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Journal of Hazardous Materials 142 (2007) 258–265

Journal of Hazardous Materials

www.elsevier.com/locate/jhazmat

Effects of insufficient air injection on methanogenic *Archaea* in landfill bioreactor

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Received 7 June 2006; received in revised form 3 August 2006; accepted 8 August 2006
Available online 12 August 2006

Abstract

In this study, methanogenic *Archaea* diversity in an aerated landfill bioreactor filled with co-disposed incineration bottom ashes and shredded incombustible wastes was monitored and analyzed as a function of time using molecular techniques. Besides, the effects of insufficient air injection on the bioreactor performance and methanogenic diversity were evaluated thoroughly. Results indicated that rapid bio-stabilization of solid waste are possible with aerated landfill bioreactor at various oxygen and oxidation reduction potential levels. Slot-blot hybridization results of leachate samples collected from aerated landfill bioreactor showed that archaeal and bacterial activities increased as stabilization accelerated and bacterial populations constituted almost 95% of all microorganisms. The results of slot-blot hybridization and phylogenetic analysis based on 16S rRNA gene revealed that *Methanobacteriales* and *Methanomicrobiales* were dominant species at the beginning while substituted by *Methanosarcina*-related methanogens close to the end of the operation of bioreactor.

Keywords: Landfill; Aeration; Incineration ash; Methanogen; Molecular methods

1. Introduction

A bioreactor landfill is a sanitary landfill site that uses enhanced microbiological processes to transform and stabilize the readily and moderately decomposable organic waste constituents within 4–8 years. The need for long-term monitoring and maintenance can be reduced if the decomposition rate is accelerated. Various enhancement techniques have been developed to enhance decomposition of organic and inorganic matter by adding supplemental water/leachate, possibly air and some nutrients to the waste [1–3].

Landfills in Japan currently receive municipal solid waste incineration (MSWI) residues and shredded low-organic wastes as main inputs [4]. The incineration residues and other shredded incombustible low-organic wastes originating especially from recycling activities are not completely stable and need further stabilization and monitoring [5]. There are only few studies evaluating the full-scale landfill bioreactor performances filled with co-disposed MSWI residues and shredded incombustible wastes [6,7]. However, microbial populations responsible for bio-stabilization of such a waste have not been discussed anywhere before.

Within a landfill environment, a complex sequence of physically, chemically and biologically mediated events occur simultaneously [8]. All stages in the aerobic and anaerobic degradation of solid waste that involved in landfills are monitored and evaluated according to the composition of landfill gas and characteristics of landfill leachate. Although much is known about the basic metabolism in landfill ecosystem, little is known about the microorganisms responsible for these processes. Only a few percent of *Bacteria* and *Archaea* have been isolated and almost nothing is known about their interactions [9]. In order to fully understand and characterize the microbial communities and activities, knowledge of their structure and diversity is necessary [10].

Molecular techniques give available information about the waste decomposition in aerobic and anaerobic processes using oligonucleotides and primers, designed to be specific for *Archaea* and *Bacteria* [11,12]. At present, ribosomal RNA

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doi:10.1016/j.jhazmat.2006.08.008

(rRNA) and DNA (rDNA) are commonly used as target nucleic acids for analysis of natural microbial communities instead of cultural methods [10]. Although only a small fraction of microorganisms in the landfills are cultivable, cultivation-independent molecular methods are crucial to identify the prevalent microorganisms responsible from decomposition of organic matter [13–15]. It is possible to investigate groups of methanogens at the order, family and genus level using group-specific 16S rRNA oligonucleotide probes [12]. In addition to 16S rDNA, amplification of the alpha-subunit gene for methyl coenzyme-M reductase (*mcrA*) was also reported as a methanogen-specific molecular marker [16]. This enzyme complex is useful for the detection of methanogenic *Archaea* in environmental studies [13].

Former studies have highlighted the methanogenic diversity in landfill bioreactors and aerobic composts [14,17–19]. However, there has been no comparable molecular investigation in an intermittently aerated landfill bioreactor. Furthermore, effects of insufficient air injection on the performances of bioreactors and diversity of methanogenic *Archaea* were not investigated in details. Thus, understanding the degradation pathway of waste and the composition of individual groups of methanogens, it is necessary to know about the microbiology of landfills. Our objective was to characterize and analyze the methanogenic *Archaea* diversity in an intermittently aerated landfill bioreactor filled with incineration bottom ashes and shredded incombustible wastes as a function of time using 16S rRNA based membrane hybridization, cloning and sequencing analysis.

2. Materials and methods

2.1. Landfill bioreactors

Aerated and control landfill bioreactors were constructed in the empty space in Yorii Landfill (Japan), which receives incineration bottom ashes and shredded incombustibles from varying sources such as municipal solid waste recycling centers and automobile recycling facilities [6,7]. There were small differences in bioreactor sizes due to limitations of construction equipments used. According to former operational experiences, wastes were landfilled in 2.5 m layers and covered with 0.5 m soil. Since on-ground storage was not allowed, the ashes and shredded incombustible wastes were not properly mixed before being landfilled and therefore not homogeneously distributed between aerated and control bioreactors. Amounts and percentages of wastes landfilled to each bioreactor are given in Table 1.

The goal was to provide sufficient air to maintain aerobic conditions without excessive drying or cooling. Injection of air into the waste mass and recycling of leachate were used to promote microbial activity in the aerated landfill bioreactor. Air injection and gas collection wells were equipped with multi-ports 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 $m^3\ min^{-1}$ and operated intermittently 8 h day^{-1} to maintain aerobic and anaerobic conditions simultaneously. The main purpose of control bioreactor was to show the effects of precipitation, infiltration and dilution because of rain water and discharges of small amount of leachate. Control landfill was operated without air injection and leachate recycling [6,7].

To identify the microbial communities in the landfill bioreactors, leachate samples were collected at days 19, 50, 60, 92, 110, 154, 175, 190, 203 and 218. They were stored frozen (−20 °C) before DNA isolation. In addition to molecular analysis, pH, oxidation reduction potential (ORP), BOD_5 and TOC were measured in collected samples according to the Standard Methods [20] to determine the interactions between microbial structure and operational performances.

2.2. DNA extraction and PCR amplification

Initially, 250 ml of leachate samples were concentrated by centrifuging at 7000 rpm at 30 min. The pellets were mechanically bead beaten for 10 s at maximum speed (Mini BeadBeater-8, Biospec Products) for DNA isolation. Cell lyses and DNA purification was performed according to the manufactures protocol with the FastDNA SPIN kit (Q-BIOgene). Primers targeting A109f [9] and 1510r [21] specific to *Archaea* domain were used to amplify 16S rRNA archaeal genes for slot-blot and cloning analysis. The PCR program for amplification of archaeal 16S rRNA genes were consisted of 34 cycles at 95 °C for 30 s, 52 °C for 40 s and 72 °C for 90 s and a final extension at 72 °C for 10 min.

2.3. Slot-blot hybridization

A set of DIG-labeled oligonucleotide probes that target most currently known methanogens was used to investigate the variations of methanogenic diversity in the landfill bioreactors in quantitative slot-blot analysis. The nucleotide sequences and positions of the probes are given in Table 2. Whole microorganisms, *Bacteria* and *Archaea* domains were detected in the DNAs isolated from leachate samples and the main groups of methanogens were relatively quantified with PCR-amplified 16S rRNA archaeal genes using domain and group-specific oligonucleotide probes.

For slot-blot analysis, total or PCR-amplified DNA samples were heated at 95 °C for 10 min and chilled on an ice bath. Then, using a Minifold II slot blotter (Schleicher and Schuell, Fredriksberg, Denmark), 5 μl of each sample was spot-

Table 1
Material distribution in the landfill bioreactors

	Aerated landfill bioreactor		Control landfill bioreactor	
	Tonne	%	Tonne	%
Shredded incombustibles	177.3	60	148.8	34
Bottom ashes	119.8	40	284.9	66
Total weight (tonne)	297.1		433.7	
Total volume (m^3)	432.6		517.7	
Waste volume (m^3)	362.4		436.8	
Waste density (tonne m^{-3}) [wet]	0.82		0.99	

Table 2
DIG-labeled oligonucleotides used in slot-blot hybridization experiment

Probe	Sequence (5′–3′)	Hybridization temperature (°C)	Target organisms	References
UNIV1390	GACGGGCGGTGTGTACAA	47.2	Universal probe	[29]
EUB338	GCTGCCTCCCGTAGGAGT	46.5	*Bacteria* domain	[11]
ARCH915	GTGCTCCCCCGCCAATTCCT	56.8	*Archaea* domain	[28]
MB310	CTTGTCTCAGGTTCCATCTCCG	50.2	*Methanobacteriales*	[12]
MB1174	TACCGTCGTCCACTCCTTCCTC	51.9	*Methanobacteriales*	[12]
MC1109	GCAACATAGGGCACGGGTCT	51.2	*Methanococcales*	[12]
MG1200	CGGATAATTCGGGGCATGCTG	55.9	*Methanomicrobiaceae*	[12]
MSMX860	GGCTCGCTTCACGGCTTCCCT	58.4	*Methanosarcinaceae*	[12]
MS821	CGCCATGCCTGACACCTAGCGAGC	62.3	*Methanosarcina*	[12]
MX825	TCGCACCGTGGCCGACACCTAGC	63.6	*Methanosaeta*	[12]

ted on a nylon membrane and UV crosslinked (Stratalinker-1800, Stratagene, La Jolla, USA). After UV crosslinking, the membrane was cut into slices and dried in ambient air. For hybridization, the membranes were put in separate 50 ml falcon tubes filled with 10 ml of prehybridization buffer and incubated for 30 min in the hybridization oven at optimum hybridization temperature (Boehringer Mannheim GmbH, Biochemica) (Table 2).

DIG-labeled oligonucleotide probes were hybridized using the DIG Easy Hybridization buffer (Boehringer Mannheim GmbH) on a rotating tube roller for at least 6 h. Then unbound oligonucleotides were washed with the DIG Wash and Block Buffer Set (Boehringer Mannheim GmbH). Afterwards, the anti-DIG alkaline phosphatase conjugate was applied to obtain an antibody–hapten complex. Subsequently, an enzyme-catalyzed color reaction with BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitroblue tetrazolium salt) produced blue precipitates on the membrane which visualize the hybridization signals.

2.4. Cloning and phylogenetic analysis

PCR products of sampling days 50, 110 and 218 were purified with QIAquick PCR purification kit (Qiagen) and cloned in competent *E. coli* JM109 cells using pGEM®-T Easy vector system (Promega) with ampicillin selection and blue/white screening according to the manufactures manual. Subsequently, inserts were screened by restriction analysis using the enzyme *MspI* (Fermentas). Plasmids of selected transformants were purified using the Wizard Plus SV Miniprep DNA purification kit (Promega).

DNA sequences were analyzed in SeqLab Sequence Laboratories (Göttingen, Germany). A similarity search, in the GenBank database, with the derived partial (app. 800 bp) 16S rRNA sequences was performed using BLAST search program available on the Internet (National Center for Biotechnology Information sequence search service). 16S rRNA sequences were aligned using the multiple alignments Clustal W programs. Neighbor-joining phylogenetic trees were constructed with the Molecular Evolutionary Genetics Analysis package (MEGA version 2.1) [22] with the Jukes-Cantor algorithm. The robustness of the phylogeny was tested by bootstrap analysis with 500 iterations.

3. Results

In municipal solid waste landfills where organic rich wastes are deposited, aeration decreases the pH by accumulation of volatile fatty acids. However, in our case since shredded incombustible wastes were deposited together with incineration residues, in the landfill bioreactor, pH increased as a result of alkaline characteristics of bottom ashes. In the control bioreactor amount of bottom ashes were relatively higher thus initial pH values above 11 were experienced which prevented the early initiation of microbial activity (Table 1). Because of extremely high pH values, no DNA was extracted from the control landfill bioreactor samples, only samples collected from the aerated bioreactor were used in molecular assays. In the aerated landfill bioreactor pH values fluctuated between 7 and 9 and provided suitable condition for microbial growth (Fig. 1a).

To monitor the degree of stabilization in landfill bioreactors, BOD_5 and TOC were used as indicator parameters. At the beginning of the operation, TOC values in the aerated landfill bioreactor was relatively high (up to 3500 g m^{-3}), and rapid reduction of TOC showed the acceleration of solid waste stabilization. BOD_5 decreased more rapidly than TOC and became negligible (<10 g m^{-3}) around day 120. TOC values also decreased to below 10 g m^{-3} after day 240 (Fig. 1c and d). Thereafter indicator parameters remained constant until end of the operation. On the other hand, the TOC and BOD_5 removal in control landfill bioreactor was conducted by precipitation, infiltration and dilution effects of rain water. Similar effects also existed in aerated bioreactor, however, TOC and BOD_5 concentrations were reduced to lower values as a result of microbiological degradation from approximately same initial concentrations.

The purpose of air injection was to provide aerobic conditions within the landfill bioreactor. However ORP values measured as low as −400 mV indicated that air injection was not sufficient. Leachate accumulated at the bottom of the bioreactor and resulted in a highly reduced anaerobic condition (Fig. 1b). After withdrawal of leachate from the bottom, high ORP values of about 100 mV were determined and values kept on increasing as air was injected (Fig. 1b). Rapid reductions in TOC and BOD_5 values obviously showed that rapid bio-stabilization of landfilled MSW incineration bottom ashes and shredded incombustible wastes are possible in an intermittently aerated landfill bioreactor within a quite short (app. 1 year) period (Fig. 1c and d).

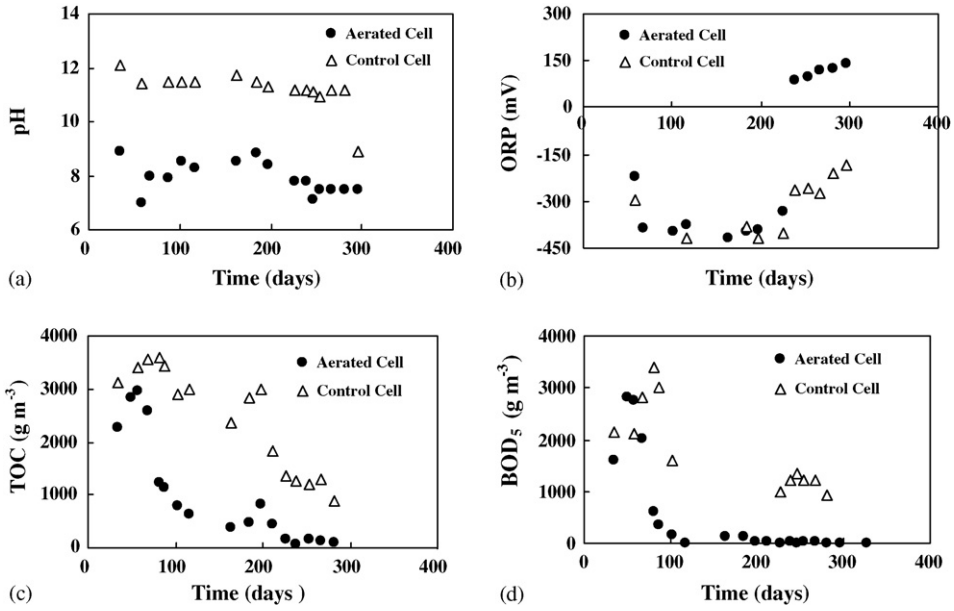

Fig. 1. Changes in pH (a), ORP (b), TOC (c) and BOD_5 (d) during operation of aerated and control landfill bioreactors.

Slot-blot hybridization results carried out using oligonucleotide probes specific for the *Archaea* and *Bacteria* domains revealed that *Bacteria* dominated in the bioreactors 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 in the aerated landfill bioreactor (data not shown).

PCR slot-blot experiments with group-specific oligonucleotide probes were performed to determine the variation in archaeal diversity during the operation of aerated landfill bioreactor. Members of the hydrogenotrophic order *Methanomicrobiales* and *Methanobacteriales* were constituted the majority of methanogens present in the landfill leachate at the beginning of the operation. Although *Methanobacteri-*

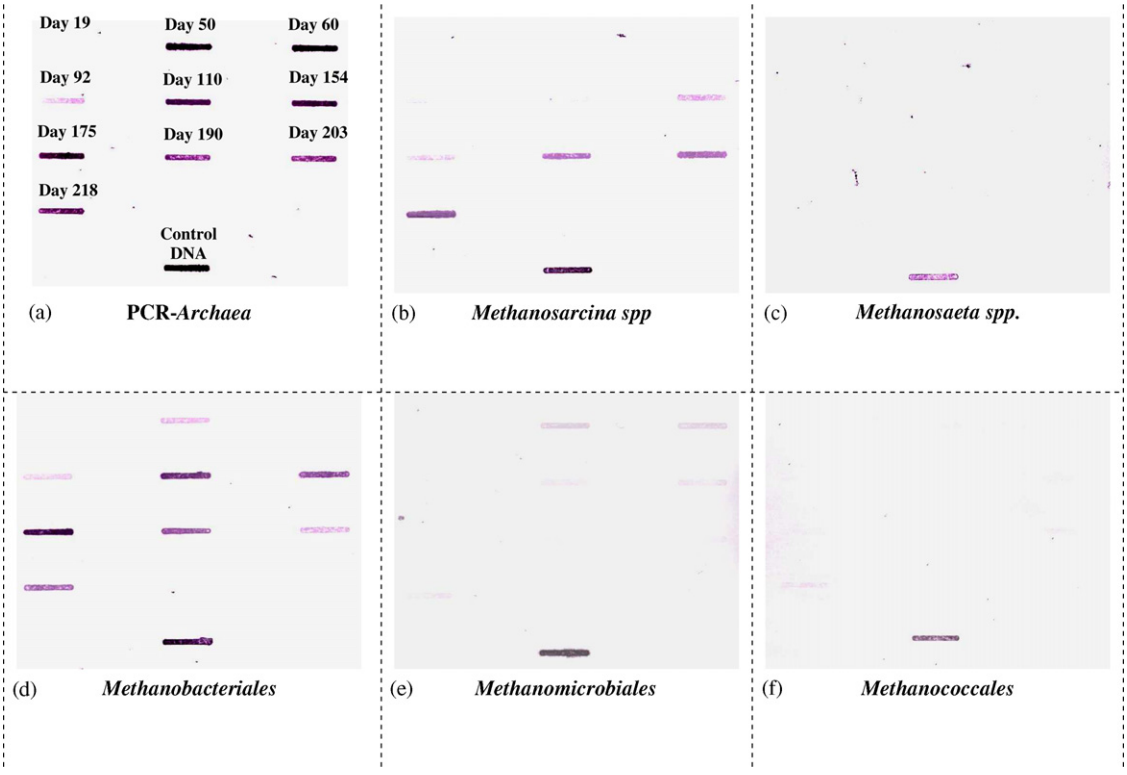

Fig. 2. Relative differences of archaeal population changes during operation of aerated landfill bioreactor. Thickness of the bands is directly proportional to the amount of target DNA. For efficient comparison DIG-labeled 1 ng pBR328 control DNA (linearized with *Bam*HI) was applied to each membrane.

Table 3
Sample source and distribution of identical 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

ales group were present at day 50 sample, their intensity appeared to increase after day 92, and this methanogenic group was very numerous in the day 110 sample. After this time H_2-utilizing *Methanobacteriales* became completely dominant methanogenic *Archaea* in all leachate samples (Fig. 2d and e). The intensive *Methanomicrobiales*-related methanogens disappeared after day 60 and gradually substituted by *Methanosarcina* species. *Methanosarcina* population gradually increased over time and became dominant after day 190 (Fig. 2b). On the other hand, only very few hybridization signals were detected with

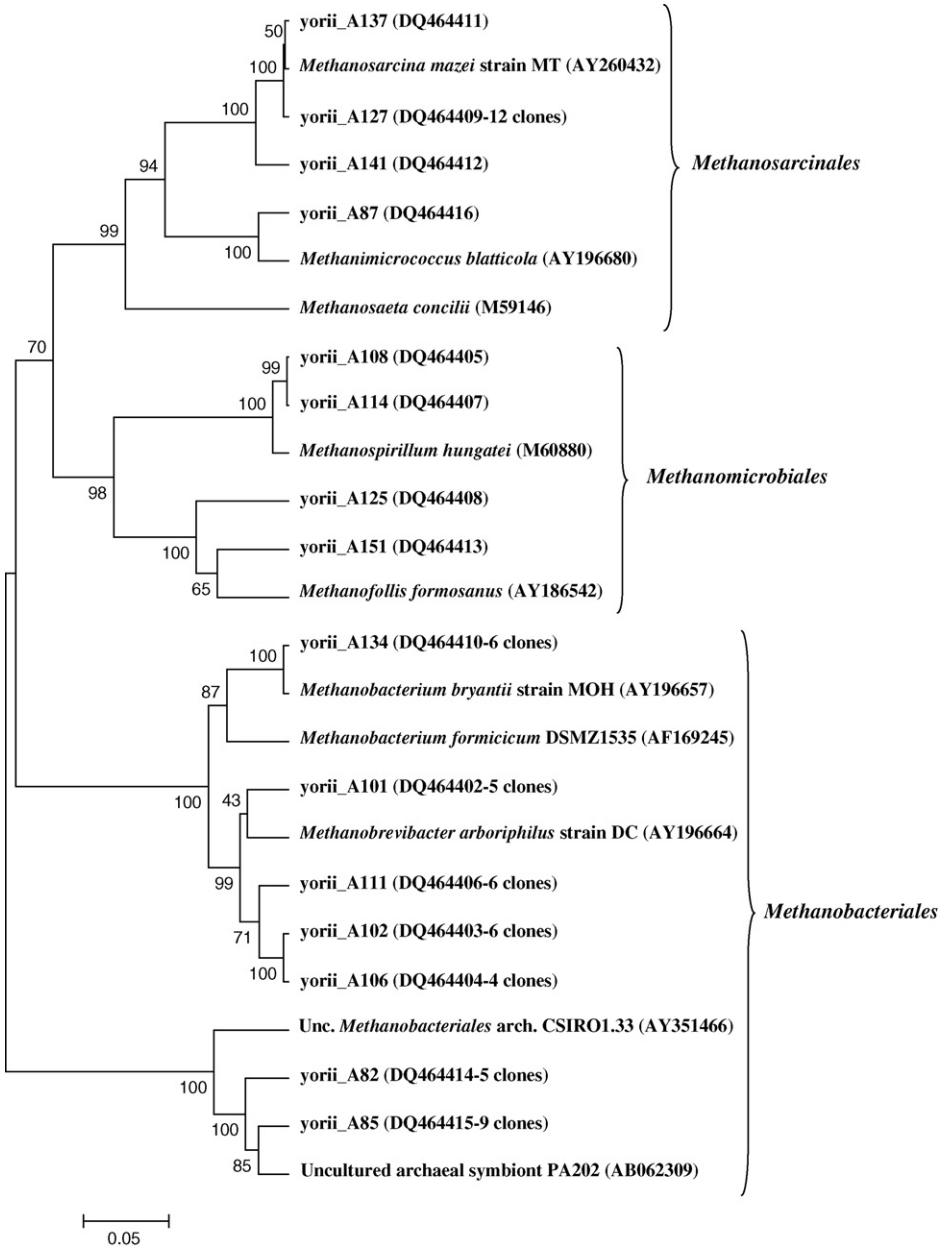

Fig. 3. A neighbor-joining trees of 16S rRNA clones from aerated landfill bioreactor. The significance of each branch is indicated by bootstrap values. The scale bar represents 0.05 inferred substitutions per nucleotide position. The number of closely related clones found among the 60 non-chimeric rDNA clones analyzed is indicated in parentheses. Accession numbers are also given in parenthesis.

oligonucleotides targeting the *Methanococcales* order of H_2-utilizing methanogens and the acetate-utilizing *Methanosaeta* species (Fig. 2c and f).

16S rDNA phylogenetic tree was created based on partial sequences obtained from DNAs isolated from leachate samples. The clone library was constructed using a PCR reaction with archaeal specific 16S rDNA-targeted primer set. 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 taken at days 50, 110 and 218. All the representative sequences were found to be closely related to 16S rDNAs of methanogens, such as orders of *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales* (Fig. 3).

Results of this phylogenetic analysis revealed that, H_2-ulitizing methanogens *Methanobacteriales* and *Methanomicrobiales* were found dominant archaeal population at the leachate sample of day 50 while members of *Methanosarcinales* order were very few. These findings indicated that hydrogenotrophic methanogens were prevalent in high abundance while the organic portion of the landfilled waste was broken down into leachate. Although *Methanosarcinales* and *Methanomicrobiales* related species were present in the leachate at day 110, H_2-utilizing *Methanobacteriales* were still representing the majority of methanogens. Similar to slot-blot hybridization experiments, 90% of the total clones (18 out of 20) belonged to the *Methanobacteriales* order at day 110 sample (Table 3). Likewise, in the next sample at day 218, as in membrane hybridization, *Methanosarcinales*-related methanogens were found dominant species in the aerated landfill bioreactor. Thirteen of the 20 clones were found belong to *Methanosarcinales* which contributed to 65% of the total clones. Six of the rest were identified as *Methanobacteriales* and only one clone was belonging to *Methanomicrobiales*. These results indicated that archaeal populations shifted from *Methanobacteriales* to *Methanosarcinales* through 9-month operation (Table 3). As in slot-blot hybridization experiments, no *Methanococcales*-related sequences were found in any of the leachate samples.

4. Discussions

As a result of incineration process, different solid and liquid residuals are generated. Significant amounts of organic carbon, heavy metals and toxic organic pollutants spread out to the environment via leachate and gas phases, requiring extensive treatment and monitoring when they are landfilled. The acceleration of MSW biodegradation not only reduces the overall monitoring costs, but also decreases the life of landfill [3]. Although many studies focused on landfill bioreactor technologies and enhancement strategies, there have been no definitive studies about the microbiology of landfill bioreactor that filled with MSWI bottom ashes and shredded incombustible wastes. In this study, aerated landfill bioreactor was operated to accelerate the decomposition of waste residuals and to understand the microbial population varieties during the stabilization periods of waste.

Most of the studies have ignored the possible presence and importance of *Archaea* in aerobic environments. Gray et al. [23] detected methanogenic *Archaea* that reside in the activated sludge of wastewater treatment plants. The presence of *Methanosarcinales*, *Methanomicrobiales* and *Methanobacteriales* groups demonstrating that methanogens can survive and being active in anoxic environments. In our case, low ORP values also allowed growing of methanogens in the intermittently aerated landfill bioreactor.

In former studies, similar to our results, bacterial populations were found more intensive than archaeal populations in full-scale landfills [14,19,24]. Mori et al. [14] indicated that 2–3% of the total DNA extracted from the leachate of a landfill site was archaeal, and this archaeal community was consisted of several species of methanogens. Huang et al. [25] investigated the phylogenetic composition of *Archaea* in the leachate of a full-scale MSW landfill that was closed several years ago. According to phylogenetic analysis of archaeal 16S rRNA gene sequences, they found that landfill leachate harbored a diverse archaeal community. Similarly, only less than 1% of the total cells were detected with *Archaea* specific Arch915 probe in 1-year-old landfill samples excavated from 1 and 3 m depths [24]. In another study, Calli and Girgin [15] investigated microbial diversity in leachate samples collected from MSW landfills at different ages using FISH (fluorescent in situ hybridization) analysis and reported that in all leachate samples, bacterial population (Eub338) was more intensive than archaeal population. Boothe et al. [18] characterized the aerobic microbial populations in landfill leachate and bulk material during an engineered aerobic bioreduction process in a test cell of a municipal landfill. 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. Despite the injection of air in landfill bioreactor intermittently we found significant amounts of methanogenic *Archaea* in our leachate samples. However, similar to former results bacterial populations were the major group of microorganisms present in the aerated landfill bioreactor (>95%).

Studies on archaeal community compositions in leachate from a landfill operated under leachate recirculation [19] and in a sea-based landfill site [14] indicated that members of *Methanomicrobiales* and *Methanosarcinales* could be significant archaeal populations in such environments. Likewise acetate-utilizing methanogens especially members of genus *Methanosarcina* that favor high acetate concentrations were found dominant in leachate samples collected from young acidogenic landfills [15]. On the other hand, a PCR-based study revealed a great diversity in the methanogenic 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 [13]. These results suggest that the methanogenic diversity may be quite dissimilar in different landfill sites as well as in different stabilization phases. However, it is also obvious that for a detailed investigation of microbial diversity in a landfill, time based monitoring under dif-

ferent operational conditions is necessary. In our study archaeal population variety were monitored in an intermittently aerated landfill bioreactor at various leachate characteristics throughout 1-year test operation.

Chen et al. [24] reported the members of the genus *Methanosarcina* as the predominant methanogen in young landfills but found negligible in mature ones. Röling et al. [26] revealed that the archaeal community was not complex, and symbiotic methanogens were detected in polluted leachate from a landfill site. However phylogenetic analysis of our samples and other landfill studies showed that far greater diversity in the methanogenic population present within the landfill material [13,27]. H_2-utilizing *Methanobacteriales* and *Methanomicrobiales* orders were found as the major methanogenic *Archaea* at the beginning of the operational period and *Methanosarcinales* order became dominant after all organic matter depleted in the aerated landfill bioreactor. The main degradation pathway for methanogenesis in intermittently aerated landfill bioreactor was hydrogenotrophy. Accumulation of leachate at the bottom of the bioreactor and highly reduced anaerobic conditions probably allowed the dominance of *Methanosarcinales* order.

Chen et al. [24] also suggested that H_2-ulitizing methanogens were the dominant archaeal population in the young and old age landfill samples. Huang et al. [25] reported that members of the hydrogenotrophic order *Methanomicrobiales* constituted the major methanogens present in the leachate of a full-scale MSW landfill that was closed several years ago. In our study, H_2-utilizing *Methanobacteriales* was found as the major methanogenic order while rapid reductions in TOC and BOD_5 values were observed. The abundance of *Methanobacteriales* and *Methanosarcinales* orders were also verified with slot-blot hybridization analysis.

5. Conclusions

Effects of insufficient air injection on the performances of landfill bioreactor and diversity of methanogenic *Archaea* were investigated using molecular techniques combined with physical and chemical parameters. The experimental results obtained from the operation of aerated bioreactor filled with municipal solid waste incineration bottom ashes and shredded incombustible wastes confirmed the viability of rapid aerobic bio-stabilization at various oxygen and ORP levels (−400 to 150 mV). Slot-blot hybridization experiments indicated that bacterial populations were the major microorganisms present in the aerated landfill bioreactor. H_2-utilizing *Methanobacteriales* and *Methanomicrobiales* orders were found as the major methanogenic *Archaea* at the beginning of the operational period using slot-blot hybridization and cloning experiments. Population diversity shifted from *Methanobacteriales* to *Methanosarcinales* order after all organic matter depleted at the end of the operation. *Methanococcales* and *Methanosaeta* species were not abundant in the aerated landfill bioreactor. In future studies, effects of different air flowrates on methanogenic population variety should be investigated in aerated and hybrid landfills.

Acknowledgements

The authors gratefully acknowledge the contributions by staff at Yorii Environmental Management Center (Saitama, Japan) and Mr. Toshiro Oshikata and Mr. Masaaki Ebihara of Taisei Corporation (Tokyo, Japan) for the construction of the test cells. This work was financially supported by Ministry of Environment, Japan (Project No.: K1535) and TUBITAK (Project Nos.: ICTAG A045, A051 and Ç106).

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