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Archaeal community diversity in municipal waste landfill sites

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Abstract Despite the pivotal role of archaea in methane production in landfills, the identity, ecology, and functional diversity of these microorganisms and their link to environmental factors remain largely unknown. We collected 11 landfill leachate samples from six geographically distinct landfills of different ages in China and analyzed the archaeal community by bar-coded 454 pyrosequencing. We retrieved 121,797 sequences from a total of 167,583 sequences (average length of 464 bp). The archaeal community was geographically structured, and nonabundant taxa primarily contributed to the observed dissimilarities. Canonical correlation analysis (CCA) suggested that the total phosphorous (TP), nitrate, and conductivity are important drivers for shaping the archaeal community. The hydrogenotrophic methanogens *Methanomicrobiales* and *Methanobacteriales* greatly dominated 9 of 11 samples, ranging from 83.7 to 99.5 %. These methanogens also dominated the remaining two samples, accounting for 70.3 and 58.8 %, respectively. Interestingly, for all of the studied Chinese landfills, 16S rRNA analysis indicated the predominance of hydrogenotrophic methanogens.

Keywords Landfill · Landfill leachate · Archaeal community · Hydrogenotrophic methanogenesis

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Introduction

Landfills harbor highly complex microbial communities with diverse metabolic activities that enable the conversion of biodegradable substances (e.g., cellulose and hemicelluloses) to methane and global carbon cycling (Barlaz et al. 1989; Kjeldsen et al. 2002; Zhao et al. 2007). A successive four-stage pathway comprising hydrolysis, acidogenesis, acetogenesis, and methanogenesis is responsible for methane production (Garrity and Holt 2001). Methanogens, the predominant archaea in landfills, perform the terminal step of methane conversion (Garrity and Holt 2001). Understanding landfill archaeal ecology might lead to more sustainable management practices through the reduction of greenhouse gas emissions and improvements in CH_4 recovery.

Methanogens are affiliated with the phylum *Euryarchaeota* within the domain Archaea. The characterized methanogens have been classified into five orders: *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcinales*, *Methanococcales*, and *Methanopyrales* (Garrity and Holt 2001). Representatives of *Methanobacteriales*, *Methanomicrobiales*, and *Methanosarcinales* have been detected in high concentrations in anaerobic digestion (AD) systems (e.g., biogas plants and municipal sludge digesters) and described as major players in these systems (Ahring 1995; Ahring et al. 2001; Karakashev et al. 2006; Krakat et al. 2010; Leclerc et al. 2004). Based on the metabolic precursors used, methanogens have been classified into two groups: acetoclastic methanogens, which strictly metabolize acetate, and hydrogenotrophic methanogens, which use H_2 or formate as an electron donor and CO_2 as a carbon source (Garrity and Holt 2001). *Methanobacteriales* and *Methanomicrobiales* are hydrogenotrophic methanogens, and *Methanosarcinales* are mixotrophic methanogens. Most of the members of the *Methanosarcinaceae* family, which belongs to the order *Methanosarcinales*, are mixotrophic methanogens that utilize

all metabolic methanogenesis pathways. The family *Methanosaetaceae* in the order *Methanosarcinales* is a strictly acetotrophic group of methanogens.

Theoretically, acetate and hydrogen account for 67 and 33 % of total methanogenesis, respectively (Conrad 1999). The observation of high concentrations of acetoclastic methanogens, such as *Methanosarcina* sp., in AD systems (Laloui-Carpentier et al. 2006; Leclerc et al. 2004; McHugh et al. 2003) initially suggested that acetoclastic methanogenesis is the predominant pathway for methane formation (Smith and Ingram-Smith 2007). However, increasing evidence suggests that hydrogenotrophic methanogenesis contributes more H_2 to CH_4 production than previously considered (Karakashev et al. 2006; Krakat et al. 2010; Nettmann et al. 2010). For example, high temperatures or inhibitory conditions (e.g., high volatile fatty acids (VFAs) and ammonia concentrations) in AD systems promote acetate oxidation and the subsequent conversion of H_2 and CO_2 to methane, increasing the contribution of H_2-derived methane production (Karakashev et al. 2006).

The coexistence of acetoclastic and hydrogenotrophic methanogens in landfills has been reported (Barlaz 1997). Studies of archaeal communities in landfills using a 16S rRNA clone library have demonstrated that methanogen groups, such as the orders *Methanomicrobiales* and *Methanosarcinales* and the genera *Methanosarcina*, *Methanoculleus*, *Methanothermobacter*, and *Methanosaeta*, are the predominant microbial communities in this niche (Chen et al. 2003; Huang et al. 2002, 2003; Krishnamurthi and Chakrabarti 2013; Laloui-Carpentier et al. 2006; Van Dyke and McCarthy 2002). However, the archaeal community structure and function, as well as the mechanism by which environmental parameters affect the methanogen population structure in landfills, are not well understood. With respect to landfill microbiology, knowledge of the identity and abundance of the indigenous microbiota is limited, reflecting the use in previous studies of a limited number of isolates and clones that did not represent the complete profile of the diversity and complex interactions of the archaeal population in landfills.

The 454 pyrosequencing technique facilitates high-throughput sequencing in a single run and has been widely applied to natural (Bolhuis and Stal 2011) and engineered (Zhang et al. 2011) habitats. Studies based on the use of this technique would improve the characterization of landfill archaeal ecology and provide insight to elucidate archaeal interactions in methane production. Therefore, the aim of the present study was to investigate the archaeal community composition in landfills using deep pyrosequencing and analyzed how environmental factor impact the archaeal community structure. We sampled 11 landfill leachate samples of different ages from six geographically distinct landfills in China using a drilling technique and analyzed the archaeal community compositions using bar-coded 454 pyrosequencing to examine archaeal diversity and compare unique dominant archaeal populations. Water chemistry was characterized by 14 environmental parameters. And then, these archaeal community diversity data was analyzed using water chemistry to answer the linkage between archaeal community structure and environmental parameters.

Materials and methods

Sampling site description

We obtained 11 samples from six refuse landfills in China (Fig. S1), including the Laohukeng landfill in Shenzhen (SZ), the Houcun landfill in Taiyuan (TY), the Jiangchungou landfill in Xi'an (XA), the Jianzigu landfill in Tangshan (TS), the Laogang landfill in Shanghai (SH), and the Yongchuan landfill in Yongchuan (YC). The XA and TY landfills are in semi-arid climates, and the TS is in a humid continental climate; the remaining landfills are located in humid subtropical climates. The information on each landfill is presented in Table 1 and Supplementary Method S1. The SZ, TY, XA, TS, SH, and YC landfills were sampled on September 8, September 12, September 15, September 20, September 28, and November 8, respectively, in 2012.

Sampling

Leachate results from the percolation of liquid through the landfill site and is a practical sample for use in direct studies of landfill microbial ecology (Kjeldsen et al. 2002; McDonald et al. 2010). Therefore, we sampled and analyzed landfill leachate to obtain an adequate representation of the archaeal community of each landfill. Previous studies have typically sampled leachate by direct sampling through a collection pipe. In this study, we used a drilling technique to collect the leachate samples. The leachates were collected from the landfills, except SH and YC, using a drilling technique based on the Chinese national standard code for the investigation of geotechnical engineering (GB50021-2001) and the technical code for geotechnical engineering of municipal solid waste sanitary landfills (CJJ176-2012). Using a high-pressure pump to a depth of 3 to 25 m, we inserted an XY-100 engineering drill (bottom inner diameter 110 mm; upper inner diameter 150 mm) (Aicheng Company, Shanghai, China) into the landfill, and a sterilized open HDPE bottle was immediately passed into the bottom of a drilled hole to collect percolated leachate samples. The collected leachate was subsequently transferred to a 1-L sterilized HDPE sample (Fisher Scientific, USA) and stored on ice for transport to the laboratory. The SH and YC landfill leachate samples were collected from the inlet of the leachate collection ponds and transferred

to a 1-L ultra-sterilized HDPE sample bottle. For the archaeal community analysis, 20 mL of the leachate sample was filtered through a sterile 0.22-μm filter (Millipore, USA), and the filter was stored in a 50-mL sterile tube.

Chemicals analysis

The leachate was stored at 4 °C and analyzed within 1 week of the collection. The leachate samples were chemically analyzed by standard methods. Total organic carbon (TOC) and total nitrogen (TN) were determined by high-temperature catalytic oxidation on a Multi TN/TC Analyzer 3100 (Element, Germany). The chloride, nitrate, and sulfate contents were determined by ICS 1100 ion chromatography (Agilent, USA) following standard method 4110-B (APHA 1998). The ammonia nitrogen (NH_3-N) concentration, biological oxygen demand (BOD_5), and chemical oxygen demand (COD) were determined according to the methods of the State Environmental Protection Administration of China (State Environmental Protection Administration 2002). The ammonia nitrogen content was determined using Nessler's reagent spectrophotometry method. BOD_5 was determined using the dilution inoculation method. The COD was determined using the dichromate method. Turbidity, pH, and conductivity were detected using a standard portable Multi-Parameter Water Quality Analyzer (Hach, USA).

The age of a landfill might be an important factor in shaping the archaeal community. A landfill is complex, and the cells are filled at different times. A cell is closed after it is filled with a specified level of refuse, and the age of each landfill is recorded. The six landfills we sampled are sanitary landfills, and records regarding the quantity and properties of the refuse, the placement time, and the corresponding compartment sites were available. It was convenient to collect corresponding landfill samples with ascertained landfilling times. The ages of the landfill samples are listed in Table 1.

DNA extraction and amplification

DNA was extracted from one half of a filter using the Ultraclean Soil DNA Isolation Kit (MO BIO Laboratories, Inc., USA) and quantified using a NanoVue Plus spectrophotometer (GE, USA). The quality of the extracted DNA was assessed by genomic DNA amplification using the 16S rRNA general primers 63 F (5′-CAG GCC TAA CAC ATG CAA GTG-3′) and 1389R (5′-GGG CGG WGT GTA CAA GGC-3′) (Marchesi et al. 1998).

High-throughput 16S rRNA gene pyrosequencing

Archaeal amplicon libraries for 454 pyrosequencing were constructed using the primers 344 F (5′-ACGGGGYGCA GCAGGCGCGA-3′) and 915R (5′-GTGCTCCCCCGCCA

Table 1 Samples' information

Code	Full name (landfill and city)	Landfill capacity (million m^3)	Climate type	pH	Cond (μS/cm)	NTU	TN (g/L)	TP (mg/L)	BOD_5 (mg/L)	COD (g/L)	BOD_5/COD	Cl^- (mg/L)	SO_4^{2-} (mg/L)	NH_3-N (mg/L)	NO_3^- (mg/L)	Landfilling ages
SZ-1	Laohukeng, Shenzhen	8.8	A	8.3	33.6	38.3	2840.0	19.0	371.8	6035.0	0.06	5081.2	1320.7	244.8	1395.1	4
SZ-2	Laohukeng, Shenzhen	8.8	A	8.3	36.1	113.0	3440.0	15.2	5241.4	15,278.4	0.34	5594.8	985.7	350.5	1569.7	4
SZ-3	Laohukeng, Shenzhen	8.8	A	7.7	22.2	60.0	1330.0	9.2	321.3	3437.6	0.09	5279.9	1078.1	183.7	1721.4	4
SZ-4	Laohukeng, Shenzhen	8.8	A	8.4	37.5	351.0	4240.0	17.7	3961.6	15,545.8	0.25	5980.8	1158.8	372.7	1442.2	10
SZ-5	Laohukeng, Shenzhen	8.8	A	8.0	15.7	39.7	1360.0	16.5	141.3	2330.0	0.06	5980.8	1158.8	128.1	1366.1	8
TY	Houcun, Taiyuan	12	B	8.3	19.5	40.4	950.0	1.0	141.8	2062.6	0.07	18,145.4	419.3	44.7	21.7	0.5
XA	Jiangchungou, Xi'an	20	B	8.0	39.6	57.4	3810.0	1.5	736.9	5003.7	0.15	4964.4	164.6	306.0	36.7	0.5
TS	Tangshan, Hebei	5.4	B	8.2	42.9	937.0	3190.0	9.0	721.1	8059.4	0.09	8784.0	917.0	289.3	67.9	0.5
SH-1	Laogang, Shanghai	80	A	7.8	38.3	919.0	3590.0	5.0	7121.6	18,181.3	0.39	357.0	99.3	367.2	20.0	0.4
SH-2	Laogang, Shanghai	80	A	8.0	19.7	124.0	1640.0	11.0	25.5	1680.6	0.02	219.0	146.6	167.0	11.9	3.0
YC	Yongchuan, Yongchuan	3.0	A	7.9	9.1	195.0	1980.0	5.0	147.9	2578.5	0.06	1108.3	397.5	566.2	584.7	0.2

Climate type A: humid, subtropical climates; climate type B: semi-arid climates

NTU turbidity, *Cond* conductivity, *TN* total nitrogen, *TP* total phosphorus, *SZ-1 to SZ-5* leachate in Shenzhen Laohukeng landfill, *TY* leachate in Taiyuan Houcun landfill, *XA* leachate in Xian Jiangxigou landfill, *TS* Tangshan landfill, *SH* Shanghai Laogang landfill, *YC* Yongchuan landfill

ATTCCT-3′) to amplify the V3–V5 region of the 16S rRNA gene (Raskin et al. 1994). Eight-nucleotide barcodes were designed for archaeal sequencing to sort multiple samples in a single 454 GS-FLX run. The archaeal communities were first amplified using high-fidelity Taq polymerase (Invitrogen, USA) under the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 amplification cycles (30 s at 95 °C, 30 s at 55 °C, and 30 s at 72 °C) and a final extension at 72 °C for 5 min. After purification and quantification, a mixture of amplicons was used for pyrosequencing on a massively parallel 454 GS-FLX sequencer (Roche) according to standard protocols.

Sequence data analysis

Data processing was conducted using Mothur v.1.31.2. The treatment of the sequences was performed according to previously described methods (Song and Wang 2015; Zhang et al. 2011). To obtain high-quality sequences, low-quality sequences without an exact match to the forward primer or to a recognizable reverse primer, with lengths shorter than 200 nucleotides, or with ambiguous base calls (Ns) were eliminated (Zhang et al. 2011). To generate operational taxonomic units (OTUs), we aligned tags using the SILVER 111 (http://www.arb-silva.de/) compatible alignment database and the align.seqs command. The remaining sequences were examined for potential chimeras, and the chimeras were subsequently removed (Edgar et al. 2011). The sequences were clustered into OTUs using a 0.03 distance limit (equivalent to 97 %), and rarefaction data and Shannon and Chao1 diversity indices were generated for each sample. The sequences were phylogenetically assigned to taxonomic classifications using an RDP naive Bayesian rRNA classifier with an 80 % confidence threshold (http://rdp.cme.msu.edu/classifier/classifier.jsp) (Wang et al. 2007). The obtained sequences are listed in Table 2.

To assess the dissimilarity in the archaeal community structures between landfills, the OTU abundances of the samples were ordered by principal component analysis (PCA) based on a Euclidean distance matrix with Hellinger transformation (Mantel 1967). Singletons were removed to eliminate bias. To determine whether the major taxonomic groups represent the whole community structure, OTUs of ≥100 sequences across all samples were compared and subjected to the same data treatment as the whole community. The results are presented as a heatmap generated using CLUSTER and visualized using the Java TreeView software.

To determine the relationship between environmental variables and the archaeal community, detrended correspondence analysis (DCA) (Hill and Gauch 1980) was first employed to determine which model (linear or unimodal) best fit the archaeal community dataset (Boer et al. 2009). This analysis was followed by a canonical correlation analysis (CCA) to test which environmental variables best explained the variation in the archaeal community structure. Significant variables for the analysis were preselected by forward model selection, and the significance of the CCA model and of the selected variables was tested by 999 Monte Carlo permutations. The environmental variables were log10-tranformed (except for pH, turbidity, and age) to normalize their distribution before the analysis. The analyses were conducted with the R program with the Vegan package, except for the significant variable selection, which was performed by the forward model selection at CANOCO (Braak and FtaŠ 2002).

Sequence accession numbers

The raw pyrosequencing data were deposited in NCBI Sequence Read Archive (SRA, http://trace.ncbi.nlm.nih.gov/Traces/sra/sra.cgi) under accession nos. SRR1514148 (SZ, TY, XA, and YC), SRS479746 (SH-1), and SRS479747 (SH-2).

Results

Water chemistry

The physiochemical parameters of the examined landfill leachates are summarized in Table 1. The values of the environmental parameters varied according to geographical location and landfilling age. Generally, the COD varied dramatically among samples and were highest in the SH-1 sample (18181.3 mg/L) and lowest in the SH-2 sample (1680.6 mg/L). Similar to the COD, the BOD_5 value was highest in SH-1 (7121.6 mg/L) and lowest in SH-2 (25.5 mg/L). The pH values were similar among all samples and ranged from 7.7 to 8.3. The total phosphorus (TP) concentration was low in the sampled landfills (1 to 19.0 mg/L). However, the TP concentration was higher in humid subtropical climate zones (SH, SZ, and YC) (5.0 to 19.0 mg/L) than in semi-arid climate zones (TY and XA) (1 and 1.5 mg/L). The total nitrogen (TN) concentration was high in all samples, with the highest value (4240.0 mg/L) observed for SZ (SZ-4) and the lowest value observed for TY (950.0 mg/L). The chloride concentration was also high in all samples, with the lowest value (219.0 mg/L) observed for SH-2 and the highest value (18145.4 mg/L) observed for TY. The sulfate concentration varied dramatically among the samples, with the highest value observed for SZ (985.7 to 1320.7 mg/L) and the lowest value observed for SH (99.3 mg/L at SH-1 and 146.6 mg/L at SH-2). A similar trend in the nitrate concentration was also observed.

General statistics

Figure S2 presents the results of the rarefaction analysis. The rarefaction curves indicate unprecedented levels of archaeal

Table 2 454 Sequence data statistics summary and archaeal diversity based on 97 % OTU clusters

Code	Raw tags	Trimmed tags	Unique tags	Total no. of OTUs	Ace	Chao	Coverage	Shannon	Simpson
SZ-1	13,689	11,574	10,568	229	1025	805	0.98	2.6	0.29
SZ-2	12,682	10,557	9426	179	798	634	0.98	2.73	0.17
SZ-3	17,579	14,330	12,877	394	1970	1439	0.97	3.28	0.21
SZ-4	8112	5466	5099	82	365	291	0.98	1.68	0.48
SZ-5	11,667	9848	8558	280	1394	1064	0.97	3.23	0.18
TY	13,394	11,537	10,495	426	2140	1461	0.96	3.2	0.21
XA	9588	7328	6375	254	1492	1019	0.96	3.66	0.08
TS	19,147	15,425	15,120	167	773	611	0.99	1.63	0.52
SH-1	17,551	12,357	12,171	221	1039	769	0.98	2.25	0.27
SH-2	13,143	10,351	9365	267	1231	964	0.97	4.07	0.05
YC	31,031	24,788	21,743	638	2868	2323	0.97	3.82	0.12

Raw tags number: the number of reads detected through pyrosequencing; trimmed tags number: the numbers of reads remaining after the removal of primers and low-quality data; unique tags number: the numbers of distinct sequences within a set of trimmed tags

complexity in the landfill samples, although none of these curves reached the curvilinear or plateau phases. Thus, these data likely represent underestimates of the different types of archaea present in each sample. We obtained a total of 167, 583 sequences averaging 464 bp in length (Table 2). After treatment, we obtained an average of 11,072±4528 archaeal sequence reads for each sample. A total of 2735 (2.4 %) of the detected sequences were singletons (the unique reads occurring once in a sample divided by the total number of different sequence reads in that sample),with a range of 1.1 to 4.5 % singletons for all the samples.

Estimation of richness and diversity

Tags differing by no more than 3 % were clustered into OTUs to calculate rarefaction and nonparametric estimators (Table 2). The average number of OTUs for all the 11 samples was 285±152. The highest archaeal richness (the number of different OTUs in a sample) and diversity (Shannon's H index, which considers the evenness of OTU distribution) were observed for the TY landfill, while the lowest richness and diversity were observed for TS.

Diversity patterns and environmental factors

The community compositions of the 11 samples were compared by PCA analysis based on the Euclidean distance between the samples (Fig. S3). The PCA revealed that the 11 samples were divided into the following three groups: group 1 (SZ-1, SZ-2, and SZ-4), group 2 (YC, TY, and SH-2), and group 3 (SH-1, XA, TS, SZ-3, and SZ-5). In addition, the two PCA axes (PC 1 and PC 2) explained 70.3 % of the variation between the samples. To determine whether the observed differences between the samples in the PCA plot were influenced by the most abundant OTUs, a Euclidian distance analysis of the relative abundance of the OTUs representing ≥100 OTUs in each sample (Table S1) was performed (Fig. 1). Five distinct clusters were evident: cluster 1 contained SZ landfill samples (SZ-1, SZ-2, SZ-3, SZ-4, and SZ-5); cluster 2 included YC and TY samples; cluster 3 comprised SH-1 and TS samples; cluster 4 included XA samples; and cluster 5 included Sh-2 samples. These results are not consistent with the PCA results, suggesting that the non-abundant taxa rather than the abundant taxa might be primarily responsible for the similarities of the landfill archaeal communities.

CCA was constructed to select significant environmental variables that explain the variation of the archaeal communities by the forward model selection. CCA rather than RDA was selected because the gradient in the detrended correspondence analysis (DCA) is longer than 3.0. The nitrate concentration, TN, TP, COD, pH, ammonium, turbidity, conductivity, and landfill age were selected as environmental variables explaining the variation in the archaeal communities (Table 3). Among these, the nitrate concentration, TP, and conductivity were the factors with a significant effect (Table 3 and Fig. 2).

Taxonomic composition

The taxonomic compositions of the archaeal communities are presented in Fig. 3, in which the order composition is shown in the upper panel and the genera composition is shown in the lower panel. Among the 11 leachate samples obtained from the six landfills, the predominant groups that we detected were *Methanomicrobiales* and *Methanosarcinales*. *Methanobacteriales* were detected in ten samples. Among these, *Methanobacteriales* (82.7 to 94.5 %) were the most predominant group in samples SZ-1, SZ-2, and SZ-4. *Methanomicrobiales* predominated the remaining landfill samples (SZ-3, SZ-5, TY, XA, TS, SH-1, SH-2, and YC), ranging from 57.6 to 97.6 %. Overall, *Methanomicrobiales*

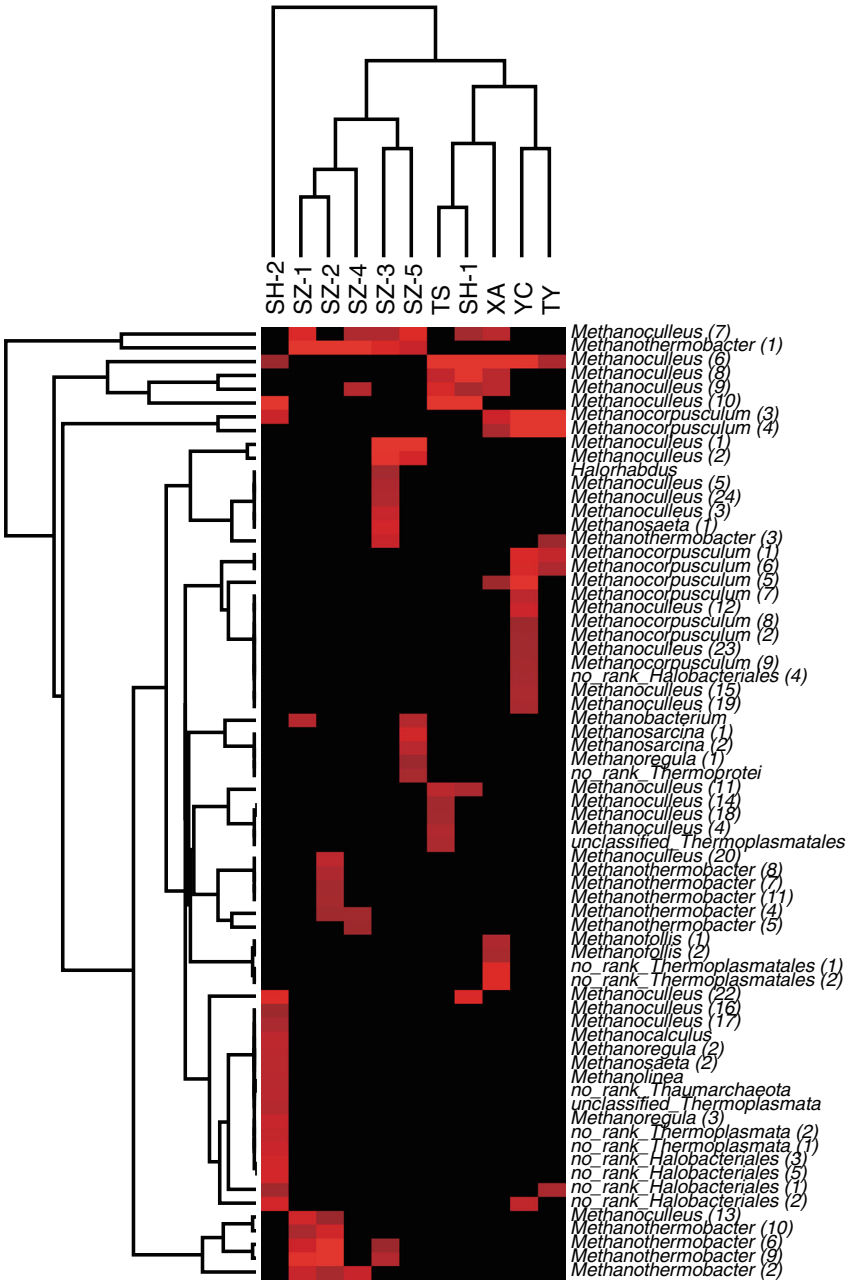

Fig. 1 Hierarchical cluster analysis of the relative abundances of specific OTUs across the samples. Only OTU numbers with a sum of ≥100 across all samples were clustered. Clustering was generated based on Euclidean distances according to the relative abundances within the data matrix and not on the observed phylogenetic relationships. The figure was generated using CLUSTER and visualized using Java TreeView. *Black* indicates no OTUs, while *red* indicates abundant OTUs. The color intensities indicate differences in OTU abundance (color figure online)

and *Methanobacteriales* absolutely dominated the SZ-1 (97.2 %), SZ-2 (99.5 %), SZ-3 (89.1 %), SZ-4 (99.3 %), TY (92.2 %), TS (96.8 %), SH-1(97.8 %), and YC (94.3 %) landfill samples. Although *Methanomicrobiales* and *Methanobacteriales* were also detected in SZ-5 (83.7 %), XA (70.3 %), and SH-2 (58.8 %), *Methanosarcinales* (10.4 %) in SZ-5, *Thermoplasmatales* (25.6 %) in XA, and *Haloarchaeales* (20.3 %) in SH-2, were also detected. *Methanosarcinaceae* were detected in all samples, and *Methanosaetaceae* were detected in all samples except TS. However, the number of detected sequences was low, at 2 to 601 for *Methanosaetaceae* and 5 to 691 for *Methanosarcinaceae*.

Methanomicrobiales and *Methanobacteriales* absolutely dominated the community in the SZ landfill samples, but this predominance was not maintained with age. By contrast, *Methanomicrobiales* completely dominated the 0.4-year-old samples (SH-1) (97.6 %) and decreased to 57.6 % in the 3.0-year-old samples (SH-2) from the SH landfill.

All the genera are listed in Table S2. *Methanoculleus* and *Methanothermobacter* were predominantly detected in SZ (SZ-1, SZ-2, SZ-3, SZ-4, and SZ-5), accounting for 72.3 to 99.0 % of the microbial community. Specifically, *Methanoculleus* and *Methanothermobacter* were completely dominant in the SZ-1, SZ-2, and SZ-4 samples, accounting for 91.4, 99.0, and 98.6 % of the microbial community,

Table 3 Conditional effects of forward model selected environmental variables determined by redundancy analysis (RDA)

Environmental variables	Lambda-A	F-ratio
Nitrate	0.27	3.3***
Conductivity	0.16	2.19*
TP	0.13	2.17*
Turbidity	0.11	1.88^{NS}
Ammonium	0.08	1.67^{NS}
pH	0.07	1.42^{NS}
Landfilling age	0.05	1.17^{NS}
COD	0.06	1.63^{NS}
TN	0.04	1.63^{NS}

Lambda-A represents the variance explanation of each variable in model

TN total nitrogen, *TP* total phosphorus, *NS* not significant

*$p<0.05$, **$p\leq0.01$, ***$p\leq0.001$, significance determined by 999 Monte Carlo permutations under full models

respectively. By contrast, SZ-3 primarily comprised *Methanoculleus* (74.1 %), *Methanothermobacter* (12.4 %) and *Methanosaeta* (4.7 %). SZ-5 comprised *Methanoculleus* (67.6 %), *Methanothermobacter* (4.8 %), and *Methanosarcina* (8.1 %). *Methanoculleus* and *Methanocorpusculum* were the most abundant groups in TY (84.7 %), TS (96.4 %), SH-1 (97.3 %), and YC (91.8 %). *Methanocorpusculum* dominated in TY (77.6 %) and YC (70.0 %). *Methanoculleus* and *Methanocorpusculum* (47.8 %) and no_rank_*Haloarchaeales* (19.6 %) were detected in SH-2, and XA comprised *Methanoculleus* (45.3 %), *Methanocorpusculum* (14.8 %), and no_rank_*Thermoplasmatales* (24.6 %).

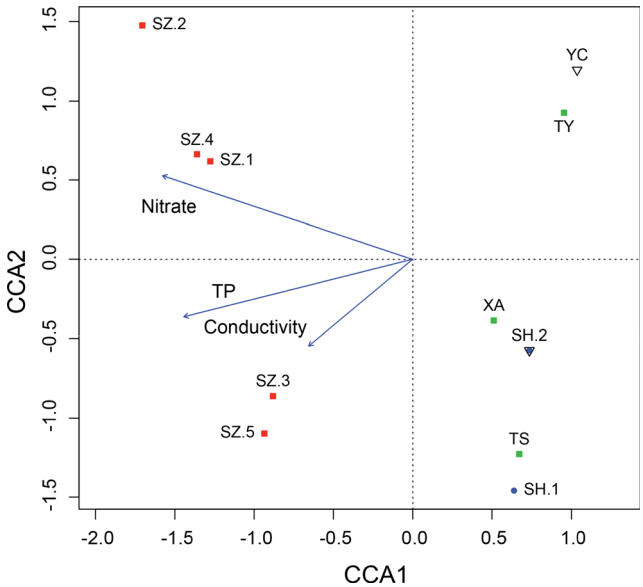

Fig. 2 The canonical correlation analysis (CCA) of the archaeal community structure based on the 16S rRNA gene sequences determined by the 454 sequencing analysis. The OTUs were used to calculate the archaeal structure based on the Euclidean distances, and the singletons were removed to eliminate bias

Notably, most of the sequences retrieved in this study were similar to those obtained from uncultured environmental clones (Table S3), indicating a vast unknown archaeal community in landfills.

Discussion

Refuse contains a high proportion of biodegradable fractions that undergo a complex series of biological and chemical reactions in landfills (Barlaz et al. 1989; Kjeldsen et al. 2002; Zhao et al. 2007). The microbial communities and physiochemical parameters are interdependent (Barlaz 1997; Barlaz et al. 1989). In the present study, we examined the physiochemical parameters and archaeal community structure and diversity in geographically distinct landfills.

Water chemistry

Although the environmental parameters varied among the sampling sites, general trends were observed. The values of the environmental parameters in each landfill leachate sample decreased with age. The values of the environmental parameters (COD, BOD_5, NH_3-N, conductivity, turbidity, TOC, TN, Cl^-, SO_4^{2-}, and NO_3^-) in young leachate samples (e.g., SH-1) were significantly higher than those in the aged leachate (e.g., SH-2) in SH and SZ landfills, reflecting continuous microbial biodegradation.

Among the physiochemical characteristics, the pH and BOD_5/COD ratio are essential indicators of refuse decomposition (initial aerobic phase (IAP), anaerobic phase (ANP), initial methanogenic phase (IMP), and stable methanogenic phase (SMP)) (Kjeldsen et al. 2002; Zhao et al. 2000). During IAP, which lasts a few days, the O_2 in the void space of the refuse is rapidly consumed, producing CO_2. After the O_2 is depleted, refuse decomposition shifts to ANP, in which hydrolytic, fermentative, and acetogenic bacteria decompose major biodegradable components, resulting in the accumulation of carboxylic acid, pH reduction (below 5.0), and an increased BOD_5/COD ratio (above 0.4). During IMP, the accumulated acid is converted to CH_4 and CO_2, and cellulose and hemicelluloses are hydrolyzed, thereby decreasing the COD and BOD_5 concentration and theBOD_5/COD ratio while increasing the pH. In SMP, methane production is dependent on the rate of cellulose and hemicellulose hydrolysis, and the BOD_5/COD ratio generally will decline below 0.1 as carboxylic acids are used as rapidly as they are produced. Meanwhile, the pH continues to increase to a steady-state value of approximately 8. In the present study, the measured BOD_5/COD ratio was less than 0.4 for all samples, and the pH values were between 7.7 and 8.3, suggesting that refuse decomposition occurred during the methanogenic phase.

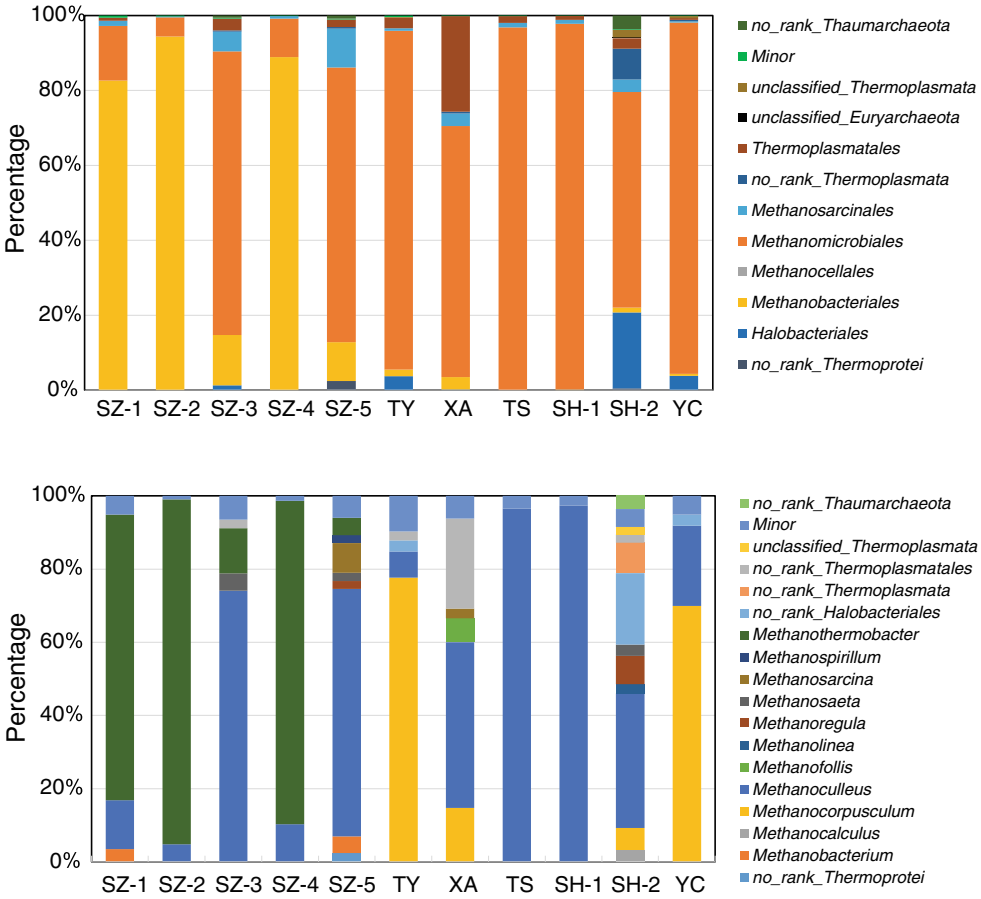

Fig. 3 Phylogenetic compositions represented as sequences from landfills (*upper panel*, archaeal order; *lower panel*, archaeal genera). The following landfill leachate samples are presented in each lane: SZ-1 to SZ-5, leachate from the Laohukeng landfill in Shenzhen; TY, leachate from the Houcun landfill in Taiyuan; XA, leachate from the Jiangxigou landfill in Xi'an; TS, leachate from the Tangshan landfill in Tangshan; SH, leachate from the Laogang landfill in Shanghai; and YC, leachate from the Yongchuan landfill in Yongchuan. The phylogenetic groups accounting for >0.50 % of the sequences are summarized in the artificial group as "others"

Refuse includes varying proportions of paper, plastic, metals, woods, food scraps and other components, and particle sizes within this agglomeration of materials range from millimeters to meters, forming heterogeneous niches (Barlaz 1997). In the present study, we observed unexpected high COD, BOD_5, and TN values in the 10-year-old SZ-4 biodegradation samples. Overall, the physiochemical results indicated a substantial difference between the different landfills and substantial changes during refuse decomposition within each landfill.

Archaeal community richness and diversity

Analyses of the tag sequence composition revealed that the archaeal diversity estimates for the landfill were 1–2 orders of magnitude greater than in the published analyses of any archaeal landfill community or landfill leachate (Chen et al. 2003; Huang et al. 2002, 2003; Krishnamurthi and Chakrabarti 2013; Laloui-Carpentier et al. 2006; Van Dyke and McCarthy 2002). Table 2 also indicates that the species diversity estimates obtained using ACE and Chao1 were greater than those in any published analyses of the archaeal communities in landfills and leachate samples (Chen et al. 2003; Huang et al. 2002, 2003; Krishnamurthi and Chakrabarti 2013; Laloui-Carpentier et al. 2006; Van Dyke and McCarthy 2002).

Archaeal community patterns and impact factors

Geographical location and environment heterogeneity are important factors in the structure of a microbial community. In this study, the archaeal community structure was found to be unique in each landfill leachate. Within one landfill, SZ, the archaeal community structure was separated. A possible reason for this separation is that the refuse involved is of various types (paper, plastic, metal, wood, food scraps, and other materials), with different particle sizes and forming heterogeneous niches (Barlaz 1997).

Environmental heterogeneity has long been hypothesized to be a very powerful factor in the structuring of a microbial community (McArthur et al. 1988). Langenheder and Ragnarsson (2007) found that environmental conditions played a major role in structuring a microbial community on a small spatial scale (<500 m). Logue and Lindstrom (2010) concluded that environmental habitat characteristics are more important than mass effects on shaping a microbial community structure. These results are consistent with those of previous studies and show that water chemistry affects a landfill

archaeal community. The nitrate concentration, TP, and conductivity affected the archaeal community structure.

TP is frequently correlated with microbial growth (Caron 1994; Downing et al. 1999), and the influence of TP on a microbial community has been demonstrated (Fisher et al. 2000). Similarly, TP concentrations from 1 to 19.0 mg/L recorded at different landfills would affect the archaeal communities of the various sites. Additionally, nitrate is an important nutrient for some microbiota. The nitrate content of soil is significantly correlated with archaeal *amoA* gene abundance (Shen et al. 2013). A landfill is an anaerobic environment in which ammonia-oxidizing archaea play an important role in the nitrogen cycle. Because the nitrate concentration varies among landfills, highest at SZ (1395.1 to 1721.4 mg/L) and lowest at SH (11.9 mg/L), it is hypothesized that ammonia affects oxidizing archaea. An effect on an archaeal community by conductivity has not been reported.

Archaeal community composition

Approximately 60 % of the CH_4 emissions to the atmosphere each year are attributed to human activity, and landfills are among the largest anthropogenic sources, accounting for approximately 10–19 % of global anthropogenic emissions (Czepiel et al. 2003). Phylogenetically diverse methanogenic archaea play a major role in methane production in landfills. Previous studies based on a 16S rRNA clone library suggested that the dominant methanogens belonged to the orders *Methanomicrobiales* and *Methanosarcinales* and the genera *Methanosaeta*, *Methanosarcina*, *Methanoculleus*, and *Methanothermobacter* (Chen et al. 2003; Huang et al. 2002, 2003; Krishnamurthi and Chakrabarti 2013). Consistently, in the present study, the detected dominant groups were *Methanomicrobiales* and *Methanobacteriales* (orders) and *Methanoculleus* and *Methanothermobacter* (genera).

Methanoculleus species have been detected in most molecular characterization studies of landfills (Huang et al. 2002, 2003; Krishnamurthi and Chakrabarti 2013; Uz et al. 2003) and other AD systems (Hori et al. 2006; Kröber et al. 2009; Nettmann et al. 2010). Regardless of the technique used, the number of detected *Methanoculleus* sequences was high, suggesting the significance of these microbes in methane production. *Methanothermobacter* are important thermophilic hydrogenotrophic methanogens, but these microbes are not frequently detected in landfills. To our knowledge, only one study has identified *Methanothermobacter* as the dominant species in diverse archaeal landfill communities (Chen et al. 2003). Interestingly, in the present study, we directly sampled the landfill refuse, rather than using leachate from the collection pipe, and we also excavated samples in situ. Little is known about the ecological role and syntrophy of *Methanothermobacter* with acetate-oxidizing bacteria. Representatives of *Methanocorpusculum* have seldom been detected in AD systems, and the ecological role of these microbes is unknown. After 1081 days in a running molasses wastewater anaerobic reactor, *Methanocorpusculum* were the dominant species with sulfate addition (3 g/L) and *Methanothermobacter* were the dominant group without sulfate addition, suggesting that sulfate might impact the structure of hydrogenotrophic methanogens (McHugh et al. 2004). The results of the present study provide the first evidence that *Methanocorpusculum* are important species in landfills.

Hydrogenotrophic methanogens were the predominant microbes in the 11 samples obtained from six geographically different landfills in this study, suggesting that hydrogenotrophic methanogens are primarily responsible for the observed methane production. This result contradicts the assumption that acetoclastic methanogenesis is the predominant pathway for methane formation (67 %) (Conrad 1999; Thauer et al. 2008). However, this observation could reflect acetate oxidation to CO_2/H_2 by syntrophic acetate-oxidizing bacteria (Ahring 1995). The oxidation of acetate to H_2 and CO_2 and subsequent conversion of these products to methane might represent a niche mechanism in anaerobic systems. Indeed, a combination of fluorescence in situ hybridization (FISH) and radiolabeled acetate analysis of samples obtained from 13 anaerobic reactors revealed that acetate oxidation was the predominant methanogenic pathway when *Methanosaetaceae* were not present (Karakashev et al. 2006). However, in the present study, although hydrogenotrophic methanogens were detected in all samples, acetoclastic *Methanosaetaceae* were also observed in samples with low sequence reads (2 to 601 sequences). The low acetoclastic *Methanosaetaceae* content might be responsible for the predominance of hydrogenotrophic methanogens (*Methanomicrobiales* and *Methanobacteriales*).

Microbial species that degrade acetate to H_2 and CO_2 in syntrophy with hydrogenotrophic methanogens such as *Clostridium* (Schnürer et al. 1996), *Thermotoga* (Balk et al. 2002), and *Acetobacterium* (Winter and Wolfe 1980) have been identified previously. It has been demonstrated recently that sulfate-reducing bacteria (SRB) (*Desulfovibrionales*, *Desulfobacterales*, and *Desulfomonadales*) oxidize acetate through the parallel reduction of sulfate during methanogenesis in decomposing refuse (Fairweather and Barlaz 1998). These authors reported extensive detection of *Clostridium* and SRB in the identical landfills and showed the important role of these bacteria in acetate oxidation. FISH analyses (Hori et al. 2006) demonstrated that *Methanoculleus* spp. were surrounded by rod-shaped bacteria, implying that these bacteria convert acetate into the basic substrates for hydrogenotrophic methanogenesis.

The studies of archaeal diversity in Chinese landfills (or landfill leachate) have demonstrated that these environments are dominated by hydrogenotrophic methanogens. *Methanothermobacter thermautotrophicus* comprised the

predominant microbes observed in refuse at depths of10, 20, and 30 m in a landfill in Taiwan (Chen et al. 2003). *Methanoculleus* dominated a closed landfill (Huang et al. 2003) and a landfill with leachate recirculation (Huang et al. 2002) in China. Cellulosic materials are the major biodegradable components of municipal solid waste (MSW) in developed countries (Barlaz 1997). The putrescible waste content in China is high, with high VFAs and a low pH (He et al. 2007). Acetoclastic methanogens are more sensitive to high VFA concentrations than are hydrogenotrophic methanogens (Conrad and Klose 2006), potentially reflecting the predominance of hydrogenotrophic methanogens in Chinese landfills.

In addition, the results obtained in the present study differ from those of several previous studies that have demonstrated that *Methanosaeta*, which belong to *Methanosarcinales*, are predominantly responsible for methane production in landfills (Laloui-Carpentier et al. 2006; State Environmental Protection Administration 2002). Although acetoclastic *Methanosaeta* have been strictly observed in landfill (leachate) samples, few studies have reported a predominance of these microbes in these environments. The characterization, using a 16S rDNA approach, of the archaeal communities in a landfill in France primarily receiving household refuse revealed that 65 % of the observed clones (190 of 292 clones) belonged to the *Methanosaetaceae* family (Laloui-Carpentier et al. 2006), likely reflecting the high content of cellulosic materials. The degradation of cellulosic material is kinetically limited by hydrolysis, resulting in low VFAs, in concentrations that promote the growth of acetoclastic methanogenic populations, such as *Methanosaeta* (Barlaz 2006). Similar results have been reported for low-acetate environments, such as rice paddies (Fey and Conrad 2000) and anaerobic waste digesters (Griffin et al. 1998). In the present study, *Methanosaeta* were observed in three samples (SZ-3, SZ-5, and SH-2) with absolutely low abundance (vs hydrogenotrophic methanogens).

Hydrogenotrophic methanogens exhibit a much higher tolerance to high temperature and stressed reactor conditions (e.g., ammonia, sulfate, and VFA) than acetoclastics (Pender et al. 2004; Schnürer et al. 1999). Thermophilic conditions may also favor the growth of rod-like or coccoid hydrogenotrophic methanogens. Fey and Conrad (2000) used the stable carbon isotope signatures of CO_2 to quantify the relative contribution of acetotrophic and hydrogenotrophic pathways and observed that higher temperatures increased the formation of methane rather than acetate from H_2/CO_2. Investigation of the methanogen population dynamics of an AD used to treat cattle manure in a laboratory-scale experiment revealed that hydrogenotrophic methanogens were the only microbial group, with higher specific methanogenic activity and unchanged cell numbers at 65 °C compared with 55 °C, whereas the activity and amounts of other methanogens were significantly reduced (Ahring et al. 2001). Cetotrophic *Methanosaetaceae* is frequently detected in AD systems under mesophilic temperatures (Laloui-Carpentier et al. 2006; McHugh et al. 2003). In the present study, hydrogenotrophic methanogens were the predominant species observed in landfills. Landfills could provide thermophilic conditions for hydrogenotrophic methanogens, reflecting the fast fermentation of refuse upon landfill initiation. Similar results were obtained using samples from a landfill in Taiwan (Chen et al. 2003) and in thermophilic biogas plants (Hori et al. 2006). High ammonium concentrations may inhibit acetate-utilizing methanogens, and homoacetogenic bacterium could subsequently convert hydrogen and carbon dioxide to methane (Schnürer et al. 1994, 1996). In the present study, the ammonia concentration ranged from 44.7 to 566.2 mg/L, much lower than the reported acetate-utilizing methanogen concentration (1.1 g/L).

A dynamic transition of the methanogenic population occurs during methane production. A distinct shift in the population structure from acetate-utilizing methanogens to hydrogen-utilizing methanogens was observed in a molasses wastewater anaerobic reactor, in which *Methanosaeta* were initially the dominant microbes and *Methanothermobacter* became dominant as the temperature increased (Pender et al. 2004). A shift in the population structure of hydrogen-utilizing methanogens was also observed. A clear shift between *Methanothermobacter* and *Methanoculleus* was observed in thermophilic methanogenic bioreactors (Hori et al. 2006). *Methanothermobacter* sp. grew favorably while *Methanoculleus* sp. began to decline when the propionate concentration increased from 0.3 to 1.4 mmol/L. Low hydrogen concentrations favor the growth of *Methanoculleus* spp. Over *Methanothermobacter* spp. The difference in hydrogen affinity between these two hydrogenotrophic methanogens will influence the specific relationship of these microbes with distinct partner bacteria. The morphotype diversity of methanogens also changes in response to changes in temperature, leading to the emergence of a morphologically mixed methanogenic population after a temperature shift from 60 to 55 °C (Krakat et al. 2010). However, few studies have described the dynamic transition of the methanogenic populations in landfills. Although the methane production pathway is unknown, experiments monitoring changes in $\delta^{13}CH_4$ and the analysis of the archaeal community structure during the mesophilic fermentation of municipal solid waste demonstrated that methanogenic metabolism changes with increasing incubation times (Qu et al. 2009). The composition of the methanogenic population in landfills differing in age by 2 and 4 years has also been characterized (Zhao et al. 2007). rDNA analysis revealed a rich assemblage of methanogens in both samples, including acetoclasts and H_2/CO_2- and formate-utilizing bacteria in the younger samples and H_2/CO_2- and formate-utilizing bacteria in the older samples. In the present study, we also compared the methanogenic populations in samples of different ages. In the SH landfill,

Methanomicrobiales accounted for 97.6 % of the total archaeal population in the 0.4-year-old samples and decreased to 57.6 % in the 3-year-old sample. By contrast, *Methanomicrobiales* and *Methanobacteriales* absolutely dominated all samples from the SZ landfill. Notably, the degradation ages of the refuse in the SZ landfills were all older than 4 years. The initiation of methanogenesis in refuse occurs at a high VFAs concentration and a low pH (5.5 to 6.25), which has been reported to inhibit methanogens (Barlaz 1997). VFA accumulation occurs in the anaerobic phase, during which hydrolytic, fermentative, and acetogenic bacteria decompose major biodegradable components (Kjeldsen et al. 2002). However, the physiochemical parameters of landfill leachate samples, particularly the pH of 7.7 to 8.4 and the BOD_5/COD ratio of 0.02 to 0.39, suggested that the landfills were maintained in the methanogenic phase. Thus, methanogen succession may occur during the refuse decomposition phase, particularly during phase transitions, such as from the anaerobic to the methanogenic phase. Therefore, refuse decomposition phases must be investigated further to obtain a better understanding of the dynamics of methanogen structure in landfills.

Previous studies based on small-subunit rRNA clone libraries have indicated that the limited number of clones sequenced does not reflect the entire profile of archaeal communities in landfills (Chen et al. 2003; Huang et al. 2002, 2003; Krishnamurthi and Chakrabarti 2013; Laloui-Carpentier et al. 2006; Van Dyke and McCarthy 2002). In this study, pyrosequencing revealed that archaeal communities in landfills are much more diverse than those reported in previous comprehensive surveys. Additionally, the results indicated that hydrogenotrophic methanogen is dominant in landfills. Clear geographical differences among the samples, based on OTU abundance, were revealed. The CCA suggested that the nitrate concentration, TP, and conductivity significantly affect the archaeal community structure. This study presents the first 454 deep sequencing surveys of archaeal communities in municipal solid waste sites and provides an overview of the richness and variability of this community in this ecological niche.

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