions is the relationship between the microbial communities as described by these culture-independent methods and community structure assessment based on culturing. Investigators exploring the wide range of microbial diversity with universal- or domain-specific 16S rRNA probes or primers have only rarely reported the isolation of novel organisms whose presence was suggested by sequence analysis (Kane *et al.,* 1993; Suzuki *et al.,* 1997).

For many decades the identification of microorganisms in environmental samples was limited to cultivation dependent methods. Only a small percentage of the microorganisms in the natural environment are cultivable (Amann *et al.,* 1995), and this in turn has limited the ability of researchers to study the general composition of microbial communities. Therefore, significant bias can enter our understanding of microbial communities in the environment when using cultivation-dependent methods. It has long been known that the direct visualization of microorganisms in a natural sample by staining and microscopy yields a population count one to two orders of magnitude higher than that measured by culturing from the same sample (Staley and Konopka, 1985; Amann *et al.,* 1995).

The identification of microorganisms after prior cultivation shows two serious disadvantages: first, environmental studies indicate that only 0.1-10% of all *Bacteria* can be cultivated. Second, 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. The consequence is not only that the main part of *Archaea* or *Bacteria* cannot be detected in environmental samples

but also that the detected microorganisms do not represent the "real" population structure. For this reason, culture independent methods, such as rRNA based molecular techniques, are important tools for examination of microorganisms in their environment.

The 16S rRNA molecule is an excellent marker to infer phylogeny because it is found in all cellular life form. It comprises highly conserved regions interspersed with more variable ones. The variable regions allow the comparison of sequences, and some of the conserved portions can be recognized as signature sequences for the domains *Archaea*, *Bacteria*, or *Eukaryote* (Head *et al.,* 1998). Conserved regions also allow the development of useful primers or probes (Giovannoni *et al.,* 1988; Stahl and Amann, 1991), which enable the amplification or identification of sequences down to species level.

The comparative analysis of ribosomal RNA (rRNA) sequences, which allowed the definition of the three domains of life (Woese *et al.,* 1990), stressed the importance of rRNA as a phylogenetic marker. At the same period, works which used molecular biology methods in combination with the new rRNA phylogeny revealed the importance of rRNA as tool for analyzing natural microbial populations (Olsen *et al.,* 1986; Pace *et al.,* 1986). Among the three existing ribosomal RNA (5S, 16S/18S, and 23S/28S), the 16S rRNA became the most widely used marker (Head *et al.,* 1998).

The applications of rRNA-based nucleic acid techniques to the analysis of treatment systems today range from a simple identification of isolates over the detection of bacterial diversity and population dynamics to attempts at fully and quantitatively describing the complex microbial communities. Several rRNA based methods have been developed to identify and quantify microorganisms in environmental samples such as polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and slot-blot hybridization. These are powerful techniques in the identification of microbial populations in combination with cloning and DNA sequencing.

II.5.1. Polymerase Chain Reaction (PCR)

Polymerase chain reaction has rapidly become one of the most widely used techniques in molecular biology for many reasons: it is a rapid, inexpensive and simple means of producing relatively large numbers of copies of DNA molecules from minute quantities of source DNA material even when the source DNA is of relatively poor quality.

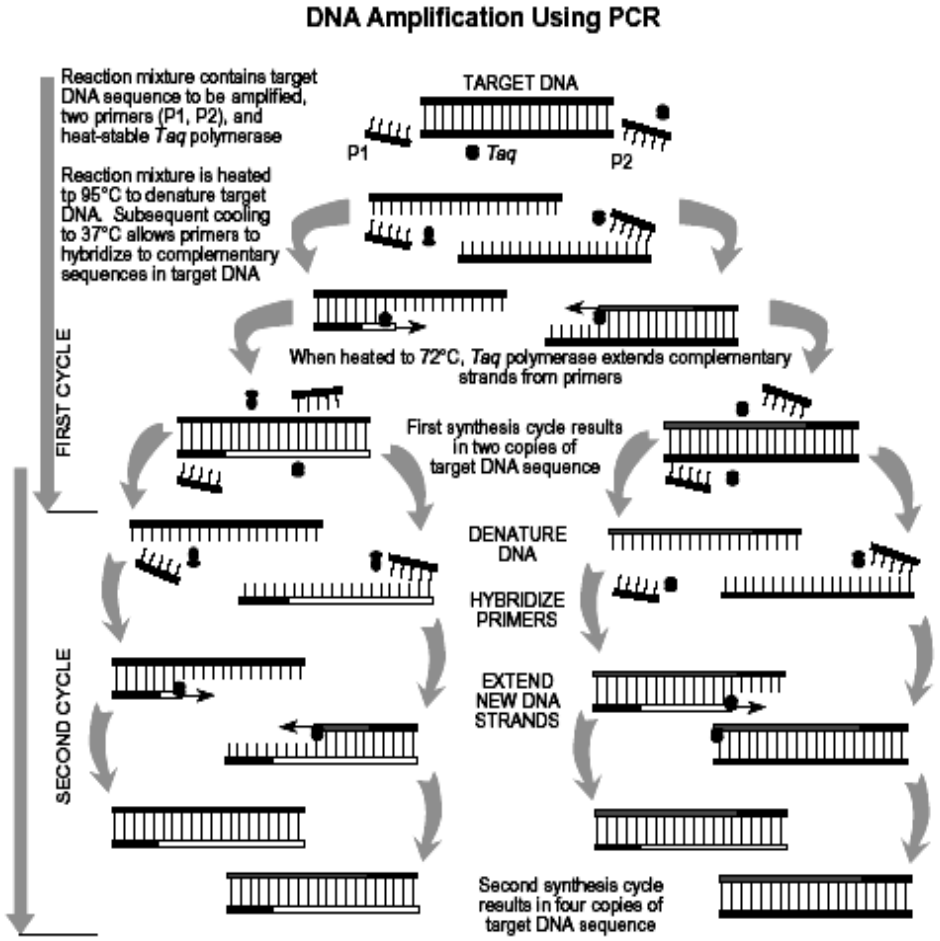


Figure II.9 Overview of polymerase chain reaction principles.

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 now 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 it's newly assembled complementary strand (Figure II.9).

All PCR really requires in the way of equipment is a reaction tube, reagents, and a source of heat. But different temperatures are optimal for each of the three steps, so machines now control these temperature variations automatically. To get more of the DNA you want, just repeat the process, beginning by denaturing the DNA you've already made. The amounts will double every time. With the cycle of rapid heating and cooling controlled automatically, nature-aided by scientist-supplied primers, polymerase, nucleotides, and chemical reagents-does the rest. Each cycle takes only 1-3 minutes, so repeating the process for just 45 minutes can generate millions of copies of a specific DNA strand. Once the primers have been characterized and obtained, PCR can do in a week work that used to take a year.

The amplification product is visualized by agarose gel electrophoresis. Although the PCR technique was originally used for genetic and clinical purposes, this technique has been used to detect and monitor microorganisms in complex environmental samples for a number of years (Bej *et al.,* 1991b). By exponentially amplifying a target sequence, PCR significantly increases the probability of detecting rare sequences in mixtures of DNA. Numerous studies have reported the detection of specific microorganisms in water, soils and sediments by PCR amplification without the need for cell cultivation (Bej *et al.,* 1991a).

II.5.2. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a gel-electrophoretic separation procedure for double stranded DNA's of equal size but with different base-pair composition or sequence (Muyzer and Smalla, 1998). In principle, the method is sensitive enough to separate DNA's on the basis of single point mutations (Sheffield *et al.,* 1989). This technique is gaining increased popularity in microbial ecology for analyzing the diversity of total bacterial communities. Briefly, the 16S rRNA genes are amplified using the appropriate primer pair, one of which has a G+C "clamp" attached to the 5' end that prevents the two DNA strands from completely dissociating even under strong denaturing conditions. During electrophoresis through a polyacrylamide gel containing denaturants, migration of the molecule is essentially arrested once a domain in a PCR product reaches its melting temperature (Figure II.10). Following staining of the

DNA, a banding pattern emerges that represents the diversity of the rRNA gene sequences present in the sample. The intensity of an individual band is a semi-quantitative measure for the relative abundance of this sequence in the population.

Other applications of these techniques include identifying 16S rRNA sequence heterogeneity (Nübel *et al.,* 1996), monitoring specific physiological groups, monitoring enrichment and facilitating isolation (Muyzer, 1999), and determining PCR biases. As an alternative to comparing DGGE profiles by eye, similarity indices may be calculated by computer analysis of scanned fingerprints or using Shannon-Weaver indices (Zoetendal *et al.,* 1998; Nübel *et al.,* 1999).

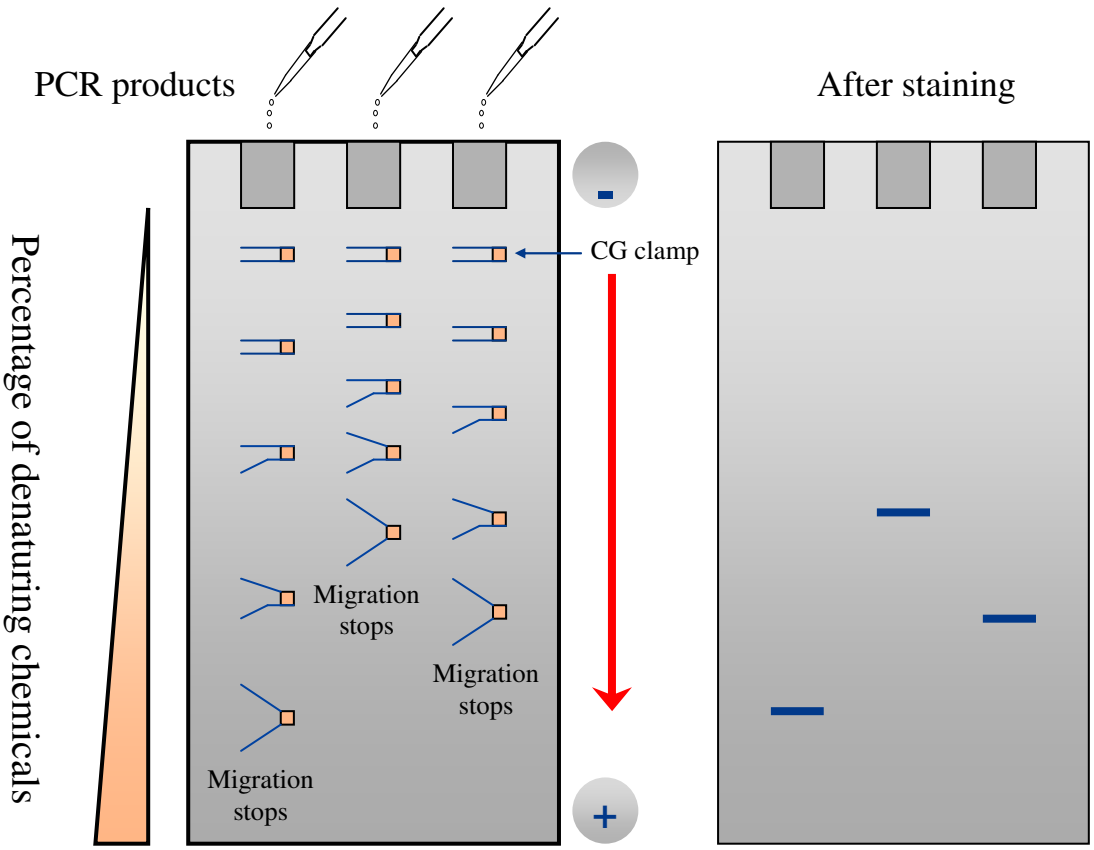


Figure II.10 Overview of denaturing gradient gel electrophoresis experiment.

Whereas DGGE analysis of rDNA PCR products is a powerful tool to analyze diversity and dynamics of microbial communities, it has severe limitations in the analysis of community structures, and is like any other method prone to specific biases:

- The method involves extraction of nucleic acids and subsequent PCR, which may both cause some bias: Not all cells lyses under the same conditions and preferential amplification of certain templates can occur (Suzuki and

Giovannoni, 1996). Therefore, different intensities of DGGE bands must not be interpreted as quantitative measures of the abundance of species relative to each other.

- Separation of DNA fragments with high resolution is restricted to a maximum size of about 500 bp. Consequently, the phylogenetic information that can be retrieved by sequencing is relatively little. In case of full identity with an rRNA sequence in a database it might be sufficient for identification, but in cases in which only distantly related sequences are available classification becomes difficult if not impossible.
- The main difficulty, however, is the "one band-one species" hypothesis. Especially in complex communities bands might originate from two or more fragments that co-migrate on the denaturing gradient gel. Furthermore, single species might result in two or more DGGE bands due to inter-operon micro-heterogeneity (Nübel *et al*., 1996).

II.5.3. Fluorescence in Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) with rRNA-targeted probes is, amongst other things, a staining technique that allows phylogenetic identification of *Bacteria* in mixed assemblages without prior cultivation by means of epifluorescence and confocal laser scanning microscopy, or by flow cytometry (Giovannoni *et al.,* 1988; DeLong *et al.,* 1989; Amann *et al.,* 1990a; Amann *et al.,* 1990b; Amann *et al.,* 1996). FISH with polynucleotide DNA probes and FISH with oligonucleotide probes targeted to mRNA has also been described by several authors (Trebesius *et al.,* 1994; Wagner *et al.,* 1998; DeLong *et al.,* 1999).

FISH of *Bacteria* has first been described more than a decade ago (Giovannoni *et al.,* 1988; DeLong *et al.,* 1989; Amann *et al.,* 1990b), and celebrated as a breakthrough for microbial ecology. However, researchers initially encountered discouraging difficulties when applying the method to environmental samples other than from highly eutrophic systems. The majority of *Bacteria* in aquatic habitats are small, slowly growing or starving, and the signal intensities of hybridized bacterioplankton cells were frequently below detection limit or lost in high levels of background fluorescence. Accordingly, an early FISH protocol stated that there was "...a good deal of room for improvement of these techniques for practical field

application." (DeLong, 1993). This still holds true to a certain extent, but several important advances, in particular new quantitative protocols (Glöckner *et al.,* 1996), brighter fluorochromes (Alfreider *et al.,* 1996; Glöckner *et al.,* 1996), commercial availability of probe labeling, advanced probe design software (Strunk *et al.,* 1999) and better instrumentation have made the method attractive also for the less "molecular" microbial ecologists, such as environmental engineers.

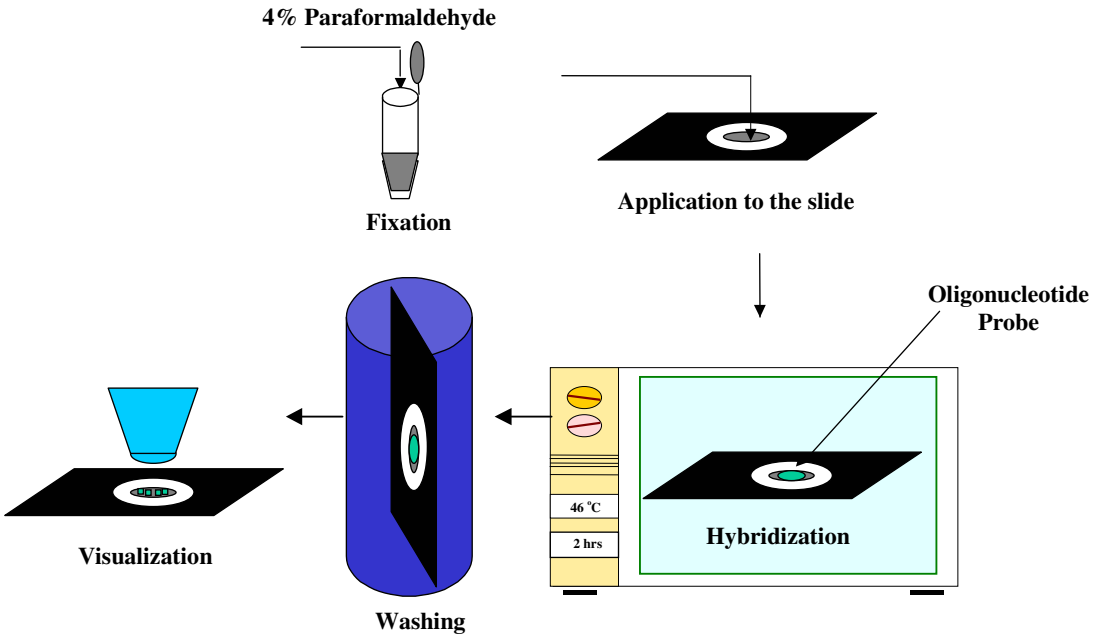


Figure II.11 Overview of fluorescence in situ hybridization steps.

The principles of in situ hybridization with fluorescently labeled, rRNA-targeted oligodeoxyribonucleotides are quite straightforward. First, the morphology of the cells in the examined sample has to be stabilized and the cell walls and membranes have to be permeabilized for the penetration of the probes. This can be both achieved with fixatives, which are usually based on aldehydes and/or alcohols. Subsequently, the probes are applied in an adequate hybridization buffer and incubated at an adequate hybridization temperature (usually between 35 and 50°C) for one to several hours. Washing steps are applied to remove unbound and part of the non-specifically bound fluorescent probe and the sample can subsequently be analyzed by epifluorescence microscopy (Figure II.11). Several probes labeled with spectrally different fluorochromes can be simultaneously used on one sample, e.g. a

fluorescein-labeled probe that emits green light upon blue excitation, together with the orange-red Cy3. The sensitive visualization of the latter requires a CCD camera.

II.5.4. Slot-Blot Hybridization

This technique is useful to measure the amount of a specific 16S rRNA in a mixture relative to the total amount of rRNA. Briefly, total DNA and RNA are isolated from the sample, bound to a filter using a dot or slot manifold device and hybridized with labeled oligonucleotide probes. The amount of label bound to the filter is a measure of the specific rRNA target present, and the relative amount of rRNA may be estimated by dividing the amount of specific probe by the amount of labeled universal probe hybridized under the same conditions. Obviously, the relative amount of rRNA sequence does not reflect the true abundance of the microbe since cells of different species have different ribosome contents and the number of ribosome within one strain will vary with growth phase. Nevertheless, the relative quantity of rRNA provides a reasonable measure of the relative physiological activity of a specific population.

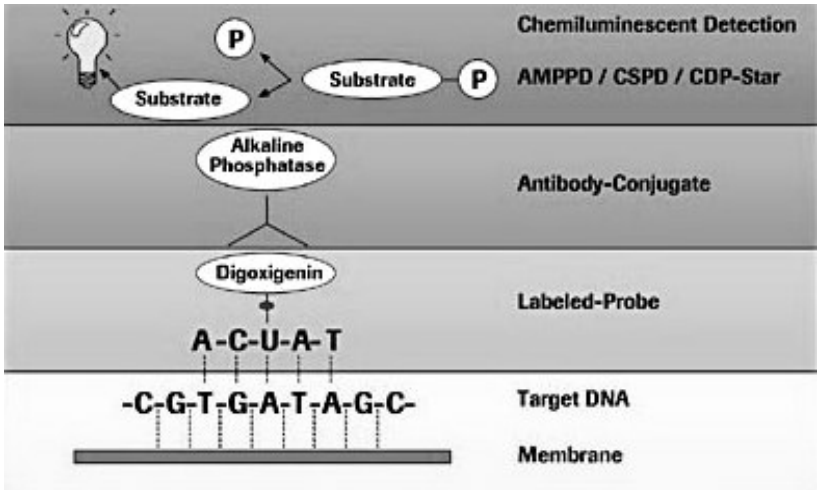


Figure II.12 Overview of slot-blot hybridization experiment.

For dot blot or slot blot hybridization total RNA or DNA is extracted from an environmental sample and immobilized ("blotted") on a nylon membrane (Stahl *et al.,* 1988). DNA oligonucleotide probes are labeled with ^{32}P (Stahl *et al.,* 1988), or – with a significant loss of sensitivity non-radioactively with digoxigenin (DIG) (Manz *et al.,* 1992). Hybridization conditions are optimized by adjusting the final wash temperature to provide adequate sensitivity and specificity relative to RNA extracted from reference

organisms. Processing of the membrane including prehybridization, hybridization, and washing takes several hours. Subsequently, bound probe is quantified by phosphorimaging or densitometry after autoradiography in case of ^{32}P or enzymatic antibody detection of probe-conferred DIG, respectively. The relative abundance of a certain 16S rRNA is expressed as a fraction of total 16S rRNA in the sample, which is determined by hybridization of a universal oligonucleotide probe (Stahl *et al.,* 1988). The DIG System is an effective system for the labeling and detection of DNA, RNA, and oligonucleotides. The protocols for labeling with digoxigenin (Figure II.12) and subsequent detection are based on well-established, widely used methods.

CHAPTER III

MATERIALS AND METHODS

III.1. MUNICIPAL SOLID WASTE LANDFILLS IN TURKEY

In this section, physical, chemical and microbial contents of leachate samples taken from various landfills which have different stabilization phases were investigated. Location of MSW landfills which leachate samples were obtained is shown in Figure III.1 and general information about the MSW landfills is summarized in Table III.1.

Istanbul is the most developed city in Turkey with a population over 8 million and it has to manage 8000 tons of solid waste everyday. The city has an average temperature of 13.7 ºC in summer and 5 ºC in winter with annual rainfall of 691 mm. Until year 1953 Istanbul's garbage was dumped into the sea. Then irregular dump areas were formed in locations like Levent-Sanayi Mahallesi, Seyrantepe, and Ümraniye-Mustafa Kemal Mahallesi. These areas are then invaded by slum districts with dump areas moving to new locations like Habibler, Ümraniye-Hekimbaşi, Yakacik, Aydinli, Halkali, Şişli-Feriköy, and Kemerburgaz-Hasdal.

Municipal solid wastes of European side of Istanbul are being landfilled at Odayeri (O) Sanitary Landfill. This landfill is in operation since 1995. Approximately, 6000 ton/day of municipal solid wastes are being removed at this landfill with 1000-1500 m^3 leachate production per day. Municipal solid wastes are disposed at 20 ha area with average 40 m waste height of 125 ha landfill surface during the last 5 years.

Kömürcüoda (K) Sanitary Landfill was constructed in the Asian side of Istanbul and under operation since 1995. It has an area of 20 ha and 3000 tons/days storage capacity. The Odayeri dump site has a daily capacity of 6100 tons receiving garbage coming from the Baruthane, Yenibosna, and Halkali transfer stations. The Asian Kömürcüoda dump site receives garbage from the Hekimbaşi and Aydinli transfer stations with a daily capacity of 2650 tons. Both sites are projected to receive and store garbage for 25 years. New dump sites are under construction.

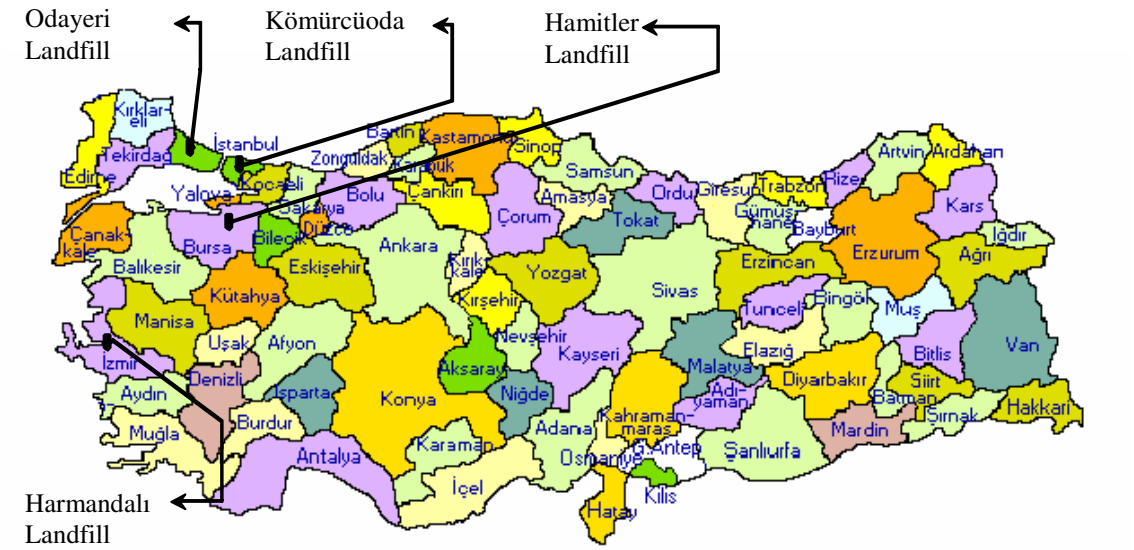


Figure III.1 MSW landfill locations.

Bursa is the fourth developed city of Turkey with a population over 1 million according to year 2000 results. Bursa-Hamitler (B) landfill has been operated since August 1995 with a 25-years design capacity. It has a disposal area of 77 ha and 20,000,000 m^3 total landfilling capacity. The domestic and non-hazardous solid wastes are allowed in the site. The flow rate of leachate from the landfill is varied between 170-260 m^3 per day (Salihoglu *et al.,* 2002).

Table III.1 General information about MSW landfills.

MSW Landfills	Location	Area	Solid Waste Inlet	Operation Commencement
Odayeri (O)	European Side of İstanbul	25 ha	6000 tons/day	1995
Kömürcüoda (K)	Asian Side of İstanbul	20 ha	3000 tons/day	1995
Bursa-Hamitler (B)	Bursa	77 ha	1200 tons/day	1995
Harmandalı (H)	İzmir	90 ha	1300 tons/day	1992

The investigated landfill site, Hamitler, leachate samples taken from, consists of 1 main valley and 4 adjacent valleys. Main valley has been operated since 2000 and has an area of 18 ha.

Izmir is the third developed city of Turkey with a population over 2 million. Due to the city's geographic location, it has an average temperature of 27.6 ºC in summer and 8.6 º C in winter. Harmandali (H) Landfill was constructed in 25 km east of Harmandali Town. It has an area of 900,000 m^2 and 1300-tons/day-storage capacity. Operation of the Harmandali Landfill was started in April 1992, after the closure of the six old landfills. Its area is 900,000 m^2, and it was designed to serve for 15 years.

The domestic, medical, and industrial solid wastes are landfilled in separate clusters in the area. According to the geological investigations conducted by Hacettepe University in Turkey, the area is far from the groundwater sites. Industrial and medical wastes are disposed in pits opened in the areas with minimum permeability. Domestic solid wastes are leveled first and then topped by soil daily. Three million tons of domestic solid wastes have been disposed of in Harmandali Landfill until the end of 2000, and a biogas utilization unit has been operated since 1996 to burn methane gas produced in the landfill. Although the biogas unit has a capacity of 1,250 m^3/hr, only 250-300 m^3/hr biogases can be collected from the landfill body (Pala and Sirin, 2001). Today, 700-800 tons of the total domestic waste amounts of 2700 tons/day in İzmir are deposited in a valley owned by government.

Leachate samples were taken from different points:

O1 Landfill section used for disposal between the years of 1995-2000;

O2 Landfill section used for disposal between the years of 2000-2002;

O3 Current Landfill section used for disposal since 2002;

K Leachate sample taken from Kömürcüoda MSW Landfill

B Leachate sample taken from Hamitler MSW Landfill.

H1 Leachate from current landfill section;

H2 Leachate from closed landfill section,

III.1.1. Chemical Characterization of Leachate Samples

The characterization of landfill leachate samples were assessed by 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), total phosphorus, phosphate (PO_4^{3-}), suspended solids (SS), volatile suspended solids (VSS), total dissolved solids (TDS), conductivity, color, chloride (Cl^-), sulfide (S^{2-}), sulfate (SO_4^{2-}) and heavy metals as iron (Fe), zinc (Zn), copper (Cu), lead (Pb), chromium (Cr) and nickel (Ni). All analyses were carried out according to the Standard Methods (APHA, 1998). The analytical methods are given in Table III.2.

Table III.2 Leachate characterization parameters and methods.

Parameter	Method	Parameter	Method
pH	Electrode	TDS & Conductivity	Electrode
Alkalinity	Titrimetric	SS & VSS	Gravimetric
COD	Closed Reflux	Color	Spectrophotometric
BOD_5	5 Day BOD Test	Cl^-	Argentometric
NH_3-N	Nesslerization	S^{2-}	Spectrophotometric
TKN	Semi-Micro-Kjeldahl	SO_4^{2-}	Spectrophotometric
Total P	Digestion-Colorimetric	Zn, Ni, Cr	AAS
PO_4^{3-}	Colorimetric	Fe, Pb, Cu	AAS

III.1.2. Biogas Production Potentials of Leachate

Rates of biogas production, and more specifically the methane yield, can potentially be a good indicator of the metabolic status of anaerobic stabilization in landfills if generated leachate were exposed to methanogenic activity test. To determine the stabilization status of leachate samples in accordance with their biodegradability, methanogenic activity tests were conducted in 100 ml batch vial bottles in triplicates. The biogas produced in these vial bottles is transferred to the manometers by a fine transfer pipe connected to the tip of the bottle. In present study, biogas measurement apparatus was used as shown in Figure III.2. The manometer of the set-up is filled with saline solution and this solution is supplied to the manometer tubes with a plastic hose connected to the holding containers. The pH of the saline solution is reduced (pH=2) to prevent the dissolution of CO_2 present in the biogas.

With this precaution, the biogas produced in the bottle is measured exactly. By reading the height of the displaced water, biogas is measured as height and multiplying it by cross sectional area of manometer tubes, volume is determined.

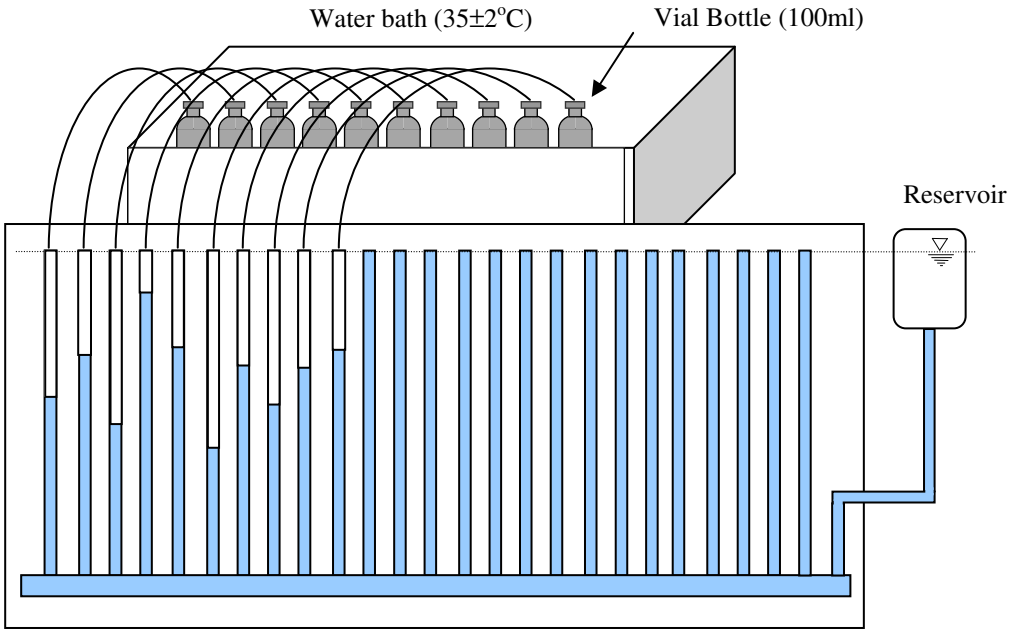


Figure III.2 Multiple manometer system.

The biogas production rate experiment begins with the preparation of dilution solution. Dilution solution is the mixture that represents content of the bottle in methanogenic phase. To calculate the volume of the dilution solution required for the test first of all the number of the cases that will be tried in the test is decided. Each case is studied triple for more accurate results and each vial bottle reactor is filled with approximately 50mL dilution solution. So, the total volume is calculated as multiplying the number of cases, 50mL vial bottle content and number of bottle per case. A spare volume should also be taken into account.

The chemical composition of the dilution solution adapted from Valcke and Verstraete (1983). After determination of the volume of dilution solution, the chemicals are dissolved in distilled water in a flask. Oxygen in this flask is removed under nitrogen purging.

Initially, equal amounts of anaerobic seed sludge acclimated to landfill leachate was added into each vial bottle. Then soluble leachate samples and dilution solution were added in appropriate amounts to provide 2000 mg/L final concentration. Synthetic solution consists of acetate, propionate and butyrate (0.7:0.15:0.15; v:v:v),

has 2000 mg/L concentration and pH of 7.0 was used as blank All the transfers were carried out under continuous nitrogen purging and the vials were sealed with butyl rubber caps and crimped with aluminum crimps. Then, the vial bottle reactors are placed into the 35 ± 2 °C water baths.

The pressure in bottles is released after 30 min incubation in water bath to provide a better starting point with identical pressure in each of the reactor. After zeroing the pressure in the bottles, they are left to 35 ± 2 °C water baths for incubation again. Biogas production, in the vial bottle reactors is measured as the height of water displacement in the column of biogas measurement apparatus. The height of the water displacement may be converted to volume of the biogas by multiplying it with the cross sectional area of the manometer tube.

The measurements are carried twice a day, in the morning and in the afternoon. Generally, biogas production in the vial bottles end after 120-140 hours. Finally, graphs of the gathered data were plotted and gas production rates of the leachate samples were compared.

III.2. AERATED LANDFILL BIOREACTOR IN JAPAN

Bioreactor test cell was constructed and monitored for one year operational period to understand the degradation rate and pathway of MSWI bottom ash and shredded incombustible waste mixes. In this part, waste characteristics, test cell design, construction and instrumentation are presented.

III.2.1. Test Cell Design and Construction

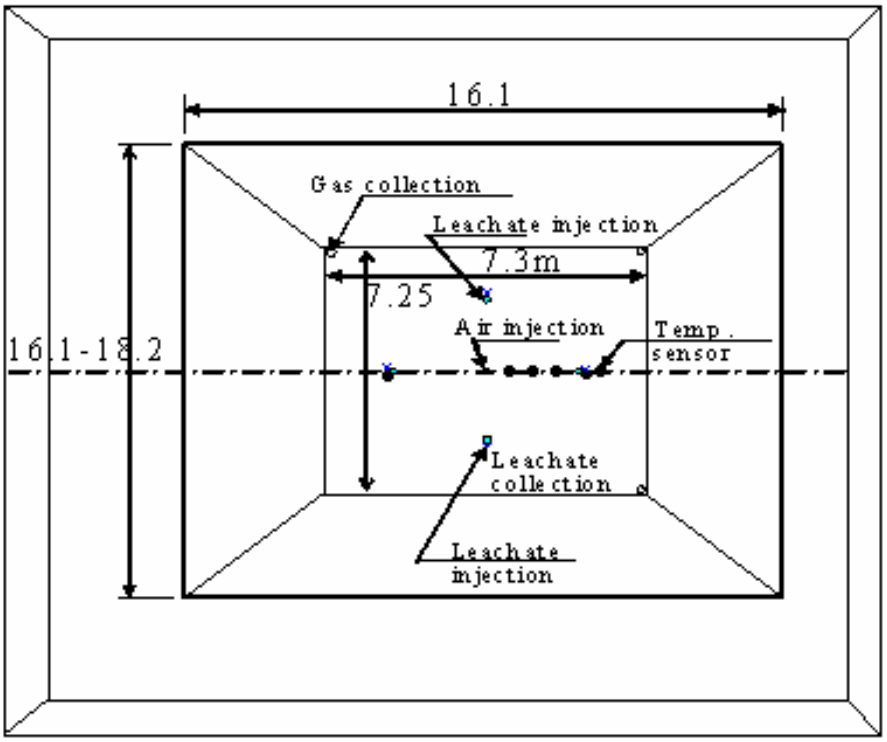
An aerated bioreactor test cell was constructed at a currently unused part of the Yorii Landfill which receives incineration bottom ashes and shredded incombustible wastes from varying sources such as MSW recycling centers and automobile recycling plants. In Yorii Landfill, wastes were landfilled in 3 m layers (2.5m waste+0.5m soil covers); therefore the height of the cell was determined by the operational practices of the landfill. The shape of the cell was like inverted truncated pyramid; bottom part approximately 7.5m x 7.5m and upper part 17m x 17m, although the exact size of the cell was different due to limitations of construction equipment (Figures III.3-4-5-6).

Waste volume in the cell was 366 m^3, and total volume was 433 m^3. Waste density (wet weight) was measured 0.811 t/m^3. Aerated bioreactor test cell was lined with 1.5 mm TPO (Thermo Plastic Olefin) geomembrane sheet and 10 mm geotextile. Total length of the leachate collection pipes (75mm, PVC) were laid in two directions and connected to a Ø 300 mm lift-up shaft was approximately 14 m (7m+7m).

Leachate injection well and gravel distribution box were installed in aerated bioreactor cell (Figure III.5). Air injection and gas collection wells were equipped with multiports allowing air and leachate injection as well as gas sampling.

Air was supplied by a vortex type blower (Hitachi VB-110-E2, 50 Hz, 12 kW). The capacity of the blower was 8.0 m^3/min, and an inverter (Hitachi L300P) had been installed for the air flow rate adjustment. The blower operation was controlled by flexible one week electronic timer (Omron H5S Time Switch). Leachate recycling in aerated bioreactor cell was achieved by a submersible type lift-up pump installed in the lift-up shaft, a 1 m^3 leachate collection tank on the surface, a submersible type leachate injection pump in the tank and an electronic flow meter. One small

submersible type pump was installed in each tank for preventing the freezing through mixing and heat discharge during cold winter days. Leachate injection pumps were controlled by a timer, and the lift-up pumps by level switches installed in the collection tank.



Blower house

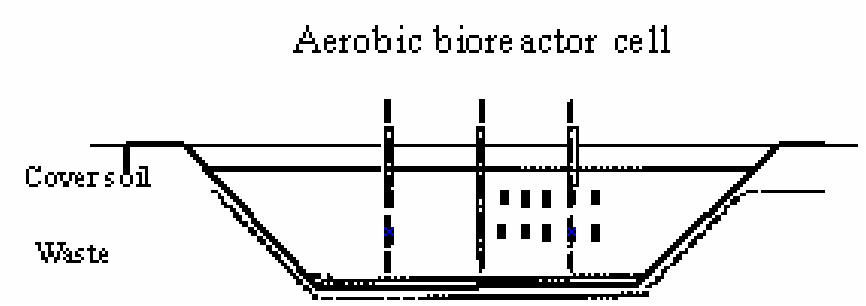
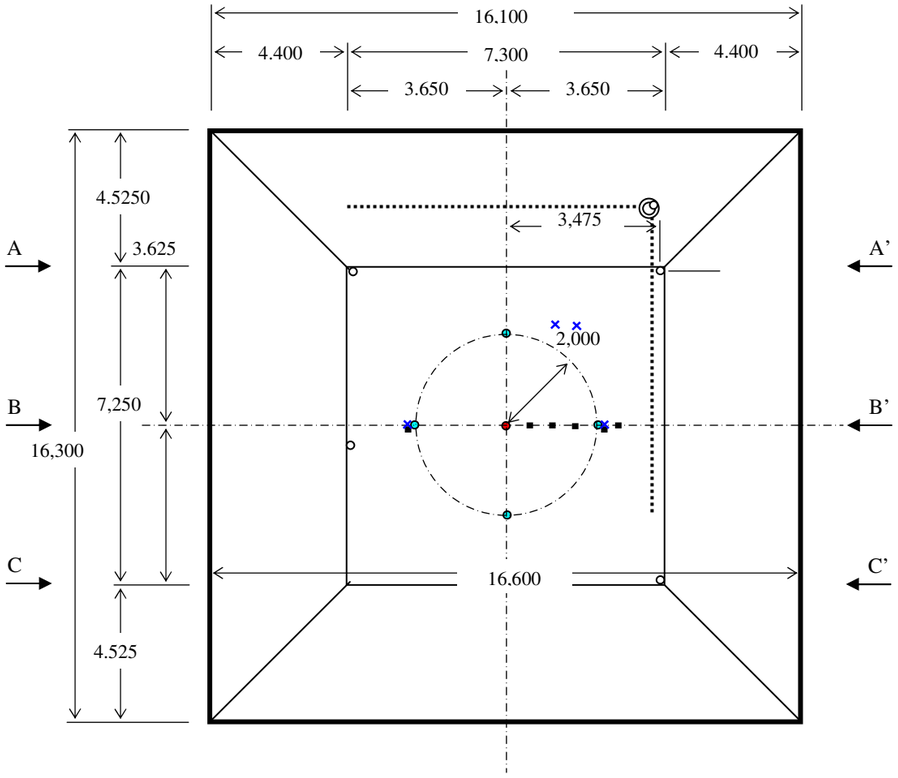
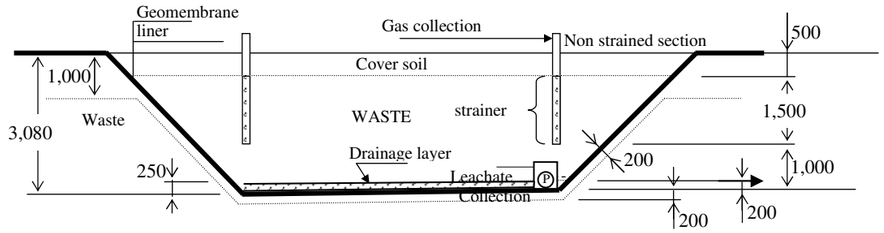


Figure III.3 General layout of the test cell.

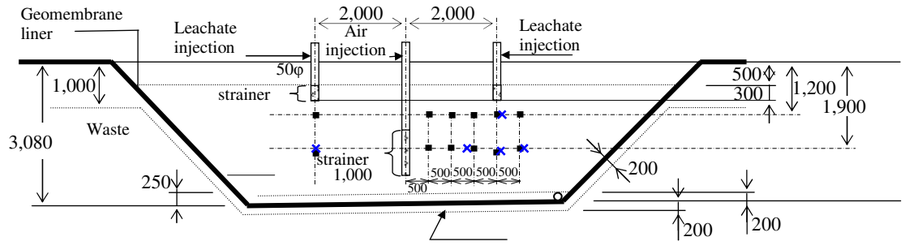


Surface Layout

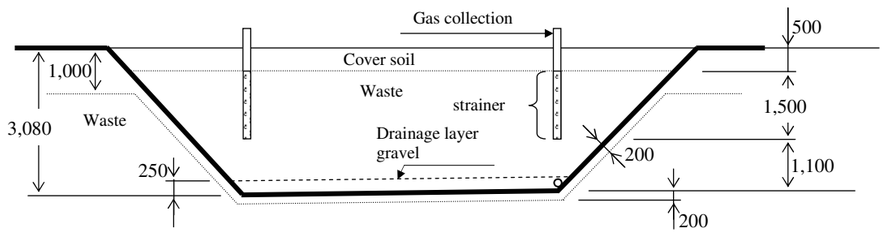
- Leachate lift-up shaft
- Leachate collection pipe
- Air injection well
- Gas collection well
- Leachate injection well
- Moisture sensor
- Temperature sensor



AA'



BB'



CC'

Figure III.4 Design details of aerated bioreactor cell.

Probe Labeling			
1st level (950mm)		2nd level (950+700mm)	
Moisture	Temperature	Moisture	Temperature
MAE11	TAE11	MAE25	TAE21
MAE13	TAE12	MAE25G	TAE22
MAE15	TAE13		TAE23
MAE15G	TAE14		TAE24
MAE16	TAE15		TAE25
	TAE16		TAE26

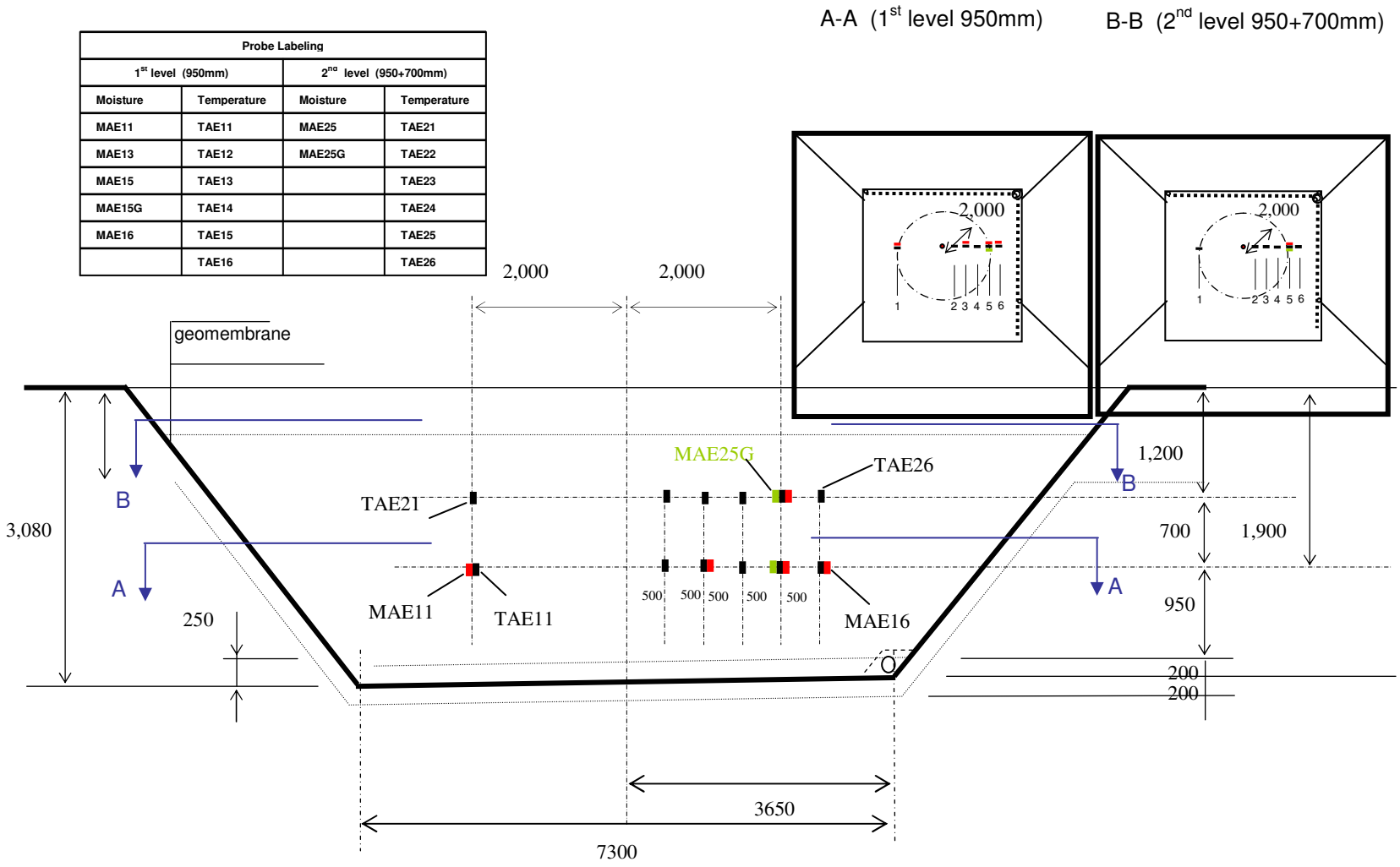


Figure III.5 Sensor locations in aerated bioreactor test cell.

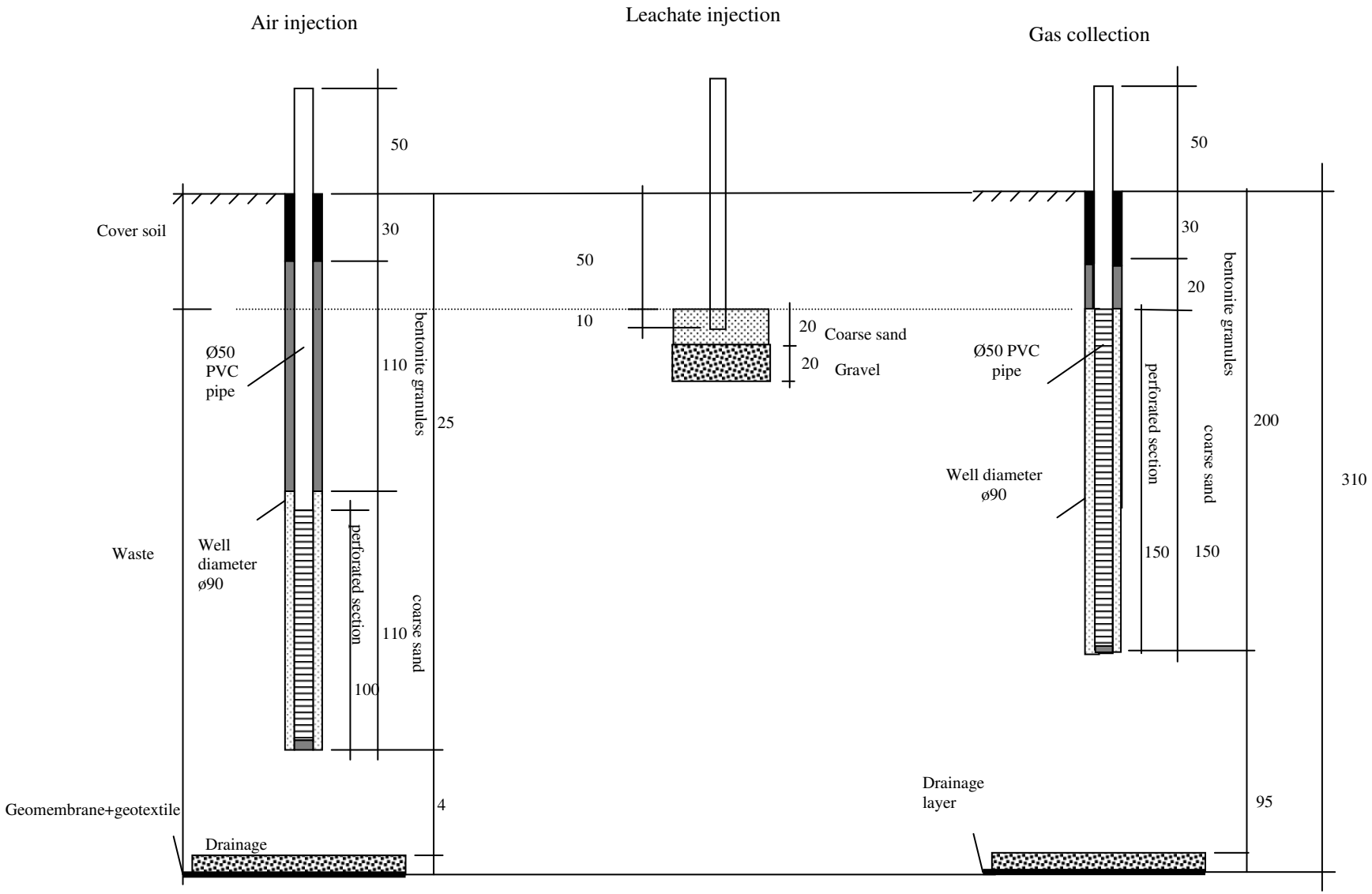


Figure III.6 Air-leachate injection and gas collection well design.

III.2.2. Monitoring Instrumentation

The cell was equipped with moisture (Theta Probe type ML1, Delta-T Devices, Cambridge, England, and gypsum block sensors, Sankei Rika, Tokyo, Japan) and temperature sensors (Type K sensors, Sankei Rika, Tokyo, Japan) for efficient monitoring and control of the bioreactor operation. In aerated bioreactor cell, 5 ADR and 2 gypsum block moisture sensors together with 12 temperature sensors were installed in two layers. First layer is 95 cm above the bottom of the cell, and the second layer is 75 cm above the first layer. Sensor numbers and installation plan is shown in Figure III.5. All the sensor cables were shielded in corrugated type cable shields for protection from damage during waste placement and other effects. A 32 channel data logger (Keyence) was used to store the data continuously on 30 min intervals.

III.2.3. Waste Characteristics and Sampling

MSWI bottom ashes and shredded incombustible wastes from different sources, such as MSW recycling centers and automobile recycling plants, were landfilled in Yorii Landfill. Unloaded ashes and shredded wastes were mixed with a backhoe as much as possible during filling process. The cell had a capacity of almost the amount of the waste received in one day, and the cell was filled on December 18, 2002. Since it was not possible to accumulate and homogenize the wastes before filling in to the cell, this result was inevitable. Table III.3 shows the ratios of ash and shredded wastes in the cell.

Table III.3 Material distribution in the bioreactor test cell.

	Bioreactor test cell	
	Ton	%
Shredded incombustibles	177,3	60
Bottom ash	119,8	40
Total Weight	297,1	
Total volume (m^3)	432.6	
Waste volume (m^3)	362.4	
Waste density (t/m^3) [wet]	0.81	

Parameters to be analyzed and sampling frequency are given in Table III.4.

Table III.4 Parameters to be monitored and sampling frequency for the aerated landfill bioreactor.

	Sampling Point	**Parameters**		**Frequency**	**number of samples**
Leachate	Recirculated leachate	pH, EC, ORP, DO, BOD, COD, TOC, Sulfide, T-N, T-P, TS, TVS, Heavy metals (total and dissolved), VOCs, Molecular weight	Anions (Cl^-, SO_4^{2-}, NO_2^-, NO_3^-, Br^-, PO_4^{3-}) Cations (Na^+, K^+, Ca^{2+}, Mg^{2+}, NH_4^+)	1/2 weeks	3
	Internal water			1/4 weeks	6-9
Gas	Gas collection pipe	CO_2, CH_4, N_2, O_2, VOCs, H_2S, (NMOC), NH_3, H_2O		1/2 weeks	10
	Internal Gas	CO_2, CH_4, N_2, O_2, H_2S, (NMOC)		1/4 weeks	6-9
	Surface emission	CO_2, CH_4, N_2, O_2, (NMOC) Flux		Not fixed	3
	In-situ respiration rate	CO_2, CH_4, N_2, O_2		1/8 weeks	Continuous monitoring at one location
Molecular Analysis	Recirculated leachate	Fluorescence in situ hybridization (FISH) Slot-blot hybridization Polymerase chain reaction (PCR) Denaturing gradient gel electrophoresis (DGGE) Cloning – Sequencing		1 month	13

III.3. MOLECULAR METHODS

III.3.1. Sample Collection and Preparation

Initially, leachate samples taken from Turkey landfills were concentrated by centrifuging for half an hour at 7,000 rpm. Concentrated sludge samples were extracted and fixed for DNA extractions and FISH experiments immediately after centrifugation. Extracted DNA samples and fixed cells were stored at - 20 °C.

For identification of microbial communities in an aerated landfill bioreactor, leachate samples were collected at different time periods (Day 19, 50, 60, 92, 110, 154, 175, 190, 203, 218, 239, 318, and 346). Subsequently, leachate samples about 1 L volumes were filtered and maintained at 4 ^{o}C prior to microbiological analyses.

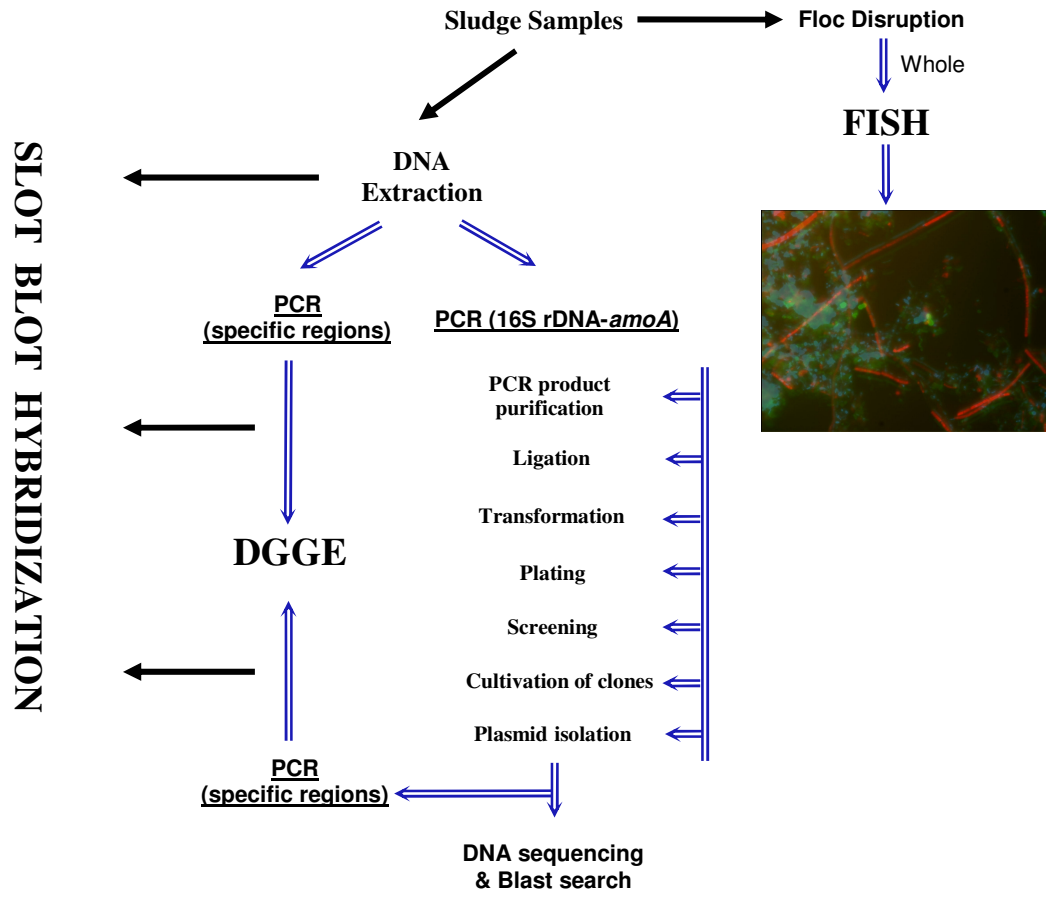


Figure III.7 Experimental identification procedure for landfill leachate samples.

In this study, microbial population diversities in landfills was monitored and analyzed by using fluorescence in situ hybridization (FISH), slot-blot hybridization, polymerase chain reaction (PCR), and denaturing gradient gel electrophoresis (DGGE) techniques. These results were compared and evaluated with cloning and DNA sequencing data to understand the function of microbial diversities during stabilization periods of sanitary and aerated bioreactor landfills. Experimental procedure followed in the identification of microorganisms in sludge samples is given in Figure III.7.

III.3.2. DNA Extraction

A lot of DNA isolation protocols are available and almost every study uses its own way of extracting DNA. In all of them included that bacterial cells have to be lysed to release DNA. The cells lysing can be done chemically, with enzymes or mechanically using bead-beating to damage the cell wall. When the microbial DNA is released, DNA-dissolving solutions and/or DNA-binding materials are used to isolate DNA from other compounds in the solution. In the last step DNA is precipitated, collected, and dissolved in water or buffer. But there are a lot of differences between different kinds of samples.

The FastDNA® kit protocol, which is the basis of our DNA extraction procedure, is an effective and widely accepted DNA isolation technique for many applications. The kit comes complete with all buffers and reagents required for DNA extractions. Through trial and error, we modified the FastDNA protocol to extract DNA from landfill leachate samples that is consistently amplified with PCR. A modified FastDNA protocol is described that adds several simple steps to the procedure, resulting in removal of amplification inhibitors.

Microorganisms in leachate samples were transferred to 2 ml polypropylene tube. The samples were centrifuged and the supernatant was discarded. 700 µl TE buffer (10 mmol/l Tris/HCl, 1mmol/l EDTA, pH 8.0), 300 µl Tris/HCl buffered phenol (pH 8.0) and approximately 0.6 g zirconium/silica beads (diameter 0.1 mm Biospec Products Inc., Bartlesville, OK) were added to the sludge samples.

The cells were disrupted by mechanically bead beaten for 10 s at maximum speed (Mini BeadBeater-8, Biospec Products) for DNA isolation. The supernatant was separated by centrifugation at 14 000 rpm for 10 min. Subsequently, 600 µl of

Binding Matrix (Bio 101) was added and the tubes were incubated at room temperature for 5 min. The matrix pellet was spinned for 1 min and the supernatant was discarded. The pellet was resuspended gently with 500 µl SEWS-M solution (Bio 101). DNA of binding matrix was eluted by gently the suspending in 100 µl DES followed by a 2-3 min incubation. It was spinned 1 min at 14000 rpm and supernatant was transferred to a new tube. The isolated DNA was used for PCR after judging the quality of the extract by agarose gel electrophoresis and ethidium bromide staining.

Agarose gel electrophoresis separates DNA fragments according to their size. To pour a gel, agarose powder was mixed with electrophoresis buffer to the desired concentration, and then heated in a magnetic stirrer until completely melted. Most commonly, ethidium bromide (final concentration 0.5 µg/mL) is added to the gel at this point to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60 ^{o}C, it was poured into a casting tray containing a sample comb and allowed to solidify at room temperature.

After the gel had solidified, the comb was removed, using care not to rip the bottom of the wells. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and just covered with TAE (Tris-acetate-EDTA) buffer. Samples containing DNA mixed with loading buffer were then pipeted into the sample wells, the lid and power leads were placed on the apparatus, and a current was applied. DNA migrates towards the anode, which is usually colored red. Gel was electrophoresed at 150 volts for 40 min. When adequate migration had occurred, DNA fragments were visualized by staining with ethidium bromide. To visualize DNA or RNA, the gel was placed on an ultraviolet transilluminator.

III.3.3. Polymerase Chain Reaction (PCR)

The utility of PCR was examined by amplifying DNA isolated from landfill leachate samples for slot blot hybridization, denaturing gradient gel electrophoresis (DGGE) and cloning. The variable regions of the ribosomal DNA and *amoA* genes were amplified to obtain more accurate phylogenetic information about landfill microorganisms. PCR primer pairs used in this study for amplification of DNA isolates are shown in Table III.5.

Table III.5 Amplification primer pairs used in polymerase chain reaction.

Primer	Target	Sequence (5' – 3')	Reference
A109 for	Archaeal forward	ACK GCT CAG TAA CAC GT	Grosskopf (1998)
1510 rev	Archaeal reverse	GGT TAC CTT GTT ACG ACT T	Ficker (1999)
27 for	Bacterial forward	AGA GTT TGA TCC TGG CTC AG	Ficker (1999)
1510 rev	Bacterial reverse	GGT TAC CTT GTT ACG ACT T	Ficker (1999)
GC515 rev	Archaeal reverse	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAT CGT ATT ACC GCG GCT GCT GGC AC	Lane (1991)
amoA – 1F	*amoA* forward	GGG GTT TCT ACT GGT GGT	Rotthauwe (1997)
amoA – 2R	*amoA* reverse	CCC CTC KGS AAA GCC TTC TTC	Rotthauwe (1997)
T7 for	pGEM-T plasmid	AAT ACG ACT CAC TAT AGG	Zoetendal (1998)
SP6 rev	pGEM-T plasmid	ATT TAG GTG ACA CTA TAG	Zoetendal (1998)

III.3.3.1. Archaeal 16S rDNA PCR

Archaeal 16S rDNA gene were enzymatically amplified with the archaeal-specific primer pairs A109 forward and 1510 reverse as previously described (Calli *et al.,* 2003). The PCR amplification reaction was performed in a Progene thermocycler (Techne, Cambridge, UK) and the following program was used: pre-denaturation (95 ^{o}C, 2 min), 34 cycles of denaturation (95 ^{o}C, 30 sec), annealing (52 ^{o}C, 40 sec), elongation (72 ^{o}C, 90 sec) and post elongation (72 ^{o}C, 10 min). The reactions were subsequently cooled to 4 ^{o}C.

Amplifications were performed in accordance with the manufacturer's recommendations by using 0.5 µM each primer, 1.5 U of Taq DNA polymerase (MBI Fermentas), 1 x PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP. The template DNA was diluted 10 times, due to humic acid inhibition, and 2 µl was added to a final volume of 50 µl.

After amplification, PCR products were analyzed on ethidium bromide-stained 1 % agarose gels. No amplified products were observed in the negative control

reaction, and expected size (1.4 kb) of amplified products were obtained when genomic DNA isolated from the landfill leachate was used as a template.

III.3.3.2. Bacterial 16S rDNA PCR

Nearly full-length bacterial 16S rDNA fragments were amplified by PCR from landfill leachate DNA extracts with the general bacterial 16S rDNA primers, 27 forward and 1510 reverse. The PCR amplification reaction was performed in a Progene thermocycler (Techne, Cambridge, UK) and the following program was used: pre-denaturation (95 ^{o}C, 5 min), 30 cycles of denaturation (95 ^{o}C, 60 sec), annealing (52 ^{o}C, 90 sec), elongation (72 ^{o}C, 90 sec) and post elongation (72 ^{o}C, 10 min). The reactions were subsequently cooled to 4 ^{o}C.

Amplifications were performed in accordance with the manufacturer's recommendations by using 0.5 µM each primer, 1.5 U of Taq DNA polymerase (MBI Fermentas), 1 x PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP. The template DNA was diluted 10 times, due to humic acid inhibition, and 2 µl was added to a final volume of 50 µl.

After amplification, PCR products were analyzed on ethidium bromide-stained 1 % agarose gels. No amplified products were observed in the negative control reaction, and expected size (1.5 kb) of amplified products were obtained when genomic DNA isolated from the landfill leachate was used as a template.

III.3.3.3. Archaeal 16S rDNA DGGE-PCR

For PCR-DGGE analysis total community DNA extracted from landfill leachate samples were PCR-amplified with archaeal 16S rDNA A109 forward and GC515 reverse primers. For the amplification of V2 – V4 region of archaeal 16S rDNA gene PCR were performed in a total volume of 50 µl containing 0.5 µM each primer, 1.5 U of Taq DNA polymerase (MBI Fermentas), 1 x PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP and 2 µl template DNA (10 times diluted).

Polymerase chain reaction was performed in Progene thermocycler (Techne, Cambridge, UK). The reaction began with an initial 95 ^{o}C denaturation for 2 min, followed by 34 cycles of 95 ^{o}C for 30 s, 52 ^{o}C for 40 s, 72 ^{o}C for 90 s, a final extension at 72 ^{o}C for 5 min and then it was held at 4 ^{o}C. After amplification, about 400 bp expected size of PCR amplified products were analyzed on ethidium bromide-stained 1 % agarose gels.

III.3.3.4. PCR Amplification of *amoA*

Primers targeting *amoA*-1F and *amoA*-2R (Rotthauwe *et al*., 1997) of ammonia-oxidizing bacteria from the β-subdivision of the group *Proteobacteria* were used to obtain amplicons of partial *amoA* sequences. PCR amplification of a 491-bp fragment of the *amoA* gene was carried out in a total volume of 50 µl in 0.5-ml tubes by means of a DNA thermocycler (Progene thermocycler-Techne, Cambridge, UK).

PCR with *amoA* primers was carried out at a cycling regime of: 4 min at 95 °C, then 35 cycles of each 1 min 95 °C, 45 sec 60 °C, and 1 min 72 °C. Final extension was carried out for 5 min at 72 °C.

Amplifications were performed in accordance with the manufacturer's recommendations by using 0.5 µM each primer, 1.5 U of Taq DNA polymerase (MBI Fermentas), 1 x PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP and 2 µl template DNA (10 times diluted).

III.3.3.5. PCR of Isolated Plasmids

Typical double-stranded PCR products are generally suitable for cloning. The quantity and quality of the amplification is then checked on an agarose gel. If a single, clear band is obtained, the PCR reaction can be used directly for cloning. Most PCR reactions, however, result in at least a few nonspecific bands that can interfere with cloning a specific fragment.

In the cloning procedure, plasmid isolation is necessary for sequencing. In this respect cloned samples were amplified with the primer pair T7 forward and Sp6 reverse. The PCR amplification reaction was performed in a Progene thermocycler (Techne, Cambridge, UK) and the following program was used: pre-denaturation (95 ^{o}C, 5 min), 30 cycles of denaturation (95 ^{o}C, 30 sec), annealing (45 ^{o}C, 30 sec), elongation (72 ^{o}C, 40 sec) and post elongation (72 ^{o}C, 5 min). The reactions were subsequently cooled to 4 ^{o}C.

PCR were performed in a total volume of 100 µl containing 0.5 µM each primer, 1.5 U of Taq DNA polymerase (MBI Fermentas), 1 x PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP and 2 µl isolated plasmid DNA (10 times diluted).

III.3.4. Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is a gel-electrophoretic separation procedure for double stranded DNA's of equal size but with different base-pair composition or sequence (Muyzer and Smalla, 1998). In principle, the method is sensitive enough to separate DNA's on the basis of single point mutations (Sheffield *et al.,* 1989).

Briefly, the 16S rRNA genes are amplified using the appropriate primer pair, one of which has a G+C "clamp" attached to the 5' end that prevents the two DNA strands from completely dissociating even under strong denaturing conditions. During electrophoresis through a polyacrylamide gel containing denaturants, migration of the molecule is essentially arrested once a domain in a PCR product reaches its melting temperature. Following staining of the DNA, a banding pattern emerges that represents the diversity of the rRNA gene sequences.

DGGE of the PCR amplified archaeal partial 16S rDNA was performed with the BioRad D-Code Universal Mutation Detection System (BioRad) in accordance with Nübel *et al*. (1996). The PCR product was loaded on to 1-mm-thick 8 % (wt/vol) polyacrylamide (ratio of acrylamide to bisacrylamide, 37.5:1) gels containing a 25 to 55 % linear denaturing gradient for archaeal 16S rDNA. The denaturing gradient gels were electrophoresed in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM Na-EDTA; pH 8.0) at 85 V and 60°C for 16 hours. Previously a voltage of 200 V was applied for 5 min.

Silver-staining and development of the gels was performed as described in Sanguinetti *et al.* (1994). The procedure consisted of an initial pre-stain fixation for 3 min in 10% ethanol, 0.5% acetic acid, staining for 10 min in fixing solution plus 0.2% silver nitrate, washing of gel in water for 2 min and development for approximately 45-60 min in 1.5% NaOH and 0.3% formaldehyde and 80 µg/l sodium borohydrate in deionized water. Following the staining, gels were fixed for a further 5 min and washed in deionized water to provide a permanent record of the experiment. Subsequent to this second fixation, gels were racked for 7 min in 25% ethanol and 10% glycerol preservation solution and covered with porous hydrophilic cellophane. Finally, gels are dried overnight at 37^{o}C.

III.3.5. Cloning and Sequencing

As DGGE gels contain many bands in one lane because of the microbial complexity of sludge samples, cloning and sequencing techniques were used to find out which band corresponded to which species. For cloning, the amplified archaeal 16S rDNA products and *amoA* products were purified with a QIAquick PCR purification kit (Qiagen) and cloned in competent *E. coli* JM109 cells by using the pGEM®-T Easy vector system (Promega) with ampicillin selection and blue/white screening, according to the manufactures manual.

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. The inserts were screened by Restriction Fragment Length Polymorphism (RFLP) analysis with the enzyme *MspI* (Fermentas) and by mobility comparison on DGGE. Plasmids of selected transformants were purified using the Wizard Plus SV miniprep DNA purification kit (Promega).

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. Sequencing analysis was carried out in a private sequence laboratory (SeqLab Sequence Laboratuaries, Göttingen, Germany) in Europe. A similarity search, in the GenBank database, with the derived partial (app. 800 bp) 16S rDNA sequences from the clones, was performed by using the NCBI sequence search service, available on the internet. 16S rDNA and *amoA* sequences were aligned by using the multiple alignment Clustal W programs (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.

III.3.6. Fluorescence in Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) is a molecular technique used for the detection of target Bacterial or Archaeal groups or species of interest in natural systems and it also allows the simultaneous analysis of multiple targets. The FISH method consists of four steps: fixation, hybridization, washing, and detection.

Specificity of probe binding to the target site depends on the hybridization and washing conditions. Hybridization probes are added to a defined, stringency determining buffer at saturation concentrations to maximize probe binding. During hybridization the samples are incubated at elevated temperature in an airtight vessel saturated with water and formamide vapors of additional hybridization buffer to avoid concentration effects due to evaporation. The washing step is performed at a slightly higher temperature and serves mainly to rinse off excess probe molecules at conditions that prevent unspecific binding.

In this study, microorganisms in leachate samples were harvested by centrifugation at 14,000 × *g* for 5 min, and each cell pellet was resuspended in 750 µl of a solution containing freshly prepared 4% paraformaldehyde (PFA) in water (Raskin *et al.,* 1994). PFA fixation solution was prepared by mixing 1 drop of 10 M NaOH, 2 g of PFA, and 16.5 ml of 3x phosphate-buffered saline with 33 ml of ddH_2O. Then PFA solution was heated to 60°C and cooled on ice, the pH was adjusted to 7.2, and finally the solution was filtered through a 0.45-µm-pore-size filter. After preparation of solution, cells were resuspended in PFA by vortexing the preparation for approximately 60 s and then were incubated at room temperature for 4 h or overnight. Next day, cells were recovered by centrifugation and washed in a solution containing 900 µl of phosphate-buffered saline (130 mM NaCl plus 10 mM sodium phosphate, pH 7.2). The final cell pellet was resuspended in a solution containing 500 µl of PBS and an equal amount of absolute ethanol and stored at 4°C.

Fixed cells were spotted on gelatin-coated [0.1% gelatin and 0.01% $KCr(SO_4)_2$] multiwell glass slides (10 wells/slide; 4 µl of sample/well) and allowed to dry at room temperature (Raskin *et al.,* 1994). The slides were then dehydrated by immersing them in 50% ethanol for 3 min, in 80% ethanol for 3 min, and then in 100% ethanol for 3 min and finally were air dried.

Hybridizations were performed at 46°C for 2 h with a hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl, pH 8.0, 0.01% SDS) containing each labeled probe (30

ng/well for Cy3 and 50 ng/well for FLUOS) (MWG Biotech, Ebersberg). Formamide was added to the final concentrations listed in Table III.6 to ensure the optimal hybridization stringency. After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer containing the same components of the hybridization buffer except the probes.

For detection of all DNA, the sludge samples were additionally stained with 4,6-diamidino-2-phenylindole (DAPI) for 10 min in the dark, finally rinsed again with distilled water, and immediately air-dried. The slides were mounted with Vectashield (Vector Laboratories) medium to prevent photo bleaching (Mertoglu *et al.,* 2005). The slides were examined with Leica DM-LB fluorescent microscope and digital images of the slides were captured with Leica DC350F digital camera.

III.3.7. Slot-Blot Hybridization

Slot blot hybridization was performed with DIG-labeled oligonucleotide to investigate the variations and activity of microbial populations during the anaerobic and aerobic degradation of incineration bottom ashes and shredded incombustible wastes. Extracted DNA was used for domain-specific probes for *Eukaryotes*, *Bacteria* and *Archaea*. All microorganisms were detected by using Universal specific oligonucleotide probe (Table III.6). PCR-amplified 16S rRNA archaeal and 16S rRNA bacterial genes were used to quantify relative differences of archaeal and bacterial populations during the operational period of aerated bioreactor test cell with relative hybridization signal intensity.

For experimental procedures, the manufacturer's protocol was followed. DNA products was heated at 95 °C for 10 min and chilled on an ice bath. Then, 5 µl of each amplification mixture was spotted on a nylon membrane using a Minifold II slot blotter (Schleicher and Schuell, Fredriksberg, Denmark) and UV-fixed (Vilber Lourmat, France). After UV-crosslinking, the membrane was cut into slices and dried to ambient air. For hybridization, the membrane slices were each pre-incubated in a 50-mL falcon tube in a hybridization oven at optimum hybridization temperature for 30 min with 10 mL of pre-hybridization buffer (Boehringer Mannheim GmbH, Biochemica).

Table III.6 Oligonucleotide probes used for FISH and slot-blot hybridization analysis.

Probe	Label	Formamide (%)	Sequence (5' --- 3')	Target organisms	References
UNIV1390	DIG	20	GAC GGG CGG TGT GTA CAA	Almost all organisms	Zheng *et al.,* 1996
EUK516	Cy3 or DIG	20	ACC AGA CTT GCC CTC C	*Eukaryote* domain	Amann *et al.,* 1990
EUB338	FLUOS or DIG	20	GCT GCC TCC CGT AGG AGT	*Bacteria* domain	Amann *et al.,* 1990
ARC915	Cy3 or DIG	20	GTG CTC CCC CGC CAA TTC CT	*Archaea* domain	Stahl and Amann, 1991
ALF1b	Cy3	35	CGT TCG Y TCT GAG CCA G	*α proteobacteria*	Manz *et al.,* 1992
BET42a	Cy3	35	GCC TTC CCA CTT CGT TT	*β proteobacteria*	Manz *et al.,* 1992
GAM42a	FLUOS	35	GCC TTC CCA CAT CGT TT	*γ proteobacteria*	Manz *et al.,* 1992
MX825	Cy3 or DIG	35	TCG CAC CGT GGC CGA CAC CTA GC	*Methanosaetaceae*	Raskin *et al.,* 1994
MS821	Cy3 or DIG	35	CGC CAT GCC TGA CAC CTA GCG AGC	*Methanosarcina*	Raskin *et al.,* 1994
MB310	Cy3 or DIG	35	CTT GCT TCA GGT TCC ATC TCC G	*Methanobacteriaceae*	Raskin *et al.,* 1994
MB1174	Cy3 or DIG	35	TAC CGT CGT CCA CTC CTT CCT C	*Methanobacteriaceae*	Raskin *et al.,* 1994
MC1109	Cy3 or DIG	35	GCA ACA TAG GGC ACG GGT CT	*Methanococcaceae*	Raskin *et al.,* 1994
MG1200	Cy3 or DIG	35	CGG ATA ATT CGG GGC ATG CTG	*Methanomicrobiales*	Raskin *et al.,* 1994
SRB385	Cy3 or DIG	35	CGG CGT CGC TGC GTC AGG	*Sulfate reducing bacteria*	Amann *et al.,* 1992
NSO1225	Cy3 or DIG	35	CGC CAT TGT ATT ACG TGT GA	*Ammonia oxidizing β-proteobacteria*	Juretschko *et al.,* 1998
NSO190	Cy3 or DIG	35	CGA TCC CCT GCT TTT CTC C	*Ammonia oxidizing β-proteobacteria*	Mobarry *et al.,* 1996
NSM156	Cy3	35	TAT TAG CAC ATC TTT CGA T	*Nitrosomonas spp.*	Mobarry *et al.,* 1996
NIT3	Cy3 or DIG	35	CCT GTG CTC CAT GCT CCG	*Nitrobacter spp*	Wagner *et al.,* 1996
NB1000	Cy3 or DIG	35	TGC GAC CGG TCA TGG	*Nitrobacter spp*	Mobarry *et al.,* 1996
NTSPA 662	Cy3 or DIG	35	GGA ATT CCG CGC TCC TCT	*Nitrospira genus*	Daims *et al.,* 2000

Hybridization and detection of oligonucleotide probes were achieved by DIG (digoxigenin) System (Boehringer Mannheim GmbH, Biochemica) according to the manufacturer's protocol. DIG-labeled oligonucleotide probes were hybridized for more than six hours with DIG Easy Hybridization buffer (Boehringer Mannheim GmbH). After washing, anti-DIG alkaline phosphatase conjugate was applied so that antibody hapten complex could be formed. Subsequent enzyme-catalyzed color reaction with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium salt) produced blue precipitates on the membrane.

CHAPTER IV

RESULTS AND DISCUSSIONS

IV.1. RAPID MOLECULAR METHODS TO EVALUATE POPULATION VARIETY IN DIFFERENT LANDFILLS

Since, landfilling of municipal solid wastes is still insufficient in Turkey, construction of new well-engineered landfills and remediation of unregulated dumping sites are urgently required. To enhance the stabilization in existing dumping sites and new landfills, the relationship between the landfill microbiology and stabilization should be certainly well understood. Therefore, in this study, in addition to chemical characterization and methane potential assays, rapid molecular methods, fluorescence in situ hybridization and slot-blot hybridization techniques were used to evaluate population variety during stabilization periods of sanitary landfills.

IV.1.1. Chemical Characterization of Leachate Samples

In this part, chemical characterization of leachate samples taken from Bursa-Hamitler (B), İzmir-Harmandali (H), Odayeri-Kemerburgaz (O) and Kömürcüoda (K) Sanitary Landfills were analyzed to determine the stabilization phases in landfills. The results from the physical and chemical characteristics of seven leachate samples were listed in Table IV.1. Physical and chemical experiments were carried out according to the standard methods for the examination of water and wastewater.

The heterogeneity of the landfill, in relation to cells that characterize the different transformation stages of biological degradation and that represent different ages, can be better accounted by the ratio of BOD_5/COD.

Table IV.1 Physical and chemical characterizations of leachate samples.

PARAMETERS	BURSA HAMİTLER LANDFILL	İZMİR HARMANDALI LANDFILL		İSTANBUL KEMERBURGAZ LANDFILL			İSTANBUL KÖMÜRCÜODA LANDFILL
	B	H1	H2	O1	O2	O3	K
pH	8.2	8.3	8.1	8.0	7.9	7.8	7.8
Alkalinity, mg /L	4460	8780	5980	14400	11800	12200	9400
COD, mg /L	7480	14100	2320	3150	3520	10470	12840
BOD_5, mg /L	4690	7340	46	126	191	5240	8410
BOD_5/COD ratio	0.63	0.52	0.02	0.04	0.05	0.50	0.65
NH_3-N, mg /L	1015	1850	1450	2780	2640	1960	2020
TKN, mg /L	1160	1935	1585	3150	3020	2325	2370
PO_4^{3-}, mg /L	11	18	10	106	111	31	40
SS, mg /L	335	652	168	126	223	907	850
VSS, mg /L	242	479	110	105	177	773	625
Total P, mg /L	-	25	15	-	-	-	-
Color, PtCo	4250	6900	5300	5800	5200	5500	5410

Table IV.1 Physical and chemical characterizations of leachate samples (continued).

PARAMETERS	BURSA HAMİTLER LANDFILL	İZMİR HARMANDALI LANDFILL		İSTANBUL KEMERBURGAZ LANDFILL			İSTANBUL KÖMÜRCÜODA LANDFILL
	B	H1	H2	O1	O2	O3	K
Conductivity, mS/cm	9840	23000	19310	28600	22830	25600	18310
TDS, mg/L	6500	15160	13050	19360	17100	17100	12160
Chloride, mg/L	3300	5450	4840	5600	5600	5560	4250
Sulfate, mg/L	122	68	59	14	15	17	13
Sulfide, mg/L	0.2	0.4	0.2	0.6	0.4	1.5	0.4
Zn, mg/L	1.4	1.3	2.1	0.9	0.6	0.3	2.1
Ni, mg/L	0.4	5.4	0.4	0.8	0.3	4.9	2.3
Cr, mg/L	0.5	4.0	0.8	2.3	2.7	8.4	0.9
Fe, mg/L	21.2	57.1	13.4	17.1	5.8	22.1	30.1
Pb, mg/L	<0.5	<0.5	<0.5	< 0.5	<0.5	< 0.5	<0.5
Cu, mg/L	1.5	0.3	0.7	0.2	0.1	0.1	0.3

The results of physical and chemical parameters showed that different stabilization stages can be formed into the same landfill while transformation may occur at the recent and old deposited landfill cells.

pH values were certainly high in all leachate samples and have varied between 7.8 and 8.3. Considerably high pH values unusual for young landfill leachate were actually due to elevated alkalinity levels which were strongly affected by extremely high ammonia concentrations. Total alkalinity has ranged between 4460 and 14400 mg/l as $CaCO_3$ while ammonium nitrogen was in the range of 1015 to 2780 mg/l. However, typical pH level and ammonium nitrogen concentration of young landfill leachate were reported in the range of 4.5 to 7.5 and 10 to 800 mg/l, respectively (Tchobanoglous *et al.,* 1993).

Fairly high BOD_5 and COD concentrations were determined in leachate samples taken from landfill units which were recently closed (B and H1) or still under operation (O3 and K) when samples were analyzed. The highest BOD_5 and COD concentrations were reported for leachate generated from landfills at acid formation phase. When landfill ages, COD and BOD_5 levels begin to decrease as volatile fatty acids are consumed (Qasim *et al.,* 1994). Since leachate sample B was collected in a rainy day, the dilution effect of rain water was strongly reflected in its quality and quite lower BOD_5 and COD concentrations were determined in this young landfill leachate (Table IV.1).

Chian and DeWalle (1977) found that many ratios of chemical properties, such as COD/TOC, BOD_5/COD, and VOA/TOC reflect the composition of the organic matter in leachate and are in turn related to the age of the landfill. In this study, to specify the degree of stabilization in the landfills, BOD_5/COD ratio in leachate samples was used as an indicator parameter. Ratios about 0.5 in samples O3 and H1 and ratios above 0.6 in samples K and B indicated high decomposition activity and acid formation phase of stabilization in relevant landfill units. The results of the slight differences between BOD_5/COD ratios of leachate samples collected from open (O3 and K) and recently closed (B and H1) landfill units may be the improved acidification promoted by anaerobic conditions in closed units. On the other hand, BOD_5/COD ratios below 0.05 in leachate samples O1, O2 and H2 were indications of almost complete stabilization in these landfill units (Table IV.1). In previous studies, the ratio of BOD_5/COD in the acid phase has been reported to be above 0.5 (Qasim *et al.,* 1994). When acidogenic landfill progresses to methane formation

phase, BOD_5/COD ratio drops below 0.1 and as maturation occurs, ratio continues to decline and may approach zero (Pacey, 1999). The calculated ratio of BOD_5 to COD based on Miller's (1974) data showed a decrease from 0.47 to 0.07 within a period of 23 years. Chian and DeWalle (1977) found the ratio decreased from 0.49 to 0.05.

Ammonia and organic nitrogen produced by decomposition of organics are stable in an anaerobic environment, and therefore represent a high percentage of the soluble nitrogen compounds in leachate (McBean *et al.,* 1995). Leachate of older landfills generally has lower concentrations and percentages of these constituents (Robinson and Maris, 1979). Unlike ammonia concentrations, phosphate levels remain generally low throughout the life of the landfill. During later stages of waste stabilization, phosphorous may be limiting (Pohland and Harper, 1985). In this study, TKN to NH_3-N ratios between 1.05 and 1.20 indicated that most of the nitrogen is in ammonium form in all leachate samples independent of stabilization degree. Because of the high moisture contents of solid waste in Turkey, most of the organic nitrogen rapidly converted to ammonium in sanitary landfills. That is, high moisture content enhances the hydrolysis process.

Due to the predominantly anaerobic conditions within the landfill, the sulfate concentration decreases rapidly as sulfate is reduced to sulfide. This tendency increases as the methanogenic phase becomes more established. Sulfate concentrations were pretty low especially in leachate collected from Istanbul Odayeri and Kömürcüoda Landfills. Another indication of the acidogenic activity in the landfill units was comparatively high concentrations of suspended and volatile suspended solids in samples O3, K, H1 and B (Table IV.1).

Heavy metal concentrations in leachate do not appear to follow patterns of organic indicators such as COD or BOD_5, nutrients, or major ions (Lu *et al.,* 1985). Heavy metal release is a function of characteristics of the leachate such as pH, flow rate, and the concentration of complexing agents. Metal solubility generally decreases with increasing pH. As given in Table IV.1, heavy metal concentrations were quite low, except for iron and there was not an obvious relation between heavy metal concentrations and stabilization degree of landfills.

IV.1.2. Biogas Potential of Leachate

In addition to chemical analysis, comparative methane potentials of leachate samples were also assessed in this study to define the degree of stabilization in the landfill units. Since methane generation rate typically increases to a peak and then certainly declines with time as landfill ages, methane potential may be a good indicator in assessing and defining the degree of landfill stabilization (Tchobanoglous *et al.,* 1993). Methane potential may be assessed with field testing and monitoring or with batch assays in laboratory using excavated landfill or leachate samples (Chen *et al.,* 2003; Hansen *et al.,* 2004). The cumulative methane productions from leachate samples, VFA mixture and seed sludge (blank) were plotted in Figure IV.1-2. Low, moderate and high biogas potentials were evaluated as methanogenic, methanogenic/acidogenic and acidogenic phases in landfills.

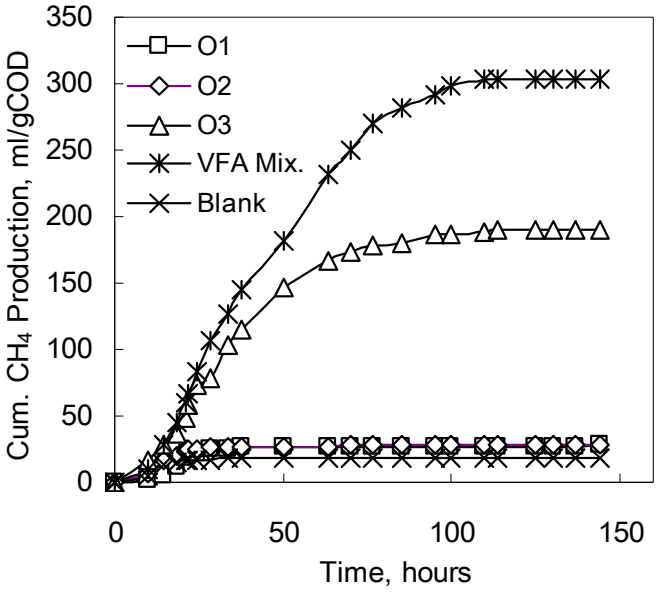


Figure IV.1 Cumulative methane production from Odayeri leachate samples.

The actual total methane gas productions were calculated by subtracting the cumulative methane gas data of blank from the others. Subsequently, comparative methane potentials presented in Figure IV.3 were calculated according to VFA mixture by assuming its methane potential as 100%. Quite high methane potentials indicating acidogenic phase of landfill stabilization were detected in leachate samples collected from young landfill units (O3, K, B and H1). On the other hand,

mature landfill leachates (O1, O2 and H2) produced significantly low amounts of methane gas (Figure IV.1-2) and represented fairly low methane potentials (Figure IV.3). These data were consistent with leachate qualities and confirming the BOD_5/COD ratios.

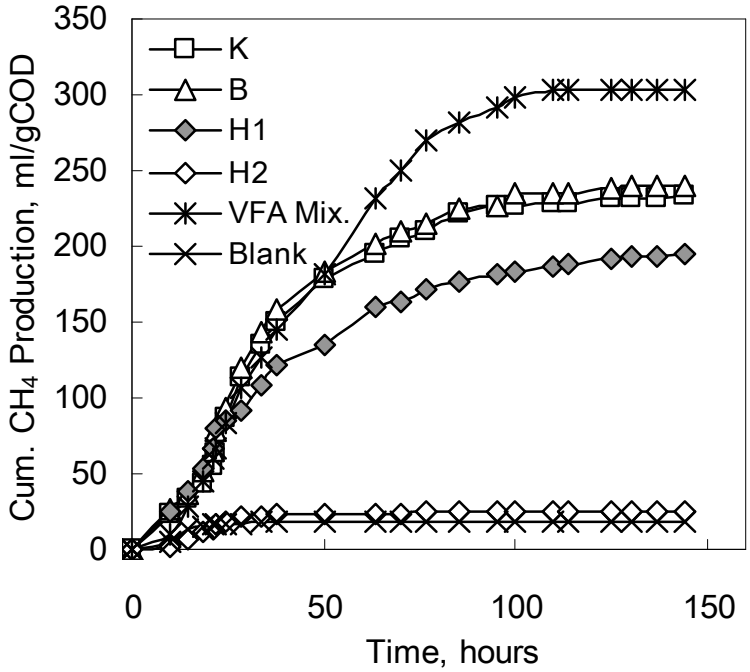


Figure IV.2 Cumulative methane production from different landfill leachate samples.

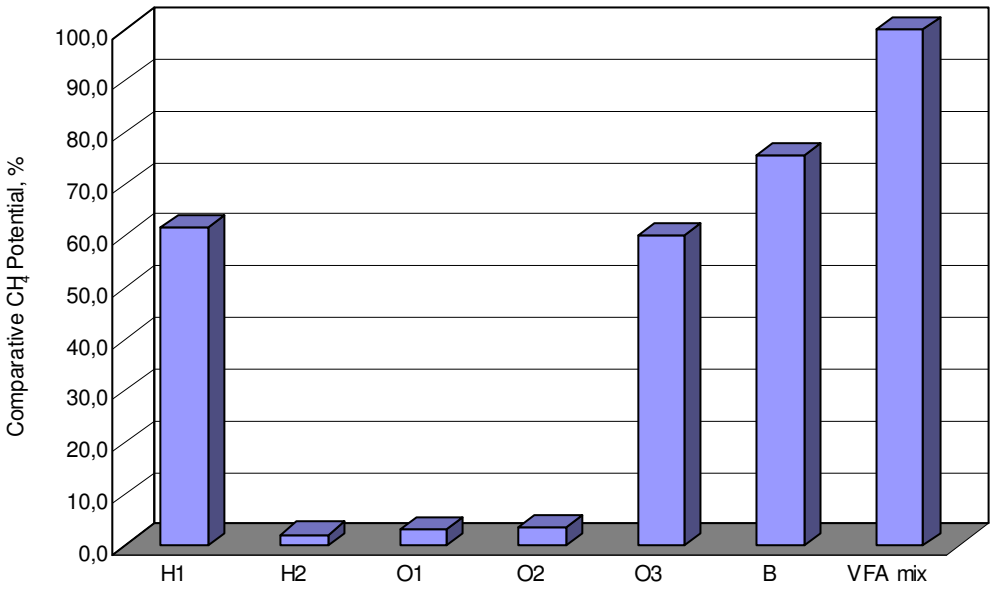


Figure IV.3 Comparative methane potentials of leachate samples.

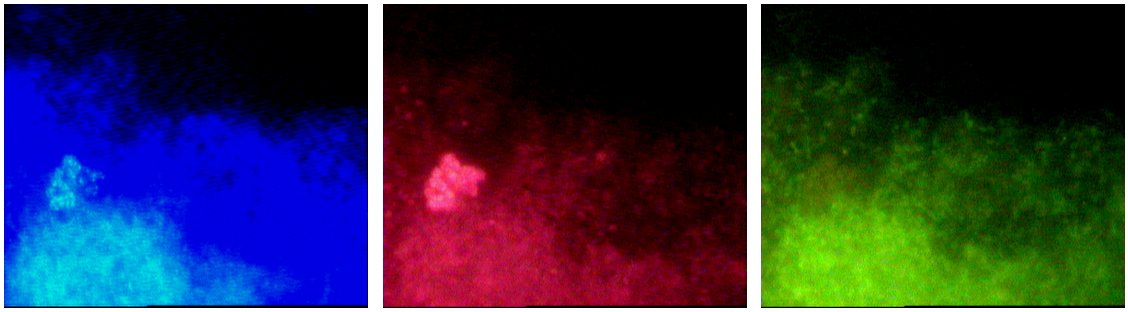
IV.1.3. In Situ Identification of Landfill Leachate

FISH analysis of leachate samples revealed that above 90% of the microorganisms (DAPI stained cells) could be detected with bacterial (Eub338) and archaeal (Arch915) specific probes in all landfill leachate samples. Bacterial population was found more intensive than archaeal population according to previous studies (Huang *et al.,* 2002; Chen *et al.,* 2003; Mori *et al.,* 2003). While methanogenic *Archaea* were detected in moderate amounts in acidogenic leachate samples (K, B, O3 and H1), they were limited in numbers in mature leachates O1 and O2 and virtually absent in leachate sample H2. High biodegradable organic matter concentrations, pH levels around 8 (Table IV.1) and relatively high methane potentials (Figure IV.3) in acidogenic leachate samples were consistent with the occurrence of methanogenic *Archaea* in young landfill units. Confirming the FISH results, mature landfill leachates having very low methane potentials and BOD_5/COD ratio were not favorable for methanogens. Low abundance (approx. 2%) of methanogenic *Archaea* in a mature landfill leachate was revealed in a previous study using quantitative oligonucleotide hybridization (Huang *et al.,* 2003). Similarly, only less than 1% of the total cells were detected with *Archaea* specific Arch915 probe in one year old landfill samples excavated from 1 and 3 m depths (Chen *et al.,* 2003). In another study, archaeal DNA represented 2-3% of the total DNA extracted from the leachate of a landfill receiving solid wastes mainly consisting of incineration ash (Mori *et al.,* 2003).

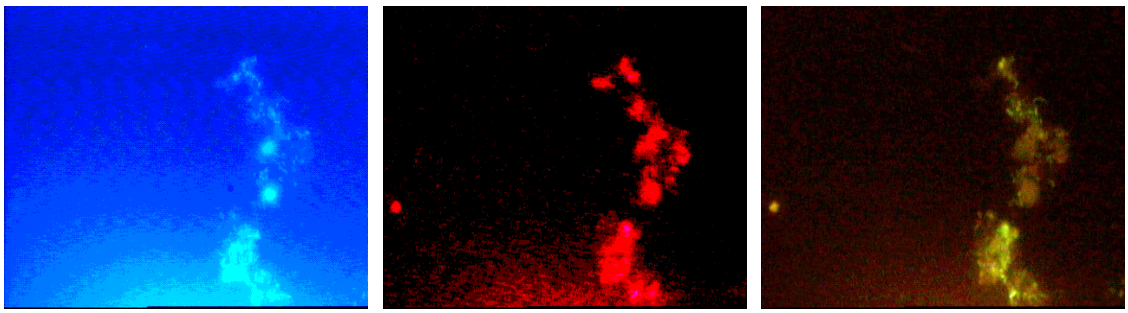
Leachate samples were hybridized with the fluorescein-labeled bacterial probe, EUB338 in order to visualize all *Bacteria*. To visualize all methanogenic *Archaea* presents in leachate samples, Cy3-labeled archaeal probe ARC915 was used in FISH analysis. Using rRNA targeted probes, two representative parts of the whole microbial community, namely acidogenic and methanogenic cells present in landfill ecosystem could be successfully observed (Figure IV.4-5).

Archaea, *Bacteria* and total microorganisms in leachate samples taken from Bursa-Hamitler (B) and İzmir-Harmandalı (H1-H2) Landfills were given in Figure IV.4. In the previous parts of the study, Hamitler Landfill and young part of the Harmandalı Landfill was characterized as immature; old part of the Harmandalı Landfill was specified as mature.

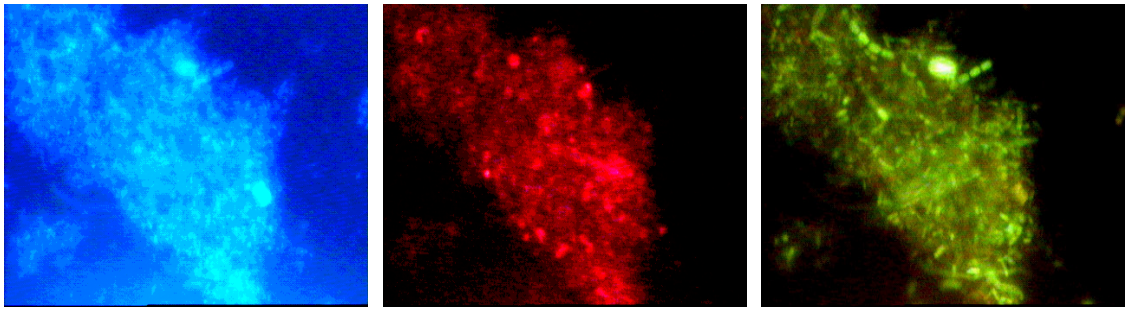
In Bursa-Hamitler Landfill (B) leachate samples, bacterial population was dominated to archaeal population. In addition, approximately 90 % of total microorganisms were belonging to bacterial domain in acidogenic sites of Harmandalı Landfill (H1). These results indicated that bacterial population is dominated to archaeal population in the early stages of landfill stabilization. On the other hand, microorganisms in old section of Harmandalı Landfill were sparse and mostly consisted of *Archaea*. This observation indicated that archaeal population was more intensive than bacterial population in mature landfills.



(a) Bursa-Hamitler -B-



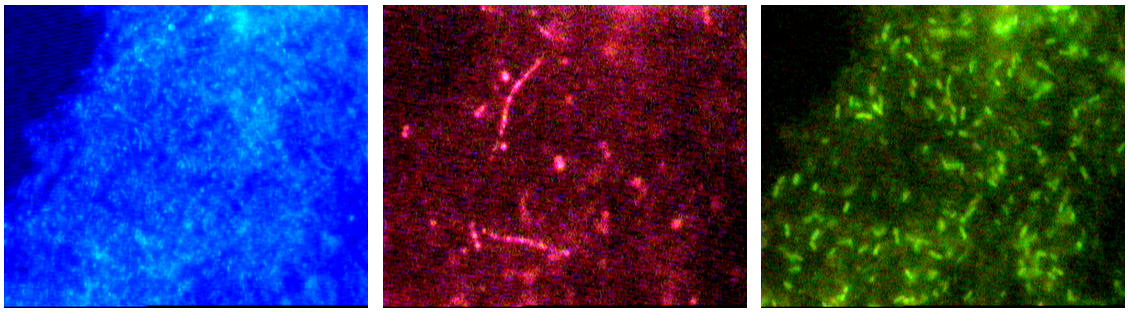
(b) İzmir-Harmandali -H2-



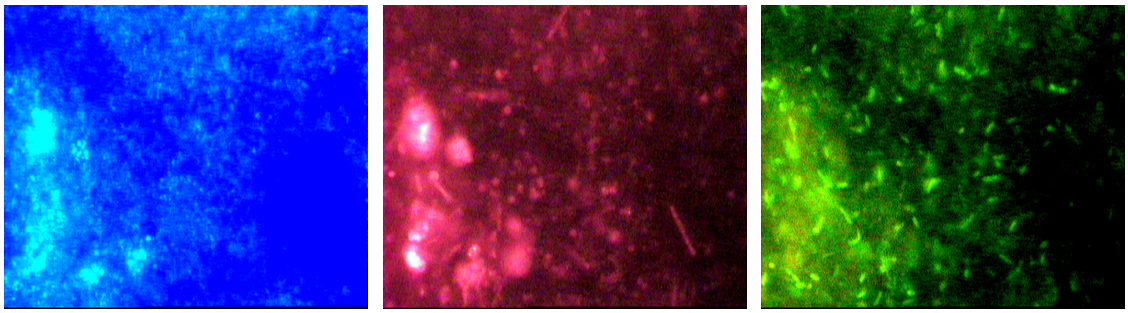
(c) İzmir-Harmandali -H1-

Figure IV.4 In situ identification results of Bursa-Hamitler and İzmir-Harmandali leachate samples. Sludge samples were hybridized with bacterial-specific probe (labeled with FLUOS-green) and archaeal-specific probe (labeled with Cy3-red). All microorganisms stained with DAPI (blue). Blue, green and red couples represent the same fields of the microscopic view.

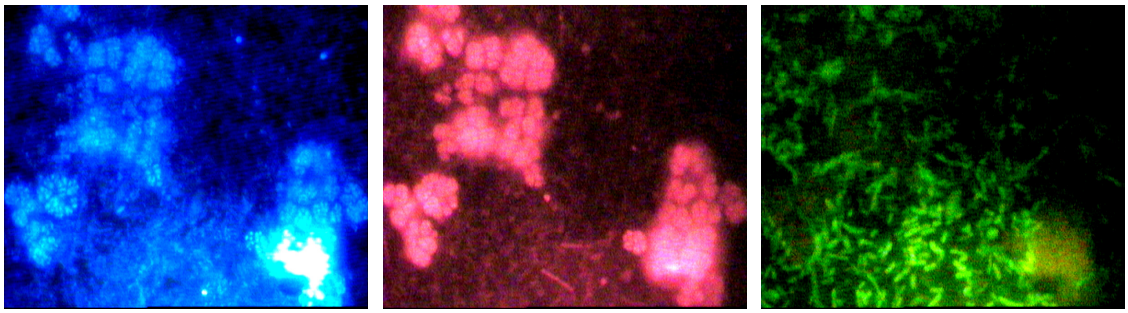
In situ identification of Odayeri-Kemerburgaz Landfill leachate samples hybridized with bacterial and archaeal specific probe were shown in Figure IV.5. As mentioned before, O-1 and O-2 leachates were characterized as methanogenic while O-3 leachate was characterized as acidogenic.



(a) Odayeri -O1-



(b) Odayeri -O2-



(c) Odayeri -O3-

Figure IV.5 In situ identification results of Odayeri leachate samples. Sludge samples hybridized with bacterial-specific probe (labeled with FLUOS-green) and archaeal-specific probe (labeled with Cy3-red). All microorganisms stained with DAPI (blue). Blue, green and red couples represent the same fields of the microscopic view.

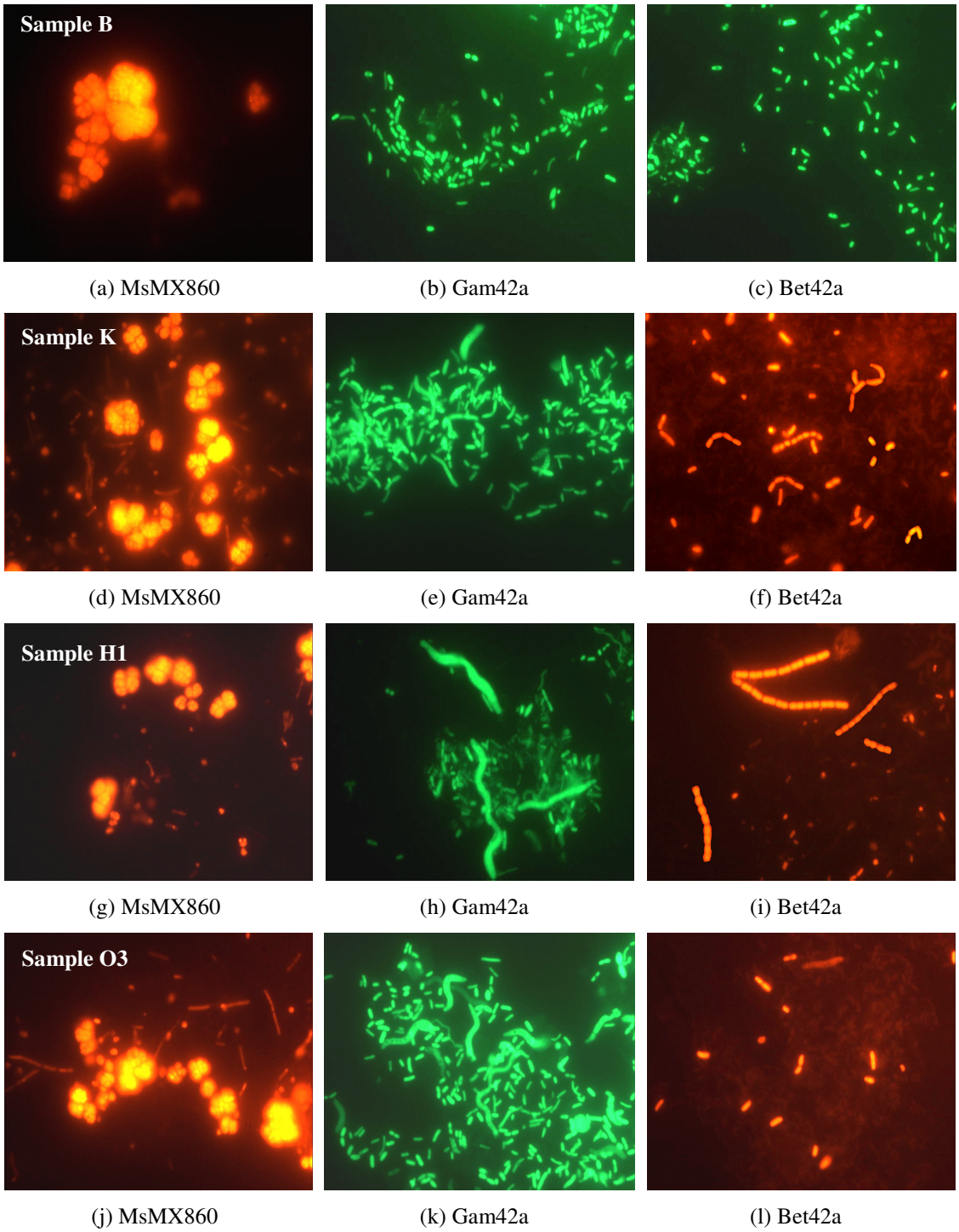
The dominant species of O-1 leachate were methanogenic *Archaea*. Some methanogenic *Archaea*, especially hydrogenotrophic methanogens have autofluorescent characteristics; therefore they could be observed in both green and red light. This observation confirms that microbial diversity of mature landfills like O-1 consists of predominantly methanogenic *Archaea*.

O-2 leachate sample had similar morphological characteristics as O-1 sample. It was also observed that both bacterial and archaeal population were in small quantities however archaeal domain was dominated to *Bacteria* in O2 sample. The reason of archaeal dominance can be explained by phase of this section in landfill that was determined as maturation in the previous parts of the study. In O-3 leachate sample, cell morphologies were significantly different from O-1 and O-2 (Figure IV.5). Due to this section of landfill is in early stages of degradation process, it comprises high amount of *Bacteria* and *Archaea* and the dominant species was *Bacteria*.

To explain the relative differences of bacterial and archaeal population changes from different landfills, FISH experiments were carried out with domain and group-specific oligonucleotide probes. In situ hybridization results of these probes revealed that acetate utilizing methanogens especially members of genus *Methanosarcina* that favor high acetate concentrations with their stringent cluster structures were dominant in all young landfill leachate samples (Figure IV.6). On the other hand, only very few, long rod and filamentous methanogenic *Archaea* belong to *Methanosaeta* genus were present in mature leachate samples O1 and O2 (Figure IV.7). These findings of FISH analysis indicated that there was an obvious correlation between the landfill stability and occurrence and abundance of *Methanosarcina* clusters (Figure IV.6 and IV.7). Likewise, members of the genus *Methanosarcina* seemed to be predominant in a young landfill sample (Chen *et al.,* 2003). In another study, the cloning analysis suggested that majority of the methanogens was acetate utilizing *Methanosaeta* in a sea-based landfill used for disposal of partially stabilized incineration ash (Mori *et al.,* 2003).

Only very few hydrogen utilizing methanogens were detected with in-situ hybridization. Therefore, their amounts were supposed to be below than lower detection limits. However, a PCR-based study revealed a great diversity in the methanogen population within the landfill and reported high numbers of hydrogen utilizing methanogens as compared with acetoclastic species in both of the young and mature landfill samples (Luton *et al.,* 2002). Based on cloning analysis, a relatively low diversity of *Archaea* was found at different depths of another moderately active landfill and similarly dominant clones were closely related to hydrogen utilizing methanogens (Chen *et al.,* 2003). In addition, studies on archaeal community compositions of young (Huang *et al.,* 2002) and old landfill leachates

(Huang *et al.,* 2003) indicated that the hydrogenotrophic *Methanomicrobiales* were the predominant archaeal community in such environments. These observations suggest that the methanogenic population diversity may be quite dissimilar in different landfill sites as well as in different stabilization phases.



(a) MsMX860 (b) Gam42a (c) Bet42a

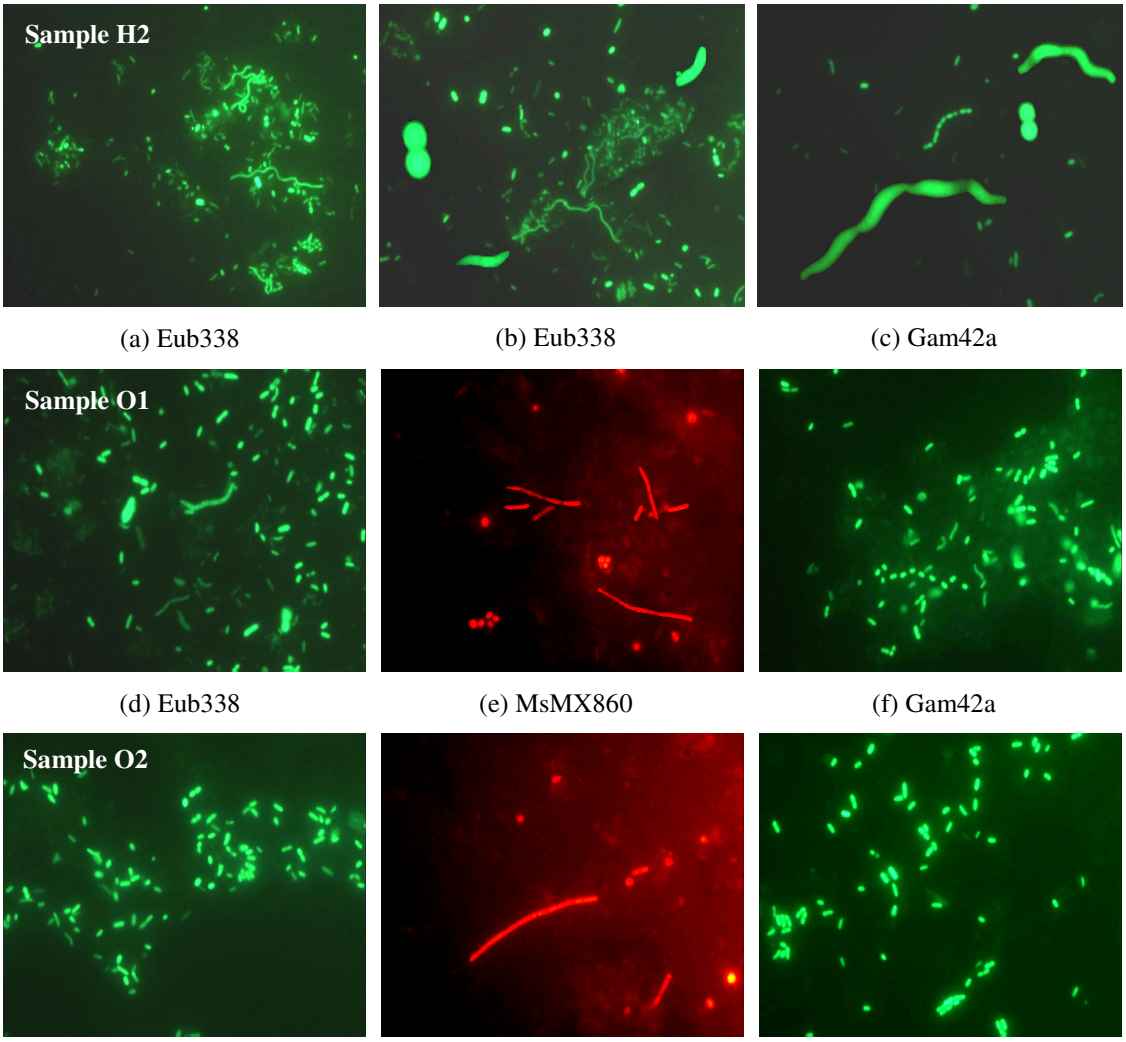
(d) MsMX860 (e) Gam42a (f) Bet42a

(g) MsMX860 (h) Gam42a (i) Bet42a

(j) MsMX860 (k) Gam42a (l) Bet42a

Figure IV.6 Fluorescence in situ hybridization photomicrographs of young landfill leachate samples hybridized with Cy3 labeled MsMx825 (red), Gam42a (green) and Bet42a (red) probes.

Since, in landfills sulfate is usually present in significant amounts (Daly *et al.,* 2000), quite low concentrations in leachate samples O and K were supposed to be an indication of enhanced sulfate reduction activity in the landfills. However, virtually no SRB were detected with SRB385 probe in none of the leachate samples, although their widespread occurrence in several municipal solid waste landfills was previously reported (Daly *et al.,* 2000).



(a) Eub338 (b) Eub338 (c) Gam42a

(d) Eub338 (e) MsMX860 (f) Gam42a

(g) Eub338 (h) MsMX860 (i) Gam42a

Figure IV.7 Fluorescence in situ hybridization photomicrographs of mature landfill leachate samples hybridized with FLUOS labeled Eub338, Cy3 labeled MsMx825 and Gam42a probes.

To identify the bacterial sub-groups, oligonucleotide probes for Gram-positive bacteria with low G+C content, *Cytophaga/Flavobacteria* group, *Verrucomicrobiales* and three subclasses of *Proteobacteria* (alpha, beta and gamma) were used. Gamma and beta *Proteobacteria* were presented as the major bacterial communities in all landfill leachate (Figure IV.6 and IV.7). However, the amounts of

beta-*Proteobacteria* in mature landfill leachates were quite lower than in young leachate samples (Figure IV.6 and IV.7). The amounts of other bacterial sub-groups were relatively low and only few numbers of Gram-positive bacteria with low G+C content and *Bacteria* belong to alpha subdivision of *Proteobacteria* were detected. *Cytophaga/Flavobacteria* and *Verrucomicrobiales* groups were virtually absent. In two recent studies, relatively high bacterial diversities were found in young and mature landfill leachates, with cloning analysis. The low-G+C gram-positive bacteria, the *Chlamydiae-Verrucomicrobia* group and the *Cytophaga-Flexibacter-Bacteroides* group were suggested to be the major bacterial groups in the young leachate of a full scale recirculating landfill (Huang *et al.,* 2004). In the second study, low-G+C gram-positive bacteria and gamma-*Proteobacteria* were reported predominant in the mature landfill leachate (Huang *et al.,* 2005).

In another study, the widespread occurrence of *Clostridium thermocellum* and *C. leptum* species in landfill leachate were demonstrated using specific amplifications of these groups from DNA extracts of landfill leachate samples (Daly *et al.,* 2000). Since, clostridia specific oligonucleotide probes were not used in this study, their occurrence and abundance could not be determined.

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.

It has been well accepted that each molecular approach introduce biases. In our case, the abundance of species level methanogenic populations were not clearly detected by in situ hybridization techniques. Diversity and activity of methanogenic population that present in old and young landfill leachate were investigated by using slot-blot hybridization techniques.

The results indicated that the application of in situ hybridization technique to leachate samples allows a good insight into the structure of microbial communities in the landfills and this technique is likely to become a favored method for routine analysis in evaluation of landfill stability and better management of stabilization processes in the landfills.

IV.1.4. Comparative Evaluation of Microorganisms in Different Landfills

Due to the potential biases introduced by the fluorescence in situ hybridization, the abundance of methanogens does not necessarily represent the true frequency of the corresponding species in the original sample. Alternatively, oligonucleotide probes can be used in slot-blot hybridizations to quantify the abundance of a certain DNA in the nucleic acids extracted directly from environmental samples (Stahl *et al.,* 1988). Although data of relative DNA abundance cannot be directly translated into cell numbers or activity, they represent a reasonable measurement of the relative physiological activity of the respective population, a very important consideration in ecological studies. Therefore, relative abundance of *Archaea*, *Bacteria* and various phylogenetic groups of methanogens was quantified by probing DNA samples isolated directly from the landfill leachate using oligonucleotide probes that target most currently known species (Griffin *et al.,* 1998; Raskin *et al.,* 1994).

In this part, slot blot hybridization with oligonucleotide probes specific for archaeal and bacterial domain were performed with total-community DNAs extracted from six different leachate samples taken from Odayeri-Kemerburgaz, Bursa-Hamitler and İzmir-Harmandali Landfills. The results showed that bacterial population was found more intensive than archaeal population in Harmandali and Hamitler Landfills, but in Odayeri samples, the abundance of archaeal population is close to the *Bacteria* (Figure IV.8). In a previous study, Huang (2003) observed that methanogens in the leachate of a closed municipal solid waste landfill accounted for only a very small fraction of the total community (approximately 2%).

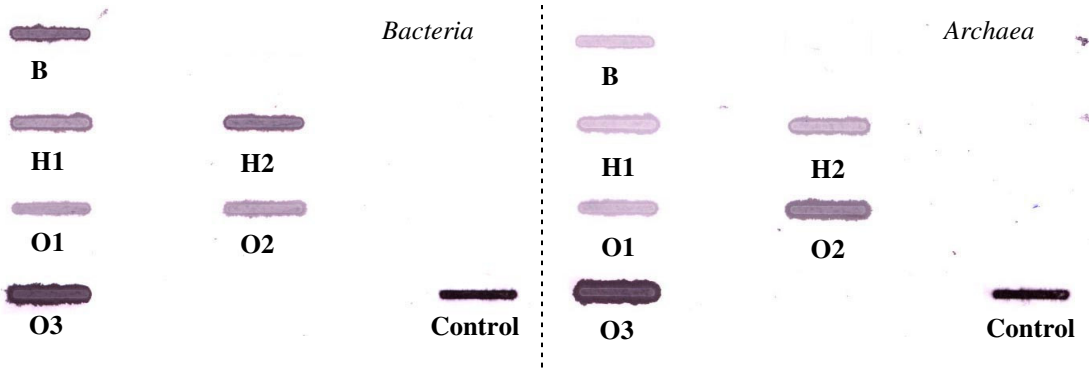


Figure IV.8 Slot blot analysis of DNA extracted from Bursa (B), İzmir-Harmandali (H) and Odayeri (O) leachate samples. Thickness of the bands is directly proportional to the amount of target DNA.

To explain the relative differences of microbial population changes from different stabilization stages of same landfill (Odayeri, O1-O2-O3), slot blot hybridization were carried out with group-specific oligonucleotide probes.

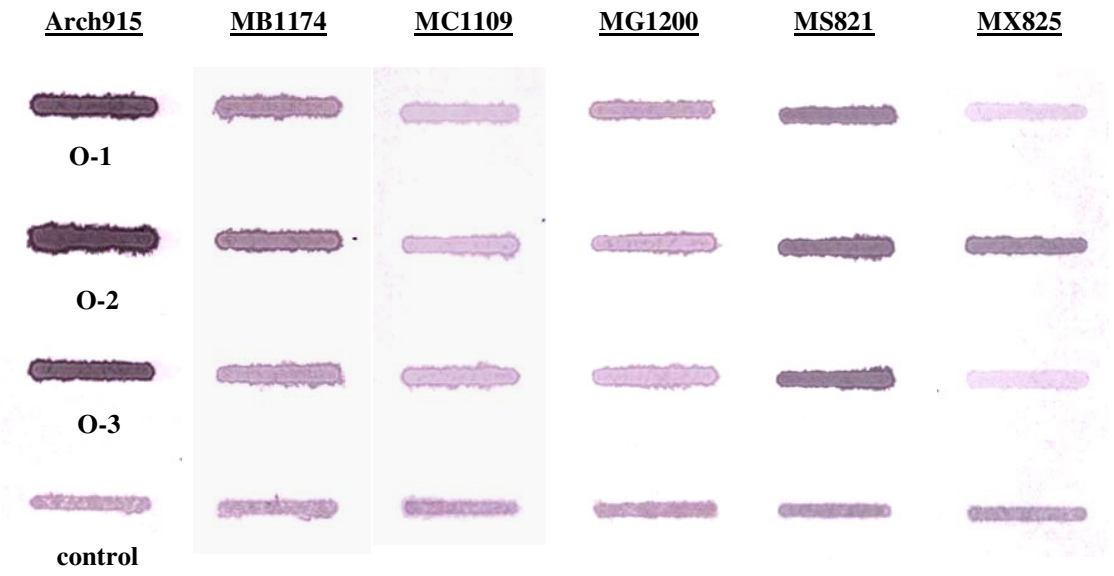


Figure IV.9 Specificities of the oligonucleotide probes used in slot blot analysis targeting *Archaea* (Arch915), *Methanobacteriales* (MB1174), *Methanococcales* (MC1109), *Methanomicrobiales* (MG1200), *Methanosarcina* (MS821), *Methanosaeta* (MX825).

Members of the hydrogenotrophic order *Methanobacteriales* constituted the major methanogens present in O1 leachate, whereas the other hydrogenotrophic methanogens belonging to *Methanococcales* and *Methanomicrobiales* were present at low levels of the total 16S rRNA archaeal gene (Figure IV.9).

Concerning the acetoclastic methanogens, *Methanosarcina spp.* were most abundant archaeal group in O2 and O3 leachate samples, whereas *Methanosaeta spp.* were detected significantly in O2 sample.

Huang (2003) indicated that cell lyses and DNA extraction are especially worth considering, since *Methanosarcina spp.* have unusual outer cell layers (polysaccharide sacculus) that might lead to difficulties in nucleic acid extraction. In our study, total-community DNA was isolated from the leachate using FastDNA® kit protocol and *Methanosarcina spp.* were observed in all leachate samples.

Raskin *et al.* (1994) designed eight probes which targeted the phylogenetically defined groups of methanogens. In this study, oligonucleotide probe hybridization experiments revealed positive signals with all methanogenic probes for all leachate samples (Figure IV.9).

IV.2. INVESTIGATION OF ARCHAEAL DIVERSITY CHANGES IN AN AERATED LANDFILL BIOREACTOR

In this part, archaeal population diversity in an aerated bioreactor test cell filled with incineration bottom ashes and shredded incombustible wastes was monitored and analyzed as a function of time during one year operational period by using molecular techniques.

IV.2.1. Aerated Landfill Monitoring

Aerated landfill bioreactor received MSWI bottom ashes and shredded incombustible wastes from varying sources such as MSW recycling centers and automobile recycling plants. In this study, chemical parameters including TS and VS, pH, alkalinity, BOD_5 and TOC concentration of the leachate were used to provide a preliminary indication of the impacts of aeration on landfill stabilization and microbial decomposition pathway in landfill bioreactor test cell.

Airflow rates, in addition to being dependent on the system configuration, are affected by the settling of the waste, which can cause substantial changes in flow paths and even decrease the air permeability. The goal was to provide sufficient air to maintain aerobic conditions without causing excessive drying or cooling.

Air injection had been started after day 49 in bioreactor test cell, and duration for the operation of the blower was set between 08:30 and 16:30 (8 hrs) due to possibility of complaints by the residents on the noise of the blower during night time (noise of the blower had been reduced to very low levels after construction of the blower house). Air flow rate had initially been set as 2.0 m^3/min (120 m^3/hr, 960 m^3/day) according to the pressure in the injection well and gas flow in the gas collection/removal pipes. This corresponded to 2.62 m^3/m^3/day (V=366 m^3). Actual discharge (rotation speed) of the blower was set at higher values and the excess air was discharged to the atmosphere. This was done to increase the temperature of the injected air during cold winter days, and it was modified according to ambient and blower outlet air temperatures.

In aerated bioreactor landfill, leachate lifted-up to collection tank on the surface was recycled to the cells by a submersible pump and the flow rate and

cumulative flow were measured by an electronic flow meter. The leachate recycle rate was set as 15 L/min and the pump was operated 15 min at 16 times in one day.

Although it was thought at the beginning of the experiments that no significant precipitation will occur, significant amounts of water had infiltrated into the cells, due to high precipitation and snow. A high temperature in the cell was causing early melting of the snow on the cell surfaces. Water levels in aerated bioreactor cell closed to 1 m caused the moisture sensors in these cells to be almost totally submerged.

IV.2.1.1. Changes in pH, TOC, BOD_5 and ORP

In municipal solid waste landfills where organic rich wastes are deposited, aeration decreases the pH by accumulation of volatile fatty acids. However, the case is opposite for most of the landfills in Japan at which either incineration residues deposited alone or together with shredded incombustible wastes because of the alkaline characteristics of bottom ash.

Aerated test cell yielding initial pH values below 9 which permitted the early initiation of microbial activity. During the degradation process pH values changed between 7 to 9 (Figure IV.10). TOC, COD and BOD_5 parameters were often used to determine the degree of stabilization in the waste. Although this criteria vary, there was some consensus that stabilized landfill leachate has a low BOD_5 or TOC values. In this study, to specify the degree of stabilization in aerated bioreactor, BOD_5 and TOC levels were used as an indicator parameter. Beginning of the operational period, TOC values in test cell was relatively high (up to 3500 mg/l), and rapid reduction of TOC showed the acceleration of solid waste stabilization. BOD_5 values in leachate had decreased faster than TOC and became negligible (<10 mg/l) after around day 120. TOC value was also decreased to below 10 mg/l after day 240 (Figure IV.10). TOC and BOD_5 values decreased mainly due to the dilution effects of rainy days and bio-reduction by the microbial activity.

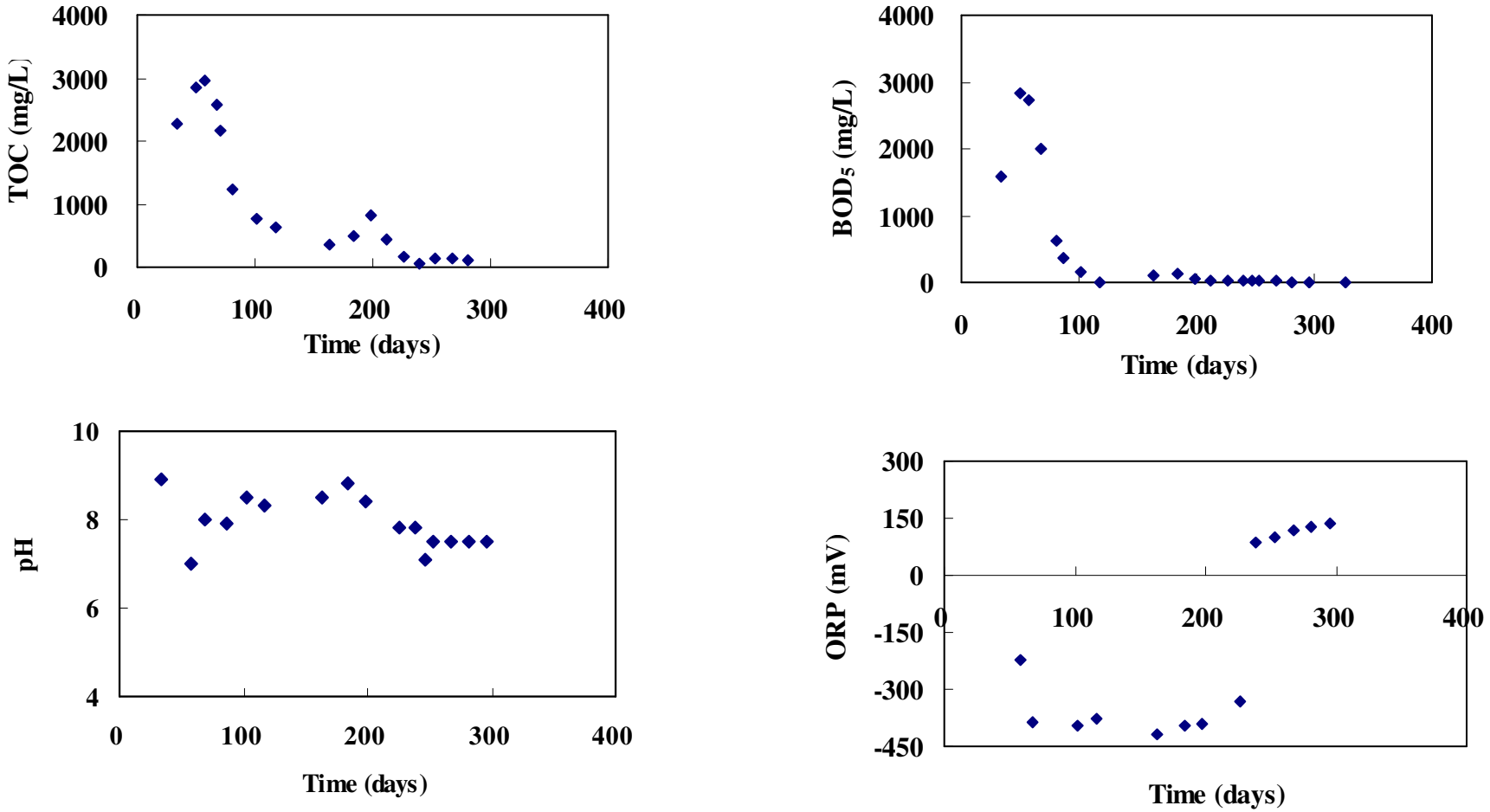


Figure IV.10 Changes in TOC, BOD_5, pH and ORP values during the operational period of aerated bioreactor test cell.

The redox potential within a landfill determines the mechanism of waste degradation. Generally, high redox potential (aerobic conditions) causes accelerated degradation of waste. Furthermore, aerobic degradation has a potential to produce fires because of the excess heat and oxygen. A two-stage process, which consists of aerobic and anaerobic phases, has recently been suggested by several researchers. Aerobic conditions in the first stage would be maintained by supplying air to the landfill. The aerobic microorganisms in the landfill would quickly metabolize the readily degradable organics first. Once the readily degradable material has been metabolized, the air supply would be shut off and anaerobic conditions would become established. The more resistant material would slowly be degraded by anaerobic mechanisms. This two-stage process would combine the advantages of both aerobic and anaerobic mechanisms, while eliminating some of the disadvantages. The overall stabilization rate is increased, while shortening the acidogenic phase which increases methane production. Another benefit is that anaerobic pathways that can degrade resistant chemicals such as PAHs are still possible. Although more research is necessary, this process appears to be the most efficient use of redox potential to accelerate degradation.

In this study, blower was operated only 8 hr/day which maintain aerobic and anaerobic conditions simultaneously in bioreactor. ORP values in aerated bioreactor test cell exhibited similar behavior ranging between -350 and -500 mV during first 226 days. Low ORP values were caused by leachate accumulation at the bottom of the test cell. Since injected air can travel in the unsaturated upper zone only, leachate accumulated at the bottom remained anaerobic. It was also assumed that waste mass in the unsaturated zone was also totally anaerobic until day 49 when air injection was started. After day 226, discharges of accumulated leachate increased the ORP values and resulted in aerobic conditions in bioreactor test cell. As a result, ORP has jumped to +85 mV on the next sampling (day 239). In this study, aerated bioreactor test cell was operated similar to the two-stage process, aerobic and anaerobic, although this process was undesirable.

The shifts in microbial populations from anaerobic to aerobic in an aerated bioreactor test cell was monitored and analyzed as a function of time during one year operational period by using molecular techniques; fluorescence in situ hybridization, slot-blot hybridization and denaturing gradient gel electrophoresis combined with cloning and DNA sequencing.

IV.2.2. Comparative Evaluations of Archaeal and Bacterial Population Diversity

Comparative evaluations of archaeal and bacterial population diversity during one year operational period, fluorescence in situ hybridization (FISH) techniques were carried out on leachate samples taken from aerated landfill bioreactor. The results of FISH experiments carried out with oligonucleotide probes specific for the archaeal and bacterial domains revealed that bacterial populations were the major microorganisms present in the reactors during the one year operational period. In spite of the air injection at bioreactor test cell, ORP values in the leachate at the bottom were around -400 mV or lower which is the indication of highly reduced anaerobic conditions. Meanwhile, the bacterial diversity has considerably changed after discharging the leachate from the cell which allowed the arrival of leachate from the unsaturated zone. Instead of previously dominant bacterial population, new morphological *Bacteria* cells were detected with in-situ hybridization experiments between days 226 and 346 (Figure IV.11-13).

The molecular phylogenetic techniques provide a basis for describing the structure of natural microbial communities at the level of species. It was found that bacterial populations were more intensive than archaeal populations according to previous studies (Huang *et al.,* 2002; Chen *et al.,* 2003; Mori *et al.,* 2003). Mori *et al.* (2003) indicated that 2-3% of total DNA extracted from the leachate in the landfill site was archaeal, and this archaeal community consisted of several species of methanogens by the cloning analysis. Huang *et al*. (2003) investigated the relative abundance of phylogenetically defined groups of methanogens and indicated that landfill leachate harbored a diverse archaeal community, by using cloning and phylogenetic analysis of archaeal 16S rRNA gene sequences.

In our study, during the first 100 days, it was observed that amounts of *Bacteria* and *Archaea* increased while TOC concentrations dropped sharply. During this period, the number of autofluorescent methanogens had gradually increased significantly from the days 20 to 100. However, group specific detection of acetoclastic methanogenic populations of *Methanosaeta* and *Methanosarcina* was not possible.

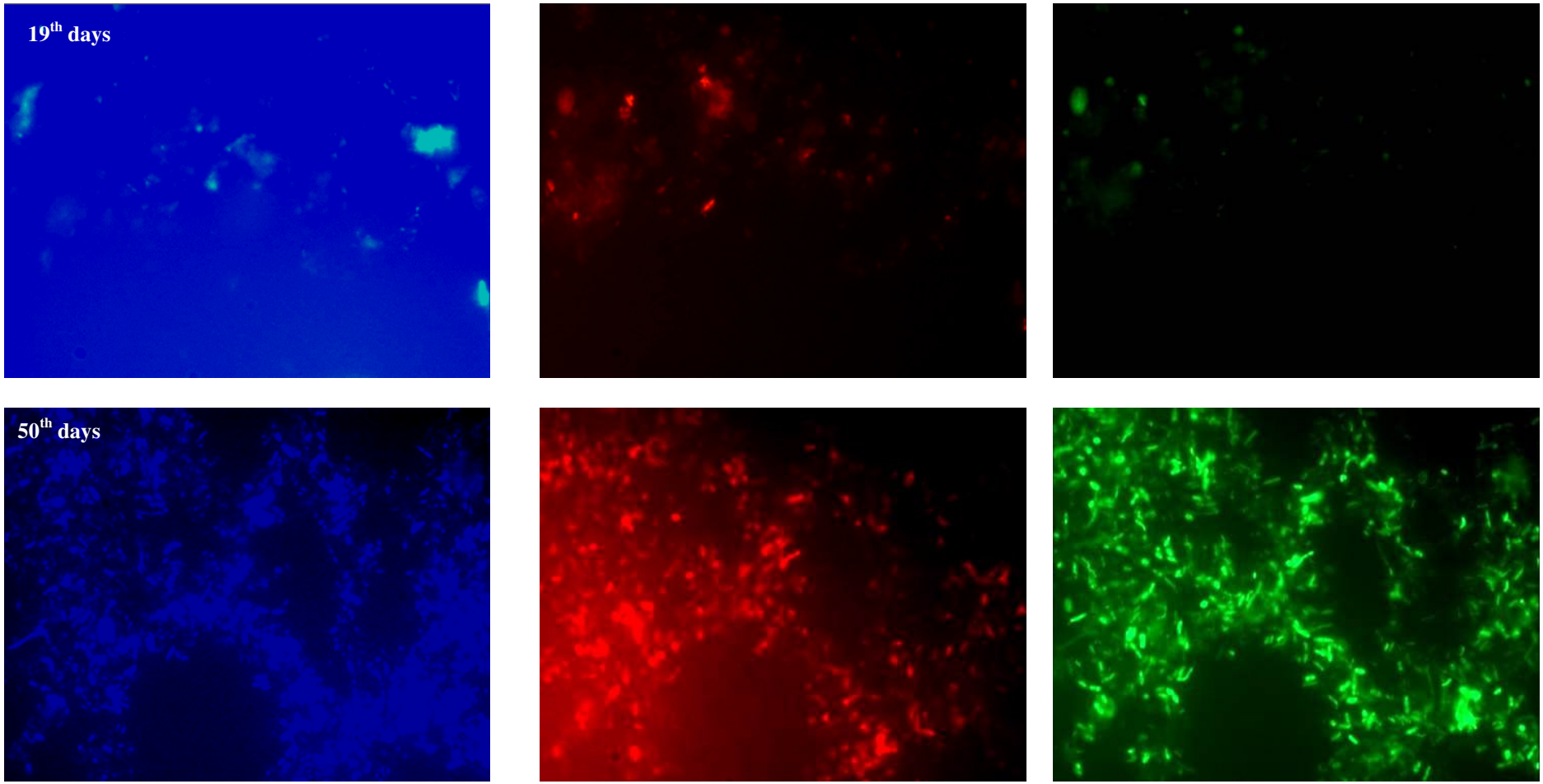


Figure IV.11 In situ identification of aerated bioreactor test cell. Samples hybridized with *Bacteria*-specific probe (labeled with FLUOS-green) and *Archaea*-specific probe (labeled with Cy3-red). Blue, green and red couples represent the same fields of the microscopic view. All microorganisms are visualized by DAPI staining (blue).

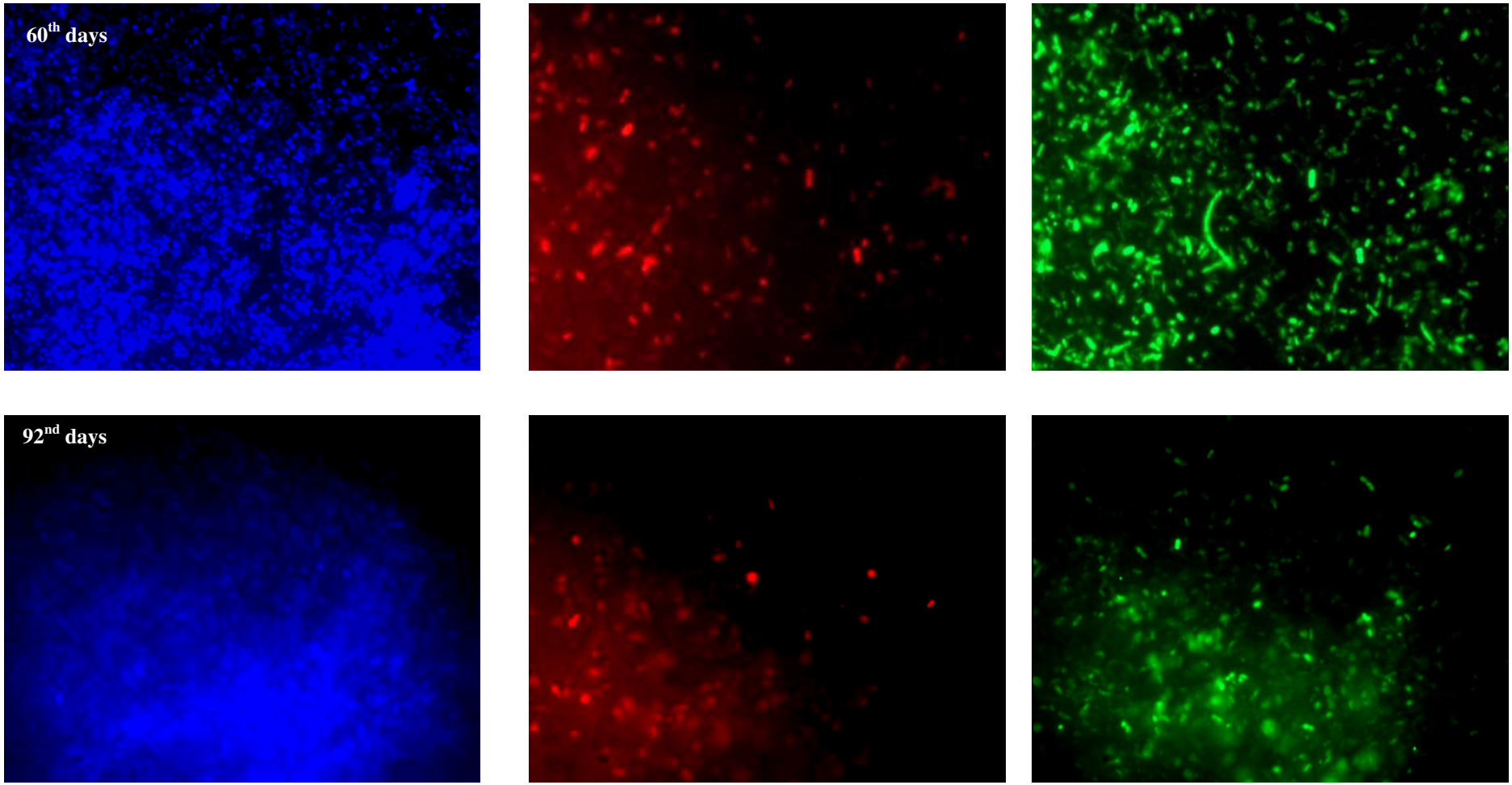


Figure IV.12 In situ identification of aerated bioreactor test cell. Samples hybridized with *Bacteria*-specific probe (labeled with FLUOS-green) and *Archaea*-specific probe (labeled with Cy3-red). Blue, green and red couples represent the same fields of the microscopic view. All microorganisms are visualized by DAPI staining (blue).

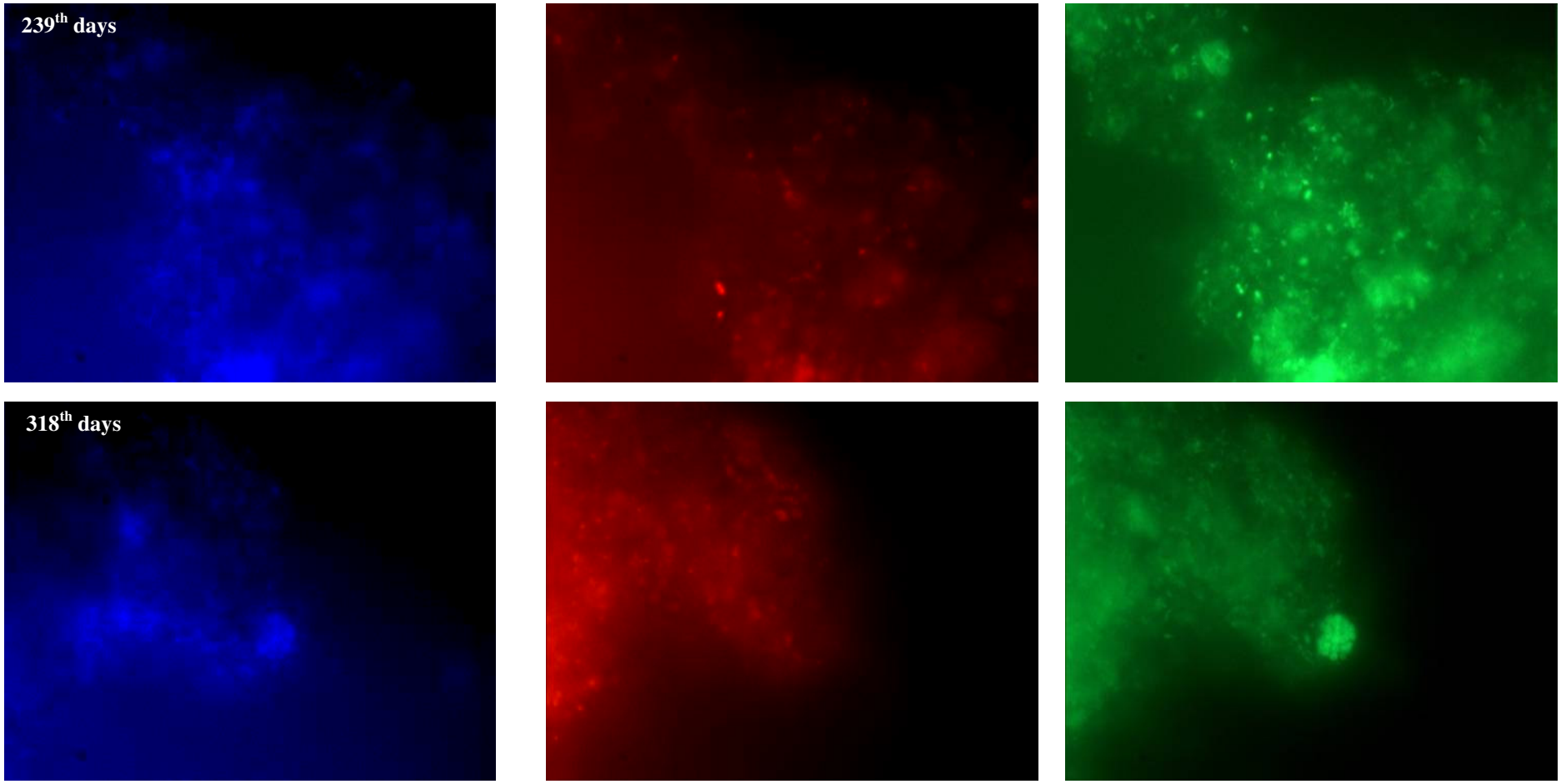


Figure IV.13 In situ identification of aerated bioreactor test cell. Samples hybridized with *Bacteria*-specific probe (labeled with FLUOS-green) and *Archaea*-specific probe (labeled with Cy3-red). Blue, green and red couples represent the same fields of the microscopic view. All microorganisms are visualized by DAPI staining (blue).

According to group-specific oligonucleotide hybridization results beta (Bet42a) subdivision of *proteobacteria* were identified as the prevalent bacterial community in all leachate samples. Relatively low intensities of gamma-*proteobacteria* (Gam42a) were also detected. However, the other bacterial sub-groups were virtually absent. Only, inconsiderable amounts of gram-positive bacteria with low G+C content and alpha-*proteobacteria* were detected randomly.

During the first 226 days, the bioreactor test cell contained rod type β-*proteobacterial* diversity. However, after complete discharge of leachate on day 226, which allowed the arrival of new leachate from upper parts of the test cells, it was clearly observed that structure of β-*proteobacterial* diversity changed from rods to cluster type. Over the time, the number of cluster-like flocs gradually increased, and finally they became predominant in the bioreactor (Figure IV.14-15).

The microbiology of leachate and bulk samples from a test cell in a municipal landfill was monitored by Boothe *et al.* (2001) during an engineered aerobic bioreduction process. It was concluded that an increase in bacterial counts was expected after initiation of aeration because introduction of oxygen would stimulate the metabolism of more energy efficient and faster growing aerobic microorganisms. In our study, bacterial numbers gradually increased in anaerobic conditions while TOC concentrations decreased dramatically during the first 100 days.

Direct information on SRB communities can be obtained by using molecular methods such as fluorescence in situ hybridization (Amann *et al.,* 1995) and denaturing gradient gel electrophoresis (Muyzer and Smalla, 1998). These methods have been used previously to investigate SRB communities in marine sediments (Devereux *et al.,* 1992), seawater (Teske *et al.,* 1996), anaerobic bioreactors (Wawer *et al.,* 1995) and petroleum hydrocarbon-contaminated aquifer (Kleikemper *et al.,* 2002). Unfortunately, hybridizations with the genus-specific SRB probes showed that only small portions of SRB genera were detected in aerated bioreactor landfill during the first 100 days.

In our study, the abundance of species level methanogens and nitrifier populations were not detected by in situ hybridization techniques. Diversity and activity of methanogenic population that present in aerated bioreactor test cell were investigated by using slot blot hybridization and other 16S rRNA based molecular methods.

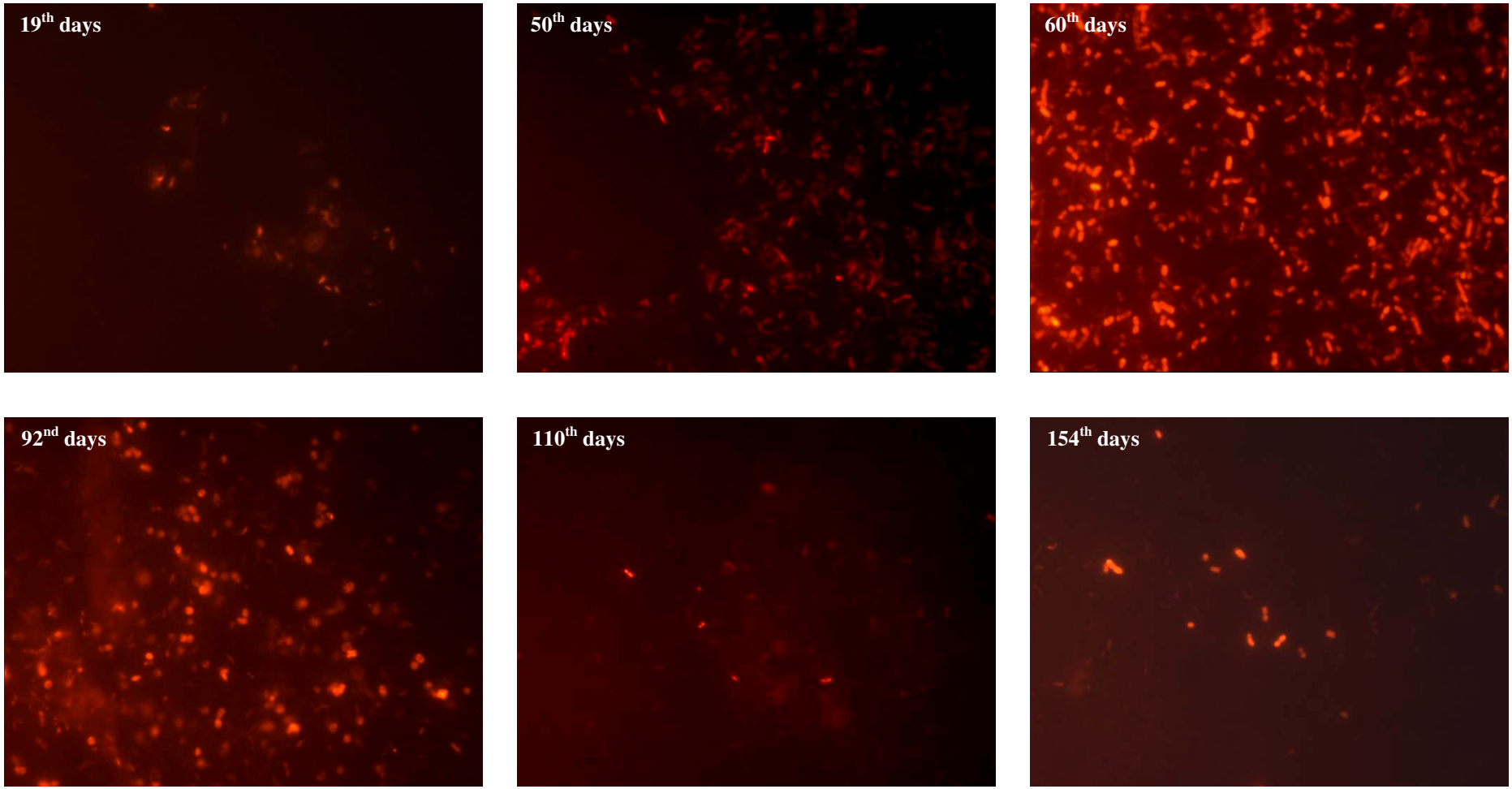


Figure IV.14 In situ identification of aerated bioreactor test cell at different time periods. Samples hybridized with β-*proteobacterial*-specific probe (labeled with Cy3-red).

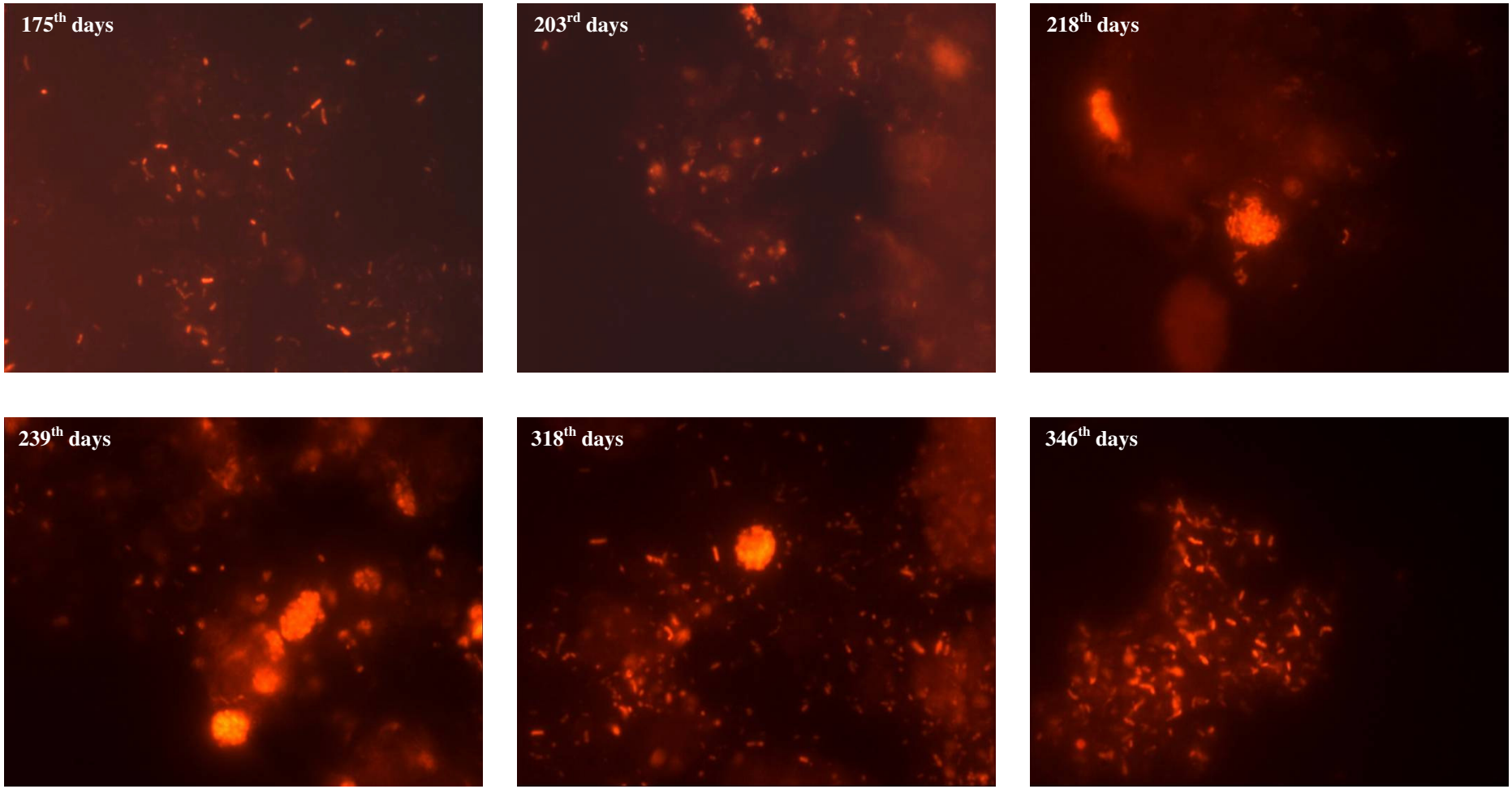


Figure IV.15 In situ identification of aerated bioreactor test cell at different time periods. Samples hybridized with β-*proteobacterial*-specific probe (labeled with Cy3-red).

IV.2.3. Identification of Methanogenic *Archaea*

IV.2.3.1. Slot-Blot Hybridization

For identification and determination of methanogens in the environment, particularly the sequences of 16S and 23S rRNA/DNA are providing valuable information and supporting powerful techniques, such as in situ or membrane probing (Ahring *et al.,* 2001). Slot-blot hybridization was successfully applied to several population analyses of wastewater treatment systems: Wagner *et al.* (1993) observed the dominating role of *proteobacterial* rRNA from the alpha, beta, or gamma subclass in activated sludge samples from aeration tanks, and a severe population shift towards the gamma subclass during cultivation-dependent analysis.

Membrane studies using total DNA extracted from environmental samples, hybridized with DIG labeled oligonucleotide probes with varying specificity for analyses of methanogenic populations were introduced by Raskin *et al.* (1994). Other studies have been published using this technique (Raskin *et al.,* 1995; Mobarry *et al.,* 1996; Sorensen *et al.,* 1997; Ahring *et al.,* 2001). In this particular field, DNA extraction from environmental samples e.g. manures or sludge may require special precautions (Raskin *et al.,* 1995).

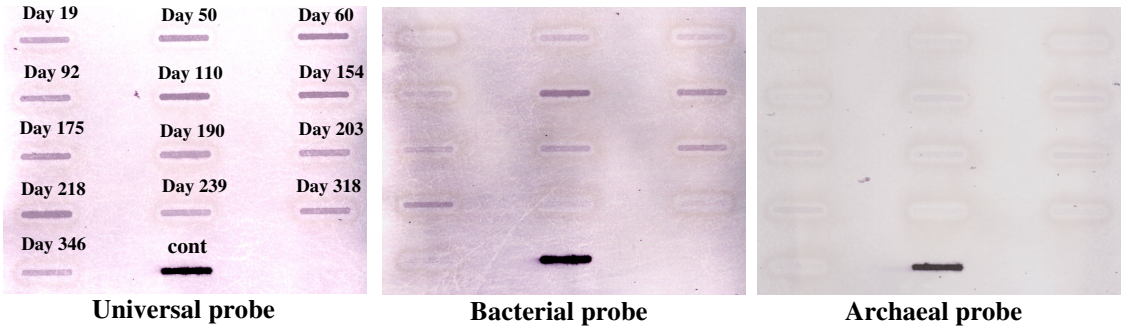


Figure IV.16 Slot-blot hybridization results. Thickness of the bands is directly proportional to the amount of target DNA.

In this part, archaeal and bacterial population diversity in an aerated bioreactor test cell filled with incineration bottom ashes and shredded incombustible wastes was monitored and analyzed as a function of time during one year operational period by using slot-blot hybridization techniques. The present study found that *Bacteria* dominated in the landfill populations at least in terms of 16S rDNA representation.

The relative abundance of *Bacteria* found by membrane hybridization was approximately 95 %. *Archaea* seemed to be a minor component of the microbial community in bioreactor test cell (Figure IV.16). Total amount of nucleic acids extracted from the aerated test cell was determined by UV-VIS Spectrophotometer with OD_{260} wavelength for applying same amounts of DNA to each well.

Low abundance (approx. 2%) of methanogenic *Archaea* in a mature landfill leachate was revealed in a previous study using quantitative oligonucleotide hybridization (Huang *et al.,* 2003). Similarly, only less than 1% of the total cells were detected with *Archaea* specific Arch915 probe in one year old landfill samples excavated from 1 and 3 m depths (Chen *et al.,* 2003). In another study, archaeal DNA represented 2-3% of the total DNA extracted from the leachate of a landfill receiving solid wastes mainly consisting of incineration ash (Mori *et al.,* 2003). In this study, because of air injection bacterial population was found more intensive during one year operational period.

To explain the relative differences of archaeal population changes during one year operational period of aerated bioreactor test cell, PCR - slot blot experiments was carried out with group-specific oligonucleotide probes. The physico-chemical parameters of the aerated bioreactor landfill leachate (Figure IV.10) indicated that the waste stabilization process completed during the first 226 days while organic portion of waste was mainly degraded while ORP values were between -300 to -500 mV.

Members of the hydrogentrophic order *Methanomicrobiales* (MG 1200) and *Methanobacteriales* (MB1174) were constituted the majority of the methanogens present in the landfill leachate during the first 60 days. Although *Methanobacteriales* group were present in the day 50 sample, the number appeared to increase after day 92, and these methanogenic group were very numerous in the day 110 sample. After this time H_2-utilizing *Methanobacteriales* were determined as the predominant methanogenic *Archaea* in leachate samples (Figure IV.17).

The *Methanomicrobiales* population had disappeared after day 60, and the *Methanosarcina* population gradually increased over the remaining samples. Acetate-utilizing methanogens, *Methanosarcina,* were detected with the MS821 oligonucleotide probe after days 154. On the other hand, only very few hybridization signals were observed from *Methanococcales* probes targeting H_2-utilizing methanogens and *Methanosaeta* probes targeting acetate-utilizing methanogens.

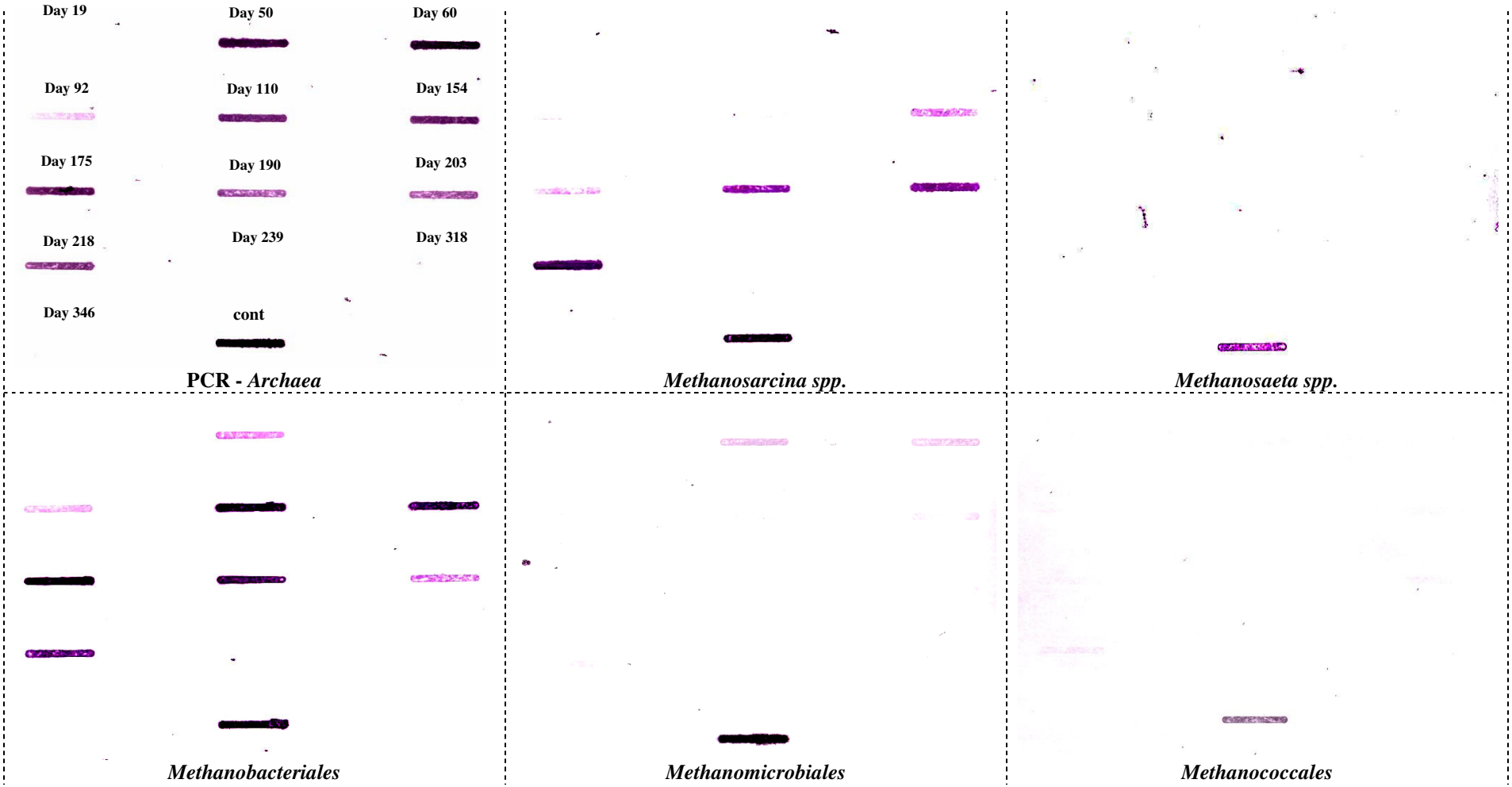


Figure IV.17 Relative differences of archaeal population changes during one year operational period of aerated bioreactor test cell. Thickness of the bands is directly proportional to the amount of target DNA.

IV.2.3.2. Denaturing Gradient Gel Electrophoresis

Denaturing gradient gel electrophoresis (DGGE) experiments is sensitive method to separate DNA's on the basis of single point mutations (Sheffield *et al.,* 1989). Our objective was to evaluate the diversity of *Archaea* in an aerated landfill bioreactor over time by using DGGE and cloning analysis. To understand the population varieties during one year operational periods, 10 samples were applied to the wells. It was observed that archaeal populations in bioreactor leachate samples had a great diversity (Figure IV.18).

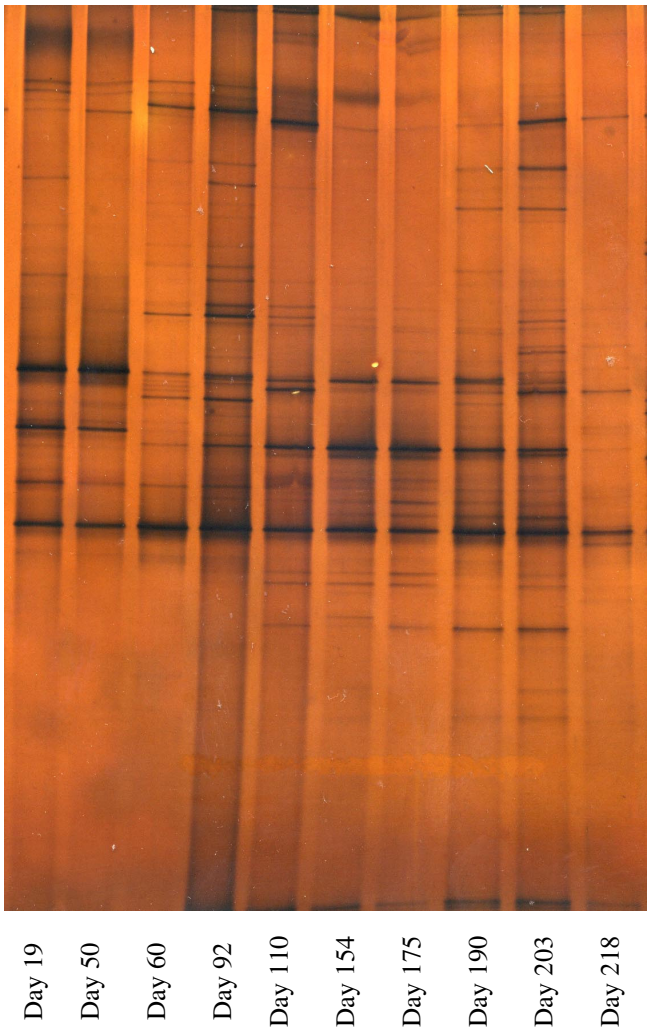


Figure IV.18 DGGE profiles of PCR amplified partial archaeal 16S rDNA.

Because of the high diverse of archaeal community in leachate samples, three different sampling day were selected for phylogenetic analysis of leachate samples, in the days 50, 110 and 218.

IV.2.3.3. Cloning and Sequencing

16S rDNA phylogenetic tree was generated from total-community genomic DNA extracted from aerated bioreactor landfill leachate, using 16S archaeal rDNA-targeted primer. Archaeal rDNA clones in the library were grouped by comparing restriction enzyme cleavage patterns, resulting in a total of 15 different RFLP types among the 60 clones examined in 3 different leachate samples. All the representative sequences were found to be closely related to 16S rDNAs of methanogens, such as *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales* order (Figure IV.19).

Studies on archaeal community compositions in leachate from a landfill (Huang *et al.,* 2002) and in a landfill site (Mori *et al.,* 2003) indicated that members of *Methanomicrobiales* and *Methanosarcinales* could be significant archaeal populations in such environments. 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 at different locations (with presumably different operational conditions). In this study, archaeal diversities in an aerated bioreactor test cell were investigated by using phylogenetic analysis in the days 50, 110 and 218 samples.

In this study, H_2-ulitizing methanogens, *Methanobacteriales* and *Methanomicrobiales* orders, were the dominant archaeal population in the day 50 leachate sample while only small fraction of *Methanosarcinales* groups were observed. These findings suggest that hydrogentrophic methanogens were the dominant *Archaea* while organic portion of waste was degraded in leachate. It was also revealed that although *Methanosarcinales* and *Methanomicrobiales* were present, H_2-utilizing *Methanobacteriales* order were still the major methanogenic *Archaea* in the day 110 leachate sample. Eighteen clones belonged to the *Methanobacteriales* order which contributed to 90 % (18 out of 20) of total clones (Table IV.2). These statistical results were also confirmed by slot blot hybridization experiments.

However, on the day 218 sample, members of the *Methanosarcinales* order seemed to predominate in the aerated landfill bioreactor. Thirteen clones belonged to the *Methanosarcinales* order which contributed to 65 % (13 out of 20) of total clones indicated that archaeal populations were shifted from *Methanobacteriales* to

Methanosarcinales order during 9 months operational period. Six and one clones belonged to the *Methanobacteriales* and *Methanomicrobiales* orders were observed, respectively (Table IV.2). *Methanococcales*-related sequences in leachate samples were not detected similar to the slot-blot hybridization and FISH experiments.

Table IV.2 Sample source and distribution of identical 16S rRNA clones in methanogenic *Archaea*.

Sample	*Methanosarcinales*	*Methanococcales*	*Methanomicrobiales*	*Methanobacteriales*
Day 50	1	0	2	17
Day 110	1	0	1	18
Day 218	13	0	1	6

In total, 60 clones were selected and classified into fifteen different sequence types or phylotypes after clone screening and sequencing. Phylogenetic analysis (Figure IV.19) showed that three (A127, A137, A141) of those fifteen phylotypes were closely related to *Methanosarcina mazei* strain MT and one (A87) phylotypes related to *Methanimicrococcus blatticola* in the acetoclastic *Methanosarcinales* order. Clone group A127 comprising 13 clones isolated from leachate sample day 218 showed 99.4% similarity to *Methanosarcina mazei* strain MT.

The remaining clones were related to hydrogen-utilizing *Methanomicrobiales* and *Methanobacteriales* order. Clone group A85 comprising 9 clones isolated from leachate sample day 50 (8 clone) and day 110 (1 clone) showed 95.6% similarity to uncultured archaeal symbiont PA202, A111 with 6 clones from day 110 sample showed 92.5% similarity to *Methanobrevibacter arboriphilus* strain DC and clone group A134 consisted of 2 clones from sample day 218 represented 99.4% similarity to *Methanobacterium bryantii* strain MOH (Figure IV.19).

In a previous study, Chen *et al.* (2003) investigated the archaeal community in samples taken from different depths of a landfill at different stabilization phases and reported that the members of the genus *Methanosarcina* as the predominant methanogen in young age samples but found negligible in mature ones. In our study, H_2-utilizing *Methanobacteriales* was found as the major methanogenic order at the beginning of the operational period in an aerated landfill bioreactor. The main reason can be generation of hydrogen from incinerated ashes. Population diversity shifted from *Methanobacteriales* to *Methanosarcinales* order after all organic matter depleted. The abundance of *Methanobacteriales* and *Methanosarcinales* orders were also verified with slot-blot hybridization analysis.

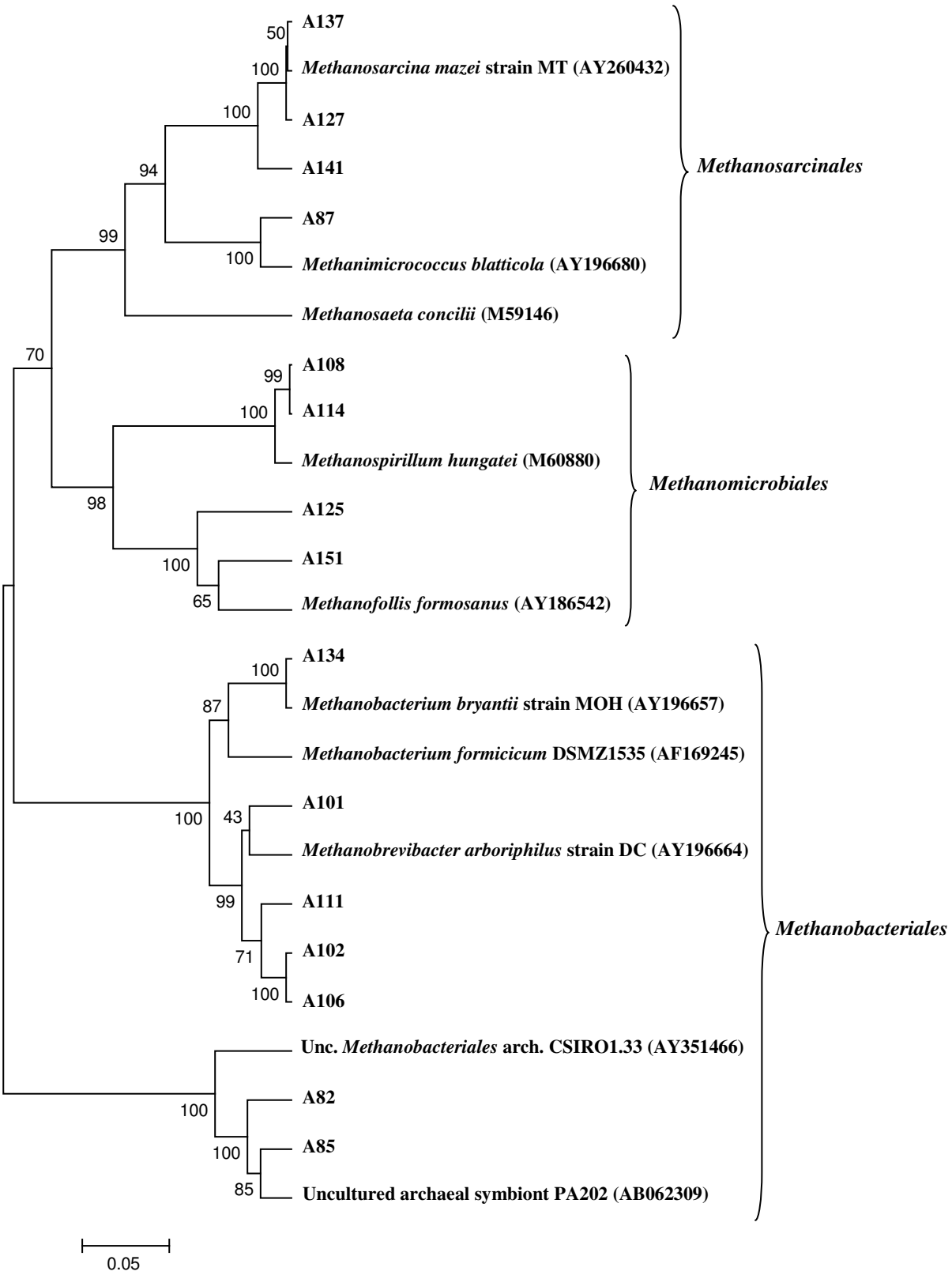


Figure IV.19 A neighbor-joining tree of 16S rRNA clones from leachate samples. The significance of each branch is indicated by bootstrap values. The scale bar represents 0.05 inferred substitutions per nucleotide position. Sample source and numbers of identical clones are given in parentheses.

IV.3. NITRIFYING POPULATION DIVERSITY IN AN AERATED LANDFILL BIOREACTOR

Although the organic part of the leachate is significantly reduced in conventional landfills, the ammonia-nitrogen accumulates because there is no degradation pathway for ammonia in anaerobic systems. The ammonia nitrogen concentrations found in leachate from bioreactor landfills are greater than those found in leachate from conventional landfills (Onay and Pohland, 1998; Barlaz *et al.,* 2002). Air injection to the landfills has been shown to enhance degradation processes in bioreactors, as aerobic processes tend to degrade organic compounds typically found in municipal solid waste (MSW) in shorter time periods.

During aerobic degradation of MSW, biodegradable materials are converted mostly to carbon dioxide and water. Little, if any, methane is produced, which may be viewed as either an advantage or disadvantage, depending on whether methane collection and use as an energy source is desired or required. Air addition has also been used as an enhancement of ammonia-nitrogen degradation during the stabilization process of bioreactors (Berge *et al.,* 2005).

It has been suggested that ammonia-nitrogen is the most significant long-term pollution problem in landfills and it is likely that the presence of NH_3-N will determine when post-closure monitoring may end (Kjeldsen *et al.,* 2002; Price *et al.,* 2003). However, operating the landfill as a bioreactor provides opportunities for in situ nitrogen transformation and removal processes. Little research has been conducted evaluating the fate of nitrogen in bioreactor landfills; however, understanding the possible nitrogen transformations is important when considering potential leachate management options.

In this study, the composition of nitrifying bacteria in an aerated landfill bioreactor test cell filled with incineration bottom ashes and shredded incombustible wastes was monitored and comparatively analyzed as a function of time during one year operational period by using molecular techniques. In addition to the physical and chemical parameters, effects of differing operational conditions on the performances of bioreactor and diversity of nitrifiers were also explored and evaluated thoroughly.

IV.3.1. In Situ Nitrogen Removal in Landfill Bioreactor

Adding air to a landfill bioreactor would be dual-purpose: removing of ammonia-nitrogen and to enhance the degradation of solid waste. During the stabilization process of bioreactor, maintaining and controlling sufficient oxygen levels within the landfill, may be difficult and may result in oxygen limitations. In our study, low ORP values for leachate samples were caused by leachate accumulation at the bottom of the test cell. Since injected air could travel in the unsaturated upper zone only, leachate accumulated at the bottom remained anaerobic. In spite of the air injection at bioreactor test cell, ORP values were around -400 mV or lower which was the indication of highly reduced anaerobic conditions (Figure IV.20). There were likely being areas in which air does not reach, resulting in anoxic or anaerobic pockets within the waste mass.

Ziehmann and Meier (1999) conducted both laboratory and pilot-scale studies evaluating the effects of cyclic air injection on the performance of bioreactor landfills. Three bioreactor systems were operated for 180 days. Results from the laboratory study showed that the leachate from the reactor in which aerobic and anaerobic conditions were alternated had lower concentrations of total organic carbon and COD than those from either the anaerobic or aerobic reactors. However, when operating the pilot-scale study, there was little difference between the cyclic and continuously aerobic reactors, suggesting that the advantages of the cyclic system seen in the small-scale studies may not be realized at field scale.

In this study, beginning of the operational period, NH_3-N values in the cell was approximately 250 mg/l, and rapid reduction in TOC and NH_3-N in aerated bioreactor cell clearly showed the acceleration of bio-stabilization (Figure IV.20). It was shown that aerobic conditions promoted not only organic matter but also nitrogen removal in bioreactor test cell. In aerated test cell, nitrate was not observed at significant concentrations as long as 226 days. This was mainly due to the fact that leachate accumulated at the bottom of the cell was in anaerobic conditions, and all nitrate produced was possibly converted to nitrogen gas.

Moreover, after discharging all the leachate from the bioreactor, new leachate accumulated at the bottom became totally aerobic as can be seen from ORP values in Figure IV.20. Parallel to change in oxidation level, nitrate nitrogen (NO_3-N) concentrations increased immediately around 30 mg/l, and indicated that it was

already being produced in aerobic zones of the bioreactor. High ORP values, acceleration of ammonia degradation and low TOC concentrations decreased the denitrification rate and these were the reason of nitrate accumulation. Nitrite values exhibited similar pattern and was detectable only after complete discharge of existing leachate at the bioreactor (Figure IV.20).

Cheng *et al.* (2004) measured the production of both nitric and nitrous oxides in Chinese agricultural soils in which high levels of fertilizer were added. Both nitric and nitrous oxide production from nitrification was observed. Production could be correlated with the pH of the system; soils that were more basic (pH > 8) resulted in the highest concentrations of nitrous oxide, while the more acidic soils produced the least. Khalil *et al.* (2004) also conducted a study evaluating the production of nitrous oxide in soils, paying particular attention to the influence of oxygen on nitrous oxide production. They found that as oxygen decreased, the mass of nitrous oxide from nitrification increased. In landfills, there may be areas in which oxygen concentrations are limiting; thus, nitrous oxide production via nitrification may result. However, in our study, nitrous oxide concentrations were not analyzed. But molecular analysis showed that nitrification was completed to the nitrate by nitrite oxidizers, *Nitrospira* and *Nitrobacter*.

Juteau *et al.* (2004) found that nitrification did not occur under thermophilic conditions. However, Lubkowitz-Baily and Steidel (1999) and Willers *et al.* (1998) found that nitrification was achievable at temperatures as high as 44^{o}C in wastewater and 50^{o}C in veal-calf slurry, respectively, although the rate of nitrification was decreased significantly at both temperature levels. In higher temperature, landfill bioreactor environments, nitrification was achieved and nitrifying bacteria that responsible for conversion of ammonium to nitrite and nitrate were detected in this study.

Several researchers have evaluated the potential use of in situ, or partially in situ, nitrification processes in landfills. Youcai *et al.* (2002) conducted a study in which a biofilter consisting of old waste (8 to 10 years old) was used to treat leachate. Aerobic portions existed at the top and bottom of the system (air was not supplied, rather was drawn in from the atmosphere via convection), while the middle of the system was anaerobic. It is important to note that these conditions (aerobic and anaerobic) were never shown experimentally, nor was the ORP measured. A removal of 99.5% of the ammonia-nitrogen in leachate was observed. Elevated concentrations

of nitrate and nitrite were measured, indicating the ammonia-nitrogen was converted biologically. Additionally, 20-30% of total nitrogen in the leachate was removed, suggesting in situ nitrification and denitrification occurred sequentially in the landfill.

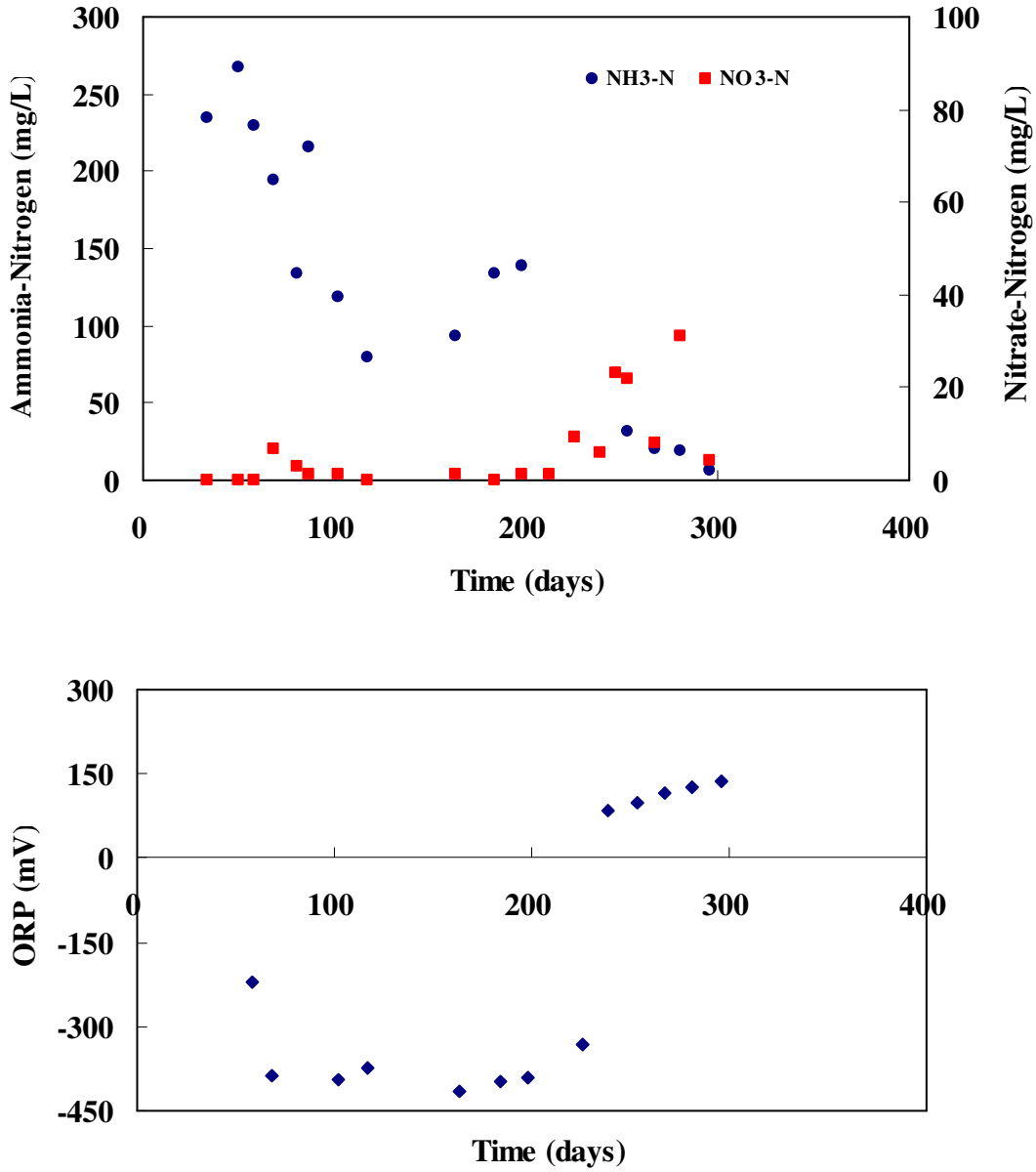


Figure IV.20 Changes in NH_3-N, NO_3-N and ORP during one year operational periods of aerated bioreactor test cell.

All these observations showed that nitrogen removal through nitrification and denitrification is possible with aerobic landfill bioreactor operation.

IV.3.2. Molecular Investigation of Nitrifying Population

IV.3.2.1. Slot-Blot Hybridization

Several methods can be used to investigate nitrifying bacterial populations in situ; cultivation-dependent analysis often leads to significantly underestimated cell counts due to the presence of unculturable species, and the very low growth rate of the nitrifiers (Watson *et al.,* 1989) makes it time-consuming. In this part, the relative abundance of nitrifying populations were determined by using PCR-slot blot hybridization with oligonucleotide probes Nso190 and Nso1225, encompass all sequenced ammonia oxidizers of the beta subclass of *Proteobacteria*, probe NIT3, which is complementary to a sequence region of all *Nitrobacter* species and Ntspa 662 and Ntspa 712, which is specific for nitrite oxidizers *Nitrospira* aggregates.

In cases where ammonia and nitrite oxidizing bacteria do not represent a large proportion of the total microbial community, it has been necessary to introduce PCR amplification steps into hybridization strategies (Ceccherini *et al.,* 1998). Although addition of PCR step(s) prior to hybridization can increase the sensitivity of slot-blot hybridization assays, this also compromises the ability to obtain quantitative results.

After starting the bioreactor operation, strong shifts in microbial community composition of ammonia and nitrite oxidizer populations were not observed. The community of ammonia and nitrite-oxidizing bacteria increased after day 50, parallel to the observed decrease of ammonia in an aerated landfill bioreactor (Figure IV.21).

A dominance of *Nitrosomonas*-like sequences was detected as a function of time during one year operational period by using slot-blot hybridization techniques targeting the 16S rRNA gene on 13 different samples. It has been reported that *Nitrosomonas* strains can survive in low oxygen concentrations, and they have been observed to constitute about one-fourth of the microbial biomass in an anoxic trickling filter biofilm (Schmid *et al.,* 2000).

It is a commonly stated hypothesis that nitrifying bacteria are poor competitors for oxygen compared with heterotrophic bacteria because of their low oxygen affinities (Sharma *et al.,* 1977; Laanbroek *et al.,* 1995). Many researchers suggest that nitrifying bacteria can be easily outcompeted by heterotrophs in the presence of organic matter (Hanaki *et al.,* 1990; van Niel *et al.,* 1993). In our study, ammonia removal was achieved with complete carbon reduction.

The two reported nitrite-oxidizing groups generally associated with environmental samples are *Nitrobacter* and *Nitrospira*, although recent research suggests *Nitrospira* is the primary nitrite oxidizer in the majority of cases (Schramm *et al.,* 1998; Juretschko *et al.,* 1998; Daims *et al.,* 2000; Daims *et al.,* 2001). In our study, using *Nitrospira* specific Ntspa-662 and Ntspa-712 probe and *Nitrobacter* specific NIT3 and Nb1000 probe, indicated that *Nitrospira* species was the dominant nitrite oxidizers (Figure IV.21). *Nitrobacter* species appeared in the bioreactor test cells where the ORP level has jumped to +85 mV on the next sampling (day 239) and significantly increased on sampling day 318.

It was hypothesized that members of the *Nitrosomonas europaea* lineage (Pommerening-Röser *et al.,* 1996) and *Nitrobacter* sp. could out-compete *Nitrosospira* sp. and *Nitrospira* sp. in habitats with high substrate concentrations because of their higher maximum growth rates, whereas *Nitrosospira* sp. and *Nitrospira sp.* were better competitors in low-substrate environments as a result of their lower K_m values (Schramm *et al.,* 1999).

Schramm *et al.* (2000) indicated that, the idea that *Nitrospira* sp. might be a typical K-strategist compared with the r-strategist *Nitrobacter* sp. (Schramm *et al.,* 1999), based on its putative higher affinities for nitrite and oxygen and its lower growth rate. In many environments, where nitrite concentrations are negligible and nitrifiers have to compete for oxygen with heterotrophic bacteria, K-strategy might provide a selective advantage.

Okabe *et al.* (2000) speculated that *Nitrobacter* spp. competes well only if both the O_2 and NO_2 concentrations are high and these results are confirmed in this study using slot blot analysis. Schramm *et al.* (2000) also indicated that when oxygen became very low or zero, cell numbers of *Nitrobacter* sp. decreased whereas *Nitrospira* sp., which was absent, reached maximum abundance.

In-situ nitrification experiments demonstrated that ammonia removal via nitrification and denitrification is feasible in an aerated landfill bioreactor test cell filled with incineration bottom ashes and shredded incombustible wastes at various oxygen and ORP levels. Results also suggest that nitrification and denitrification may occur simultaneously in an intermittent aerated bioreactor test cell (even under low biodegradable C:N conditions), rather than requiring two separate cells containing two different in-situ environments (i.e. anoxic and aerobic).

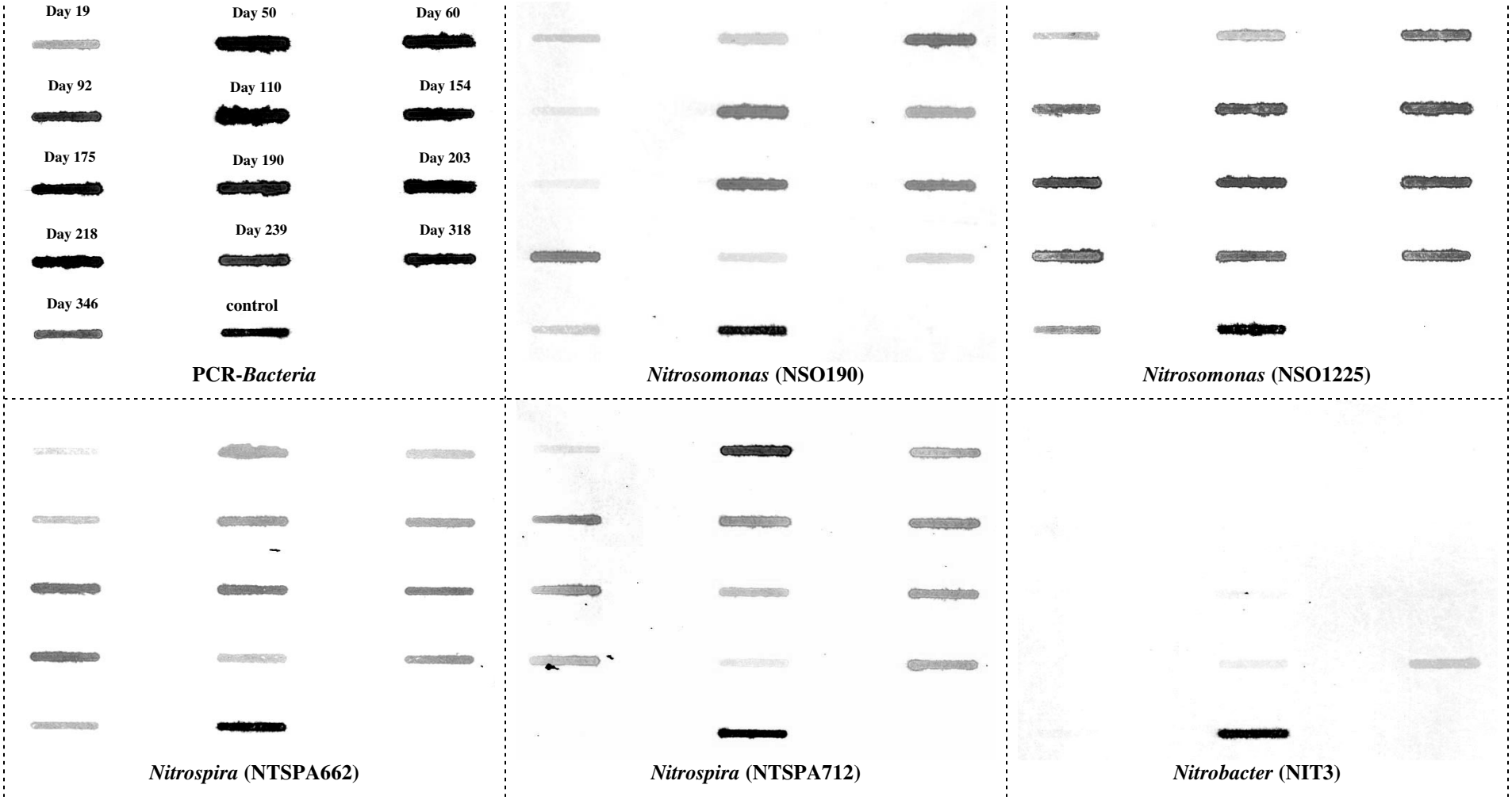


Figure IV.21 Relative differences of nitrifying bacterial population changes during one year operational period of aerated bioreactor test cell. Thickness of the bands is directly proportional to the amount of target DNA.

IV.3.2.2. Cloning and Sequencing

It is well known that ammonia concentration, pH, temperature and oxygen supply affect nitrification rate (Kowalchuk and Stephen, 2001). Thus, different operational conditions can affect the nitrification activities or nitrifying populations during one year operational period in an aerated landfill bioreactor.

In this part, the ammonia-oxidizing communities in the leachate samples during the stabilization process were investigated over time by using alternative molecular approaches. An alternative approach is to use a translated gene as a molecular marker (Sinigalliano *et al.,* 1995; Wawer *et al.,* 1997; Wagner *et al.,* 1998). Ammonia oxidizing bacteria (AOB) convert ammonia to nitrite in a two step process, where the first step is the conversion of ammonia to hydroxylamine catalyzed by the enzyme ammonia monooxygenase (Hooper *et al.,* 1997). Rotthauwe *et al.* (1997) have developed a primer pair (a*moA*-1F and a*moA*-2R), which amplify a 491 bp fragment of the ammonia monooxygenase subunit A gene (*amoA*). *amoA* is present in all autotrophic AOB and is believed to contain enough information to make phylogenetic inferences based on its sequence (Rotthauwe *et al.,* 1997; Purkhold *et al.,* 2000).

RFLP analysis of the *amoA* gene was used to investigate diversity of the involved ammonia-oxidizing bacteria under different operational conditions. Fragments of *amoA* gene coding for the active subunit of ammonium monooxygenase were amplified from sludge community DNA by PCR. Amplified *amoA* fragments from community DNA of the bioreactors at different time points were digested simultaneously with *MsPI*. The restriction reaction is checked by electrophoresis with 1x TBE buffer on 2% agarose gel with a separation range from approximately 20 to 500 bp.

It was clearly shown that RFLP patterns of *amoA* amplified DNA from sampling day 92, 218 and 239 were highly similar fragments except for some minor banding differences on sampling day 92 (Figure IV.22-23). The *amoA* RFLP of this population was represented by the banding pattern derived from one of the cloned sequences (see below, Figure IV.24). However, different RFLP pattern of *amoA* were observed after long periods of aerobic conditions (from day 239 to 318). These RFLP patterns originate from different *amoA* sequences.

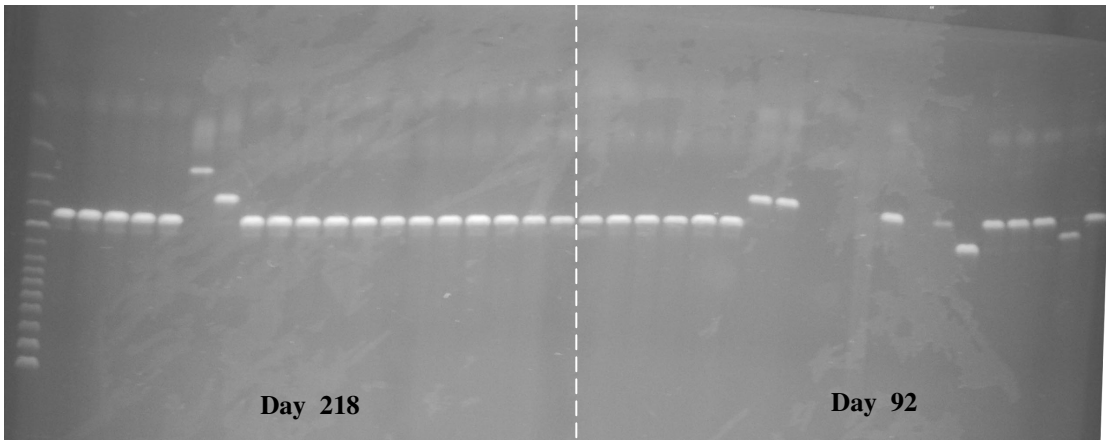


Figure IV.22 Gel with *MspI* restriction patterns of analyzed *amoA* clones in an aerated landfill bioreactor from different time periods.

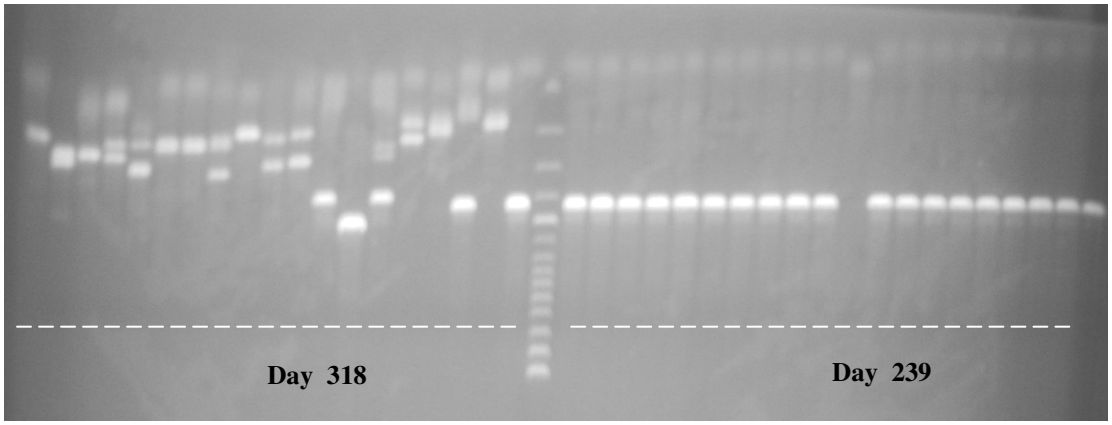


Figure IV.23 Gel with *MspI* restriction patterns of analyzed *amoA* clones in an aerated landfill bioreactor from different time periods.

From each sampling day, approximately 20 clones picked up and screened by restriction fragment length polymorphism (RFLP) analysis before the sequencing analysis. *amoA* phylogenetic tree was generated from total-community genomic DNA extracted from aerated bioreactor landfill leachate, using *amoA* gene-targeted primers. *amoA* clones in the library were grouped by comparing restriction enzyme cleavage patterns, resulting in a total of 6 different RFLP types among the 80 clones examined in 4 different leachate samples. All the representative sequences were found to be closely related to *Nitrosomonas europaea* like bacteria (Figure IV.24).

Phylogenetic analysis (Figure IV.24) showed that two (amo2 and amo34) of those six phylotypes were closely related to a*moA* anoxic biofilm clone S3 and one phylotypes (amo35) related to uncultured beta *proteobacterium* UMTRA_602-L5. Two phylotypes (amo22 and amo41) were closely related to *Nitrosomonas nitrosa* and one phylotypes (amo54) related to *Nitrosomonas sp. Nm148*.

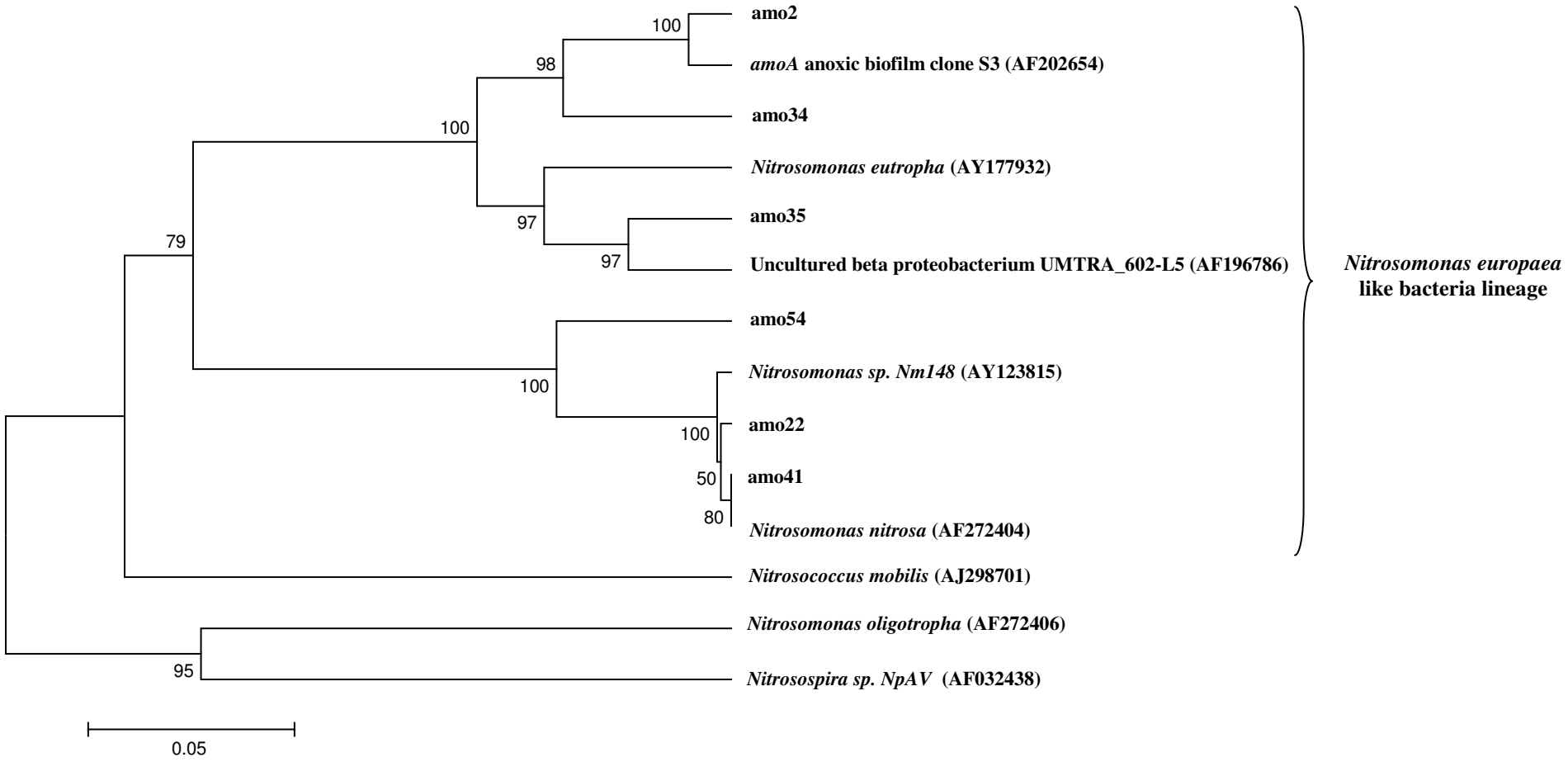


Figure IV.24 A neighbor-joining tree of *amoA* clones from leachate samples. The significance of each branch is indicated by bootstrap values. The scale bar represents 0.05 inferred substitutions per nucleotide position. Sample source and numbers of identical clones are given in parentheses.

Clone group amo22 comprising the most abundance population about 48 of 60 clones isolated from leachate sample day 92, 218 and 318 showed 96.2% similarity to *Nitrosomonas nitrosa* species. It has been established that *Nitrosospira* species are more common than *Nitrosomonas* species in the soil environment (Kowalchuk *et al.,* 1997; Juretschko *et al.,* 1998).

Nitrosospira-like *amoA* sequences were detected from both natural and manipulated organic and mineral soils, and also at the early stages of composting process. *Nitrosomonas*-like *amoA* sequences were found only from nutrient-rich mineral agricultural soil and from compost samples.

Schmid *et al.* (2000) reported that *Nitrosomonas* strains can survive in low oxygen concentrations, and they have been observed to constitute about one-fourth of the microbial biomass in an anoxic trickling filter biofilm. In our study, ammonia removal via nitrification and denitrification was achieved in an aerated landfill bioreactor test cell at various oxygen and ORP levels (even -400 mV ORP levels). Our results also suggest that *Nitrosomonas* strains can survive in bioreactor landfill and can compete with heterotrophs in the presence of organic matter.

To understand a better view of nitrification in an aerated landfill bioreactor test cell we carried out *amoA* sequence analysis to investigate the AOB diversity. The *amoA* gene codes α subunit of ammonia monooxygenase, which is the key enzyme of all aerobic AOB. Six different DNA bands were analyzed to monitor AOB diversity. It was shown that a novel *Nitrosomonas*-like sequence group was observed the most dominantly detected ammonia oxidizers in an aerated landfill bioreactor during one year operational periods.

CHAPTER V

CONCLUSION

In this study, microbial population diversities in sanitary and bioreactor landfills was monitored and analyzed by using fluorescence in situ hybridization, slot-blot hybridization, polymerase chain reaction and denaturing gradient gel electrophoresis analysis. These results were compared and evaluated with cloning and DNA sequencing data to understand the function of microbial diversities during stabilization periods of landfills. The results indicated that the application of in situ hybridization techniques to leachate samples allows a good insight into the structure of microbial communities in the landfills and these techniques are likely to become a favored method for routine analysis in evaluation of landfill stability and better management of stabilization processes in the landfills.

Conventional landfill studies showed that high methane potentials indicating acidogenic phase of landfill stabilization were detected in leachate samples collected from young landfill units (O3, K, B and H1). On the other hand, mature landfill leachates (O1, O2 and H2) produced significantly low amounts of methane gas and represented fairly low methane potentials. These data were consistent with leachate qualities and confirming the BOD_5/COD ratios.

Methanogenic population diversity may be quite dissimilar in different landfill sites as well as in different stabilization phases. Acetate utilizing methanogens especially members of genus *Methanosarcina* that favor high acetate concentrations were dominant in all young landfill leachate samples. On the other hand, only very few, long rod and

filamentous methanogenic *Archaea* belong to *Methanosaeta* genus were present in mature leachate samples.

In an aerated landfill bioreactor TOC, BOD_5 and NH_3-N values decreased mainly due to the dilution effects of rainy days and bio-reduction by the microbial activity. The results showed that rapid bio-stabilization of landfilled MSW incineration bottom ash and shredded incombustible wastes are possible with aerated landfill bioreactor. Results also suggest that nitrification and denitrification may occur simultaneously in an intermittent aerated bioreactor test cell (even under low biodegradable C:N conditions), rather than requiring two separate cells containing two different in-situ environments (i.e. anoxic and aerobic).

In situ hybridization results have indicated that archaeal and bacterial activities increases with the acceleration of degradation process. Slot-blot hybridization experiments carried out with oligonucleotide probe specific for the archaeal and bacterial domains have indicated that bacterial populations were the major microorganisms present in the bioreactor test cell against archaeal species. The results also revealed that *Methanobacteriales* and *Methanomicrobiales* were dominant species at the beginning of bioreactor while *Methanosarcina* species were considerably dominant at the end of the one year operational period. *Methanococcales* and *Methanosaeta* species were not abundant in an aerated bioreactor test cell.

In-situ nitrification experiments were conducted demonstrating that ammonia removal via nitrification and denitrification is feasible in an aerated landfill bioreactor test cell at various oxygen and ORP levels. *Nitrosomonas-like* ammonia oxidizing and *Nitrospira* related nitrite oxidizing bacteria were responsible for nitrification and intensively present nitrifiers in an aerated landfill bioreactor during one year operational periods.

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3. **Researcher**, TUBITAK ICTAG-C106 *Identification of nitrifier diversity and activity in biological wastewater treatment systems* (2004-2006)
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6. **Researcher**, TUBITAK ICTAG-A036 *Investigation of Nitrifying Bacteria Diversity in a Biological Nitrogen Removal System by using 16S rRNA/rDNA based Microbial Identification Techniques* (2003-2004)
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