View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Elsevier - Publisher Connector

Bioresource Technology 119 (2012) 373–383

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor from start-up to steady-state conditions

Juliana Cardinali-Rezende [a], Luís F.D.B. Colturato [b], Thiago D.B. Colturato [b], Edmar Chartone-Souza [a], Andréa M.A. Nascimento [a,*], José L. Sanz [c]

[a] *Departamento de Biologia Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, Belo Horizonte, MG 31.270-901, Brazil*
[b] *Methanum Environmental and Energy Solutions, Belo Horizonte, Minas Gerais, Brazil*
[c] *Department of Molecular Biology, Universidad Autónoma de Madrid, c/Darwin 2, Madrid 28049, Spain*

HIGHLIGHTS

- ▶ We identify and quantify microbiota of a full-scale MSW anaerobic reactor.
- ▶ Shifts in the diversity and abundance were observed from start-up to steady-state.
- ▶ Hydrogenotrophic methanogens dominated the methane production in the MSW reactor.
- ▶ *Methanosarcina*, *Methanimicrococcus* and *Methanosaeta* were identified in the reactor.
- ▶ Quantification of bacterial and archaeal by FISH and qPCR differ.

ARTICLE INFO

Article history:
Received 1 March 2012
Received in revised form 27 May 2012
Accepted 28 May 2012
Available online 9 June 2012

Keywords:
Municipal solid waste anaerobic reactor
16S rDNA clone library
Real-time PCR
DGGE
FISH/CARD-FISH

ABSTRACT

The prokaryotic diversity of an anaerobic reactor for the treatment of municipal solid waste was investigated over the course of 2 years with the use of 16S rDNA-targeted molecular approaches. The fermentative *Bacteroidetes* and *Firmicutes* predominated, and *Proteobacteria*, *Actinobacteria*, *Tenericutes* and the candidate division WWE1 were also identified. Methane production was dominated by the hydrogenotrophic *Methanomicrobiales* (*Methanoculleus* sp.) and their syntrophic association with acetate-utilizing and propionate-oxidizing bacteria. qPCR demonstrated the predominance of the hydrogenotrophic over aceticlastic *Methanosarcinaceae* (*Methanosarcina* sp. and *Methanimicrococcus* sp.), and *Methanosaetaceae* (*Methanosaeta* sp.) were measured in low numbers in the reactor. According to the FISH and CARD-FISH analyses, *Bacteria* and *Archaea* accounted for 85% and 15% of the cells, respectively. Different cell counts for these domains were obtained by qPCR versus FISH analyses. The use of several molecular tools increases our knowledge of the prokaryotic community dynamics from start-up to steady-state conditions in a full-scale MSW reactor.

© 2012 Elsevier Ltd. All rights reserved.

1. Introduction

The environmentally friendly management of municipal solid waste (MSW) has become a global challenge because of limited resources, an ever-increasing population, rapid urbanization and industrialization. An enormous quantity of MSW is produced daily in the European Union (400,000 tons). However, the separate collection of MSW fractions has increased significantly, and the biomethanization (anaerobic digestion, AD) of the organic fraction (OF) of the MSW has become an effective solution (Mata-Alvarez et al., 2000).

Among various biological treatments, the AD of OF-MSW in anaerobic digesters is frequently the most cost-effective. This process results in the reduction of the organic content of the waste, the production of an energy-rich biogas (CH_4, CO_2, and traces of N_2, H_2S and O_2) and a solid residue with a high nutrient content, which can be recycled to recuperate degraded environments. The potential of biogas production throughout Europe could reach in 2020 77.9 billion m^3 of methane, being 10 billion m^3 from biodegradable fraction from municipal solid wastes. (http://www.aebiom.org/IMG/pdf/Brochure_BiogasRoadmap_WEB.pdf).

In 1995, the first biomethanization plant for MSW began operation in Europe. Since then, many plants have opened in countries where the collection of MSW has been established, such as Spain, Germany, France, Denmark, and Belgium (IEA, 2003). Approximately 15% of the OF-MSW is biologically treated in Europe, and AD represents approximately 20% of all biological treatment capacity (http://www.waste-management-world.com). Spain produces 26.2 million tons of MSW

* Corresponding author.
E-mail address: amaral@ufmg.br (A.M.A. Nascimento).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.05.136

per year, which amounts to 1.7 kg for person per day (INE, 2009). A total of 24 MSW biomethanization plants are in operation in this country, which includes seven close to Madrid. Recently, two plants commenced operation in Valdemingómez Technology Park (Las Dehesas), with five anaerobic digesters, and in Paloma, both of which are under the responsibility of the municipality of Madrid. These plants are considered the largest and most modern in Europe and are an international model for waste treatment. It is estimated that both plants will be responsible for the processing of 370,000 tons of MSW and the production of 34 million m^3 of biogas per year, which will reduce the use of other energy sources (http://www.madridiario.es/2008/Noviembre/medioambiente/gasnatural/118868/plantsbiometanizacion-Valdemingómez-empiezan-rodaje.html). The concomitant production of electricity, heat and biogas from biomass is an environmentally and economically attractive option.

Previous culture-independent studies of the microbial communities in pilot-scale anaerobic reactors for the treatment of OF-MSW have been performed. Tang et al. (2004) comparing the communities from a thermophilic MSW digester without and with micro-aeration, using complementary molecular techniques, observed that *Firmicutes* dominated and *Methanosarcina* decreased while *Methanoculleus* increased as a result of micro-aerations. Nayak et al. (2009) showed temporal shifts in the archaeal and bacterial community by (DGGE) and the predominance of *Methanosarcinales* and *Methanomicrobiales* by *mcrA* gene libraries. Cardinali-Rezende et al. (2009) studying the microbial community of the MSW before, during and after the AD into a mesophilic reactor, by 16S rRNA and *mcrA* genes clone libraries, observed that *Firmicutes*, *Proteobacteria*, and *Bacteroidetes* were represented in all libraries, and only hydrogenotrophic *Methanomicrobiales* (*Methanoculleus* genus) and *Methanobacteriales* (*Methanosphaera* and *Methanobacterium* genera) were identified. The same was observed by Sasaki et al. (2011) in a thermophilic digester, where *Methanoculleus* sp. predominated. Supaphol et al. (2011) observed in the mesophilic anaerobic co-digestion of mixed waste the predominance of *Firmicutes*, *Actinobacteria* and *Proteobacteria* and the shifts of predominance of hydrogenotrophic to aceticlastic methanogens (*Methanosaeta*) from early to end stages of the AD. Bertin et al. (2012) using DGGE analysis in a mesophilic continuous anaerobic reactor fed initially with cattle manure and later with OF-MSW, also observed that *Firmicutes* and *Bacteroidetes* predominated. However, in contrast to the earlier works only two aceticlastic *Methanosarcina* sp. were identified.

Despite the increasing number of full-scale MSW plants in Europe, the prokaryotic composition and dynamics of a full-scale MSW anaerobic digester have been scarcely explored. The performance of AD is linked closely to the structure of the digester's microbial community, and an investigation of its prokaryotic diversity can therefore provide relevant insights. In this study, we sought to illuminate the bacterial and archaeal community dynamics of a full-scale MSW anaerobic digester (Las Dehesas Biomethanization Plant, Madrid) from the start of operations (start-up) to steady-state conditions. To achieve this goal, we applied molecular approaches such as denaturing gradient gel electrophoresis (DGGE), amplified ribosomal DNA restriction analysis (ARDRA), real-time quantitative polymerase chain reaction (qPCR), fluorescence in situ hybridization (FISH) and catalyzed reporter deposition (CARD)-FISH.

2. Methods

2.1. Reactor conditions

A full-scale, 12-m anaerobic digester for the treatment of OF-MSW commenced operation in February 2009 at the Las Dehesas Biomethanization Plant (Madrid, Spain). The reactor operates at 35 °C, an approximate pH of 7 and a total concentration of 30–40% solids (w/v). The average residence time is 100 days when the reactor is filled. The biogas is extracted from the top of the reactor and injected into the bottom to promote mixing in the reactor tank. Approximately 50–100 tons of fresh OF-MSW is added daily. $CaCO_3$ and water, together with the steam necessary to raise the temperature to approximately 35 °C, are added as needed.

2.2. Sampling and chemical analysis

The digested MSW (DMSW) samples were aseptically extracted from the digester with bottles. The samples were collected on May 21, 2009 (DMSW1), October 22, 2009 (DMSW2), February 18, 2010 (DMSW3), and May 11, 2011 (DMSW4). The first three samples were extracted during the start-up phase of the reactor, which took two years. The first sampling (DMSW1) was performed about one year after the beginning of the operation of the reactor, when 50% of the reactor was filled. The last sample was removed under steady-state conditions while the reactor was completely filled with OF-MSW.

The samples were analyzed for temperature, pH, alkalinity, ammonia content, total suspended solids (TSS) and volatile suspended solids (VSS) according to the Standards Protocols (APHA, 2005). Alkalinity, ammonia content, TSS and VSS were analyzed according to the method numbers 2320B, 2130B, 2540D and 2540E, respectively. The volatile fatty acids (VFAs) were measured by gas chromatography (Varian STAR 3400 CX) using FFAP capillary column (split ratio 1:40, temperature of the column, injector and detector: 250, 140 and 250 °C, respectively).

The general scheme of the experimental design is depicted in Fig. 1. Detailed methodology is described below.

2.3. Pretreatment of the DMSW samples and the total DNA extraction

The samples were pretreated to obtain intact DNA. For this process, 0.91 g of DMSW (wet weight) was resuspended in 10 mL of 0.5 M EDTA (Disodium Ethylenediaminetetraacetate), pH 8.0 and the suspension was stirred at 4 °C for 1 h according to Sánchez-Andrea et al. (2011). Next, the samples were centrifuged (10,000 rpm in an Eppendorf 5430 for 1 h), and the sediments were again resuspended in EDTA by vortexing. The previous steps were repeated twice, and the pellet was resuspended in 10 mL of EDTA. A 1-mL aliquot of the suspension was sonicated with five 30-s cycles (LABSONIC M; Sartorius Stedim Biotech) and allowed to settle for 1 h at 4 °C. The supernatant (900 μL) was centrifuged for 15 min at 12,000 rpm. The supernatant was discarded, and the pellet was resuspended in 1 mL of a 1:1 solution of 0.9% NaCl and 50 mM EDTA, pH 8.0, and stirred overnight at 4 °C according to Sánchez-Andrea et al. (2011). The total DNA of all of the samples was extracted using the FastDNA SPIN kit for soils (Bio 101) according to the manufacturer's instructions. For real-time PCR, the DNA from the DMSW1, DMSW2 and DMSW4 samples was extracted simultaneously.

2.4. PCR amplification of 16S rRNA genes

The 16S rRNA genes of the Bacteria and Archaea domain from the DMSW samples were PCR amplified for cloning (samples DMSW1, 2 and 4) and for DGGE (samples DMSW1, 2 and 3) analyses. The primers and PCR conditions used are listed in Table S1. For the DGGE analysis, the partial bacterial and archaeal 16S rDNA amplicons were re-amplified with the same primer pairs (with a GC-clamp in the 341F and 622F primers).

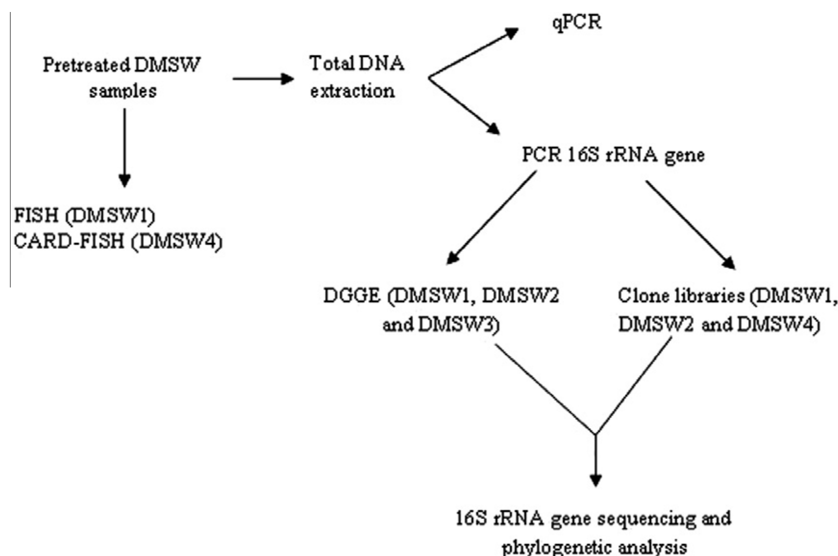

Fig. 1. Flow chart of experimental methods.

2.5. Denaturing gradient gel electrophoresis (DGGE)

PCR products of the bacterial and archaeal 16S rDNA fragments from the DMSW1, DMSW2 and DMSW3 samples were separated by DGGE (DcodeSystem, BioRad, Germany). The 6% polyacrylamide gels (w/v, 37.5:1 acrylamide and bisacrylamide) were prepared with denaturing gradients that ranged from 30% to 60% (in which the 100% denaturant contained 7 M urea and 40% v/v formamide) and were run at 60 °C and 80 V for 15 h. The bands of interest were excised, and their DNA was re-amplified by PCR for sequencing according to the PCR conditions described (Table S1).

2.6. Clustering analysis from the DGGE patterns

For the clustering analysis, the profiles of the bacterial (B1, B2 and B3) and archaeal (A1, A2 and A3) 16S rDNA fragments, which corresponded to the DMSW1, 2 and 3 samples, were converted into a binary matrix in which the digit 1 represented the presence of the band and the digit 0, its absence. The similarity matrix was generated by Euclidean distance, which was used to construct the dendrogram with the UPGMA algorithm (Ryan et al., 1995). The data analysis was performed using PAST (Paleontological Statistics Software Package) (Hammer et al., 2001).

2.7. Libraries and ARDRA analysis

For a further comparison between the DMSW samples, clone libraries were constructed from the DMSW1, DMSW2 and DMSW4 samples and analyzed. The sizes of the amplicons (1465 and 1467 bp for the bacteria and archaea, respectively) were confirmed by 0.8% (w/v) agarose gel electrophoresis. The bands were excised, and the DNA was purified from the gel slices using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare UK Limited). For cloning, the purified amplicons were cloned into the pGEM®-T Vector (Promega) according to the manufacturer's instructions and transformed into chemically competent *Escherichia coli* DH5α. Plasmids from the clones that contained the 16S rDNA inserts were extracted using a standard alkaline lysis method (Sambrook et al., 1989). The plasmid inserts were screened by ARDRA with the restriction endonuclease *Bfu*Cl (New England BioLabs Inc., New England) according to the manufacturer's instructions. The fragments were separated by 2.5% (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining (0.5 μg/mL). The clones were grouped according to their restriction patterns, which defined the different operational taxonomic units (OTUs). Two clones of each OTU were subsequently sequenced.

2.7.1. 16S rRNA gene sequencing and phylogenetic analysis

Sequencing of the bacterial and archaeal 16S rDNA from the clone libraries (BL1, BL2 and BL4 for the bacteria and AL1, AL2 and AL4 for the archaea) and of the bacterial and archaeal DGGE bands was performed in an ABI model 377 sequencer (Applied Biosystems) using standard protocols. All of the sequences were compared with sequences in the Ribosomal Database Project (RDP) using Library Compare and the sequences in GenBank using BLASTN. Prior to these comparisons, the 16S rRNA gene sequences were base-called, checked for quality, aligned, and analyzed with Phred v.0.020425, Phrap v.0.990319 and Consed 12.0. RDP's CHECK-CHIMERA program and VecScreen program (NCBI) were used to detect chimeric DNAs and vectors, respectively. The phylogenetic relationships were inferred by MEGA 4.1 using the neighbor-joining method and Kimura's 2-P model of sequence evolution. The nucleotide sequences generated in this study were deposited in GenBank under the accession numbers JX101959-102021 for the 16S rRNA gene sequences and JX102022-102039 for the DGGE bands.

2.8. Primer design for quantitative real-time PCR (qPCR)

The 16S rRNA gene sequences specific for the methanogenic archaea *Methanosaeta* sp., *Methanosarcina* sp., *Methanobacteriales* and *Methanomicrobiales* were retrieved from the NCBI database and aligned automatically using the CAP3 Sequence Assembly Program (http://pbil.univ-lyon1.fr/cap3.php) to form a contig. Primers specific for each group (Table S1) were designed using the Universal Probe Library Assay Design Center (Roche Applied Science – https://www.roche-applied-science.com). The primer specificity was tested using the GenBank BLASTN database and qPCR, which consisted of observing the melting and amplification curves.

2.9. Standard DNA for qPCR analysis

A pool of the total DNA that was extracted from the different samples (DMSW1, DMSW2 and DMSW4) was used to establish

absolute quantification standards. The pooled DNA was amplified using conventional PCR in a Mini-cycler™ PTC-100 (MJ Research Inc., Waltham, MA). The specific primers for the bacteria, archaea and methanogenic archaea and the PCR conditions are listed in Table S1. The amplicons were purified using 20% polyethylene glycol (PEG 20%) prepared in 5 M NaCl (Sambrook et al., 1989). The DNA concentration was determined using a Nanodrop ND-1000 spectrophotometer. The 16S rDNA copy number for each group was calculated per amplicon by the URI Genomics & Sequencing Center (http://www.uri.edu/research/gsc/resources/cndna.html). The standards were diluted in nuclease-free water and stored in single-use aliquots at −80 °C. An 8.10-fold serial dilution of the standards (in triplicate) were used for qPCR to generate the standard curve.

2.10. qPCR

Quantitative standard curves were constructed for the following groups: the *Bacteria* and *Archaea* domains and the methanogenic archaea *Methanomicrobiales*, *Methanobacteriales*, *Methanosarcina* sp. and *Methanosaeta* sp. The real-time PCR was conducted in an ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA). The reactions, at a final volume of 10 μL, consisted of 5 μL of SYBR Green PCR master mix (QIAGEN, USA), 0.5 μL of each primer (2.5 mM), 1.0 μL of the template (total DNA or dilutions for the standard curve) and sterile H_2O. The quantitative measurement of the samples using real-time PCR was performed in triplicate together with the standards (that were specific for each microbial group) to generate a standard curve. The real-time PCR amplification was performed with 40 cycles of denaturation (20 s at 95 °C), annealing (20 s at 60 °C), and elongation (120 s at 72 °C). The primer set used for measuring the 16S rRNA copy number of the *Bacteria* and *Archaea* domain, *Methanobacteriales*, *Methanomicrobiales*, *Methanosarcina* sp. and *Methanosaeta* sp. is listed in Table S1. The results were analyzed in an ABI PRISM 7900HT SDS (Applied Biosystems, Foster City, CA, USA). The absolute quantitative analysis of the bacterial, archaeal and methanogenic archaeal communities was obtained, and the number of cells per gram of the DMSW samples for each microbial group was calculated considering 4 and 2.5 copies of the 16S rRNA gene per cell bacteria/archaea and methanogenic archaea, respectively (Klamppenbach et al., 2001).

2.11. Fixation of the DMSW samples and standardization of the cell concentrations for FISH

The DMSW1 and DMSW4 samples were collected from the digester and immediately fixed in 4% paraformaldehyde in 1X phosphate-buffered saline solution (PBS) (130 mM NaCl and 10 mM Na_2PO_4/NaH_2PO_4, pH 7.2–7.4) for 4 h at 4 °C for Gram-negative bacteria. The samples were then washed in 1X PBS and centrifuged, and 2.9 g of the sample was re-suspended in 8.5 mL of a 1:1 solution of 1X PBS and ethanol and stored at −20 °C.

2.12. FISH and CARD-FISH analyses

The hybridizations were performed for the DMSW samples following the protocol described for FISH (Amann et al., 1990) and CARD-FISH (Pernthaler et al., 2002). The probes used in this work are listed in Table S1. The oligonucleotide probes were labeled with the cyanin dye Cy3 for the FISH and horseradish peroxidase HRP (Alexa488) for the CARD-FISH analyses. *E. coli* ATCC 12435, *Methanosaeta concilli* DSM2139 and DSM 863 were used as positive controls, and the NON338 *Methanobacterium bryantii* probe was used as a negative control. The total cells present in the samples were enumerated by direct counting of 4′,6′-diaminphenylindol- (DAPI, 1 mg/mL) stained cells when possible. A total of 20 fields for each sample were examined for each probe under a Zeiss Axiovert 200 microscope. The number of microbial cells was calculated and converted to cells per gram of sample.

3. Results and discussion

3.1. Physicochemical characterization of the MSW and their digested samples

The start-up phase of the reactor persisted for almost 2 years. The characteristics of the feed (MSW) and the digested (DMSW) materials are listed in Table 1. Although differences in the TSS and VSS contents of the digested samples can be ascribed to the heterogeneity of the feed material, there was a significant reduction of TSS (50–62%) and VSS (67–71%) during the treatment, which indicates an efficient degradation of organic matter in the anaerobic reactor. The increase in the concentrations of VFA (acetate, propionate and butyrate) with time throughout the study period is likely due to the higher acidogenesis rate versus methanogenesis (the feed material is added daily and includes rapidly degraded carbohydrates). Despite the high VFA amounts detected, the pH values were maintained between 7 and 8 due to $CaCO_3$ addition and ammonium released during the MSW treatment. The average concentration of CH_4 in the biogas ranged from 57% to 60% and is a good indicator of the stability of the process. The production of H_2S was not detected in the reactor, which permits use of the biogas without pre-treatment in engines. Although ammonia was generated in the reactor, its toxicity was overcome via the recirculation of ambient, air-dried biogas back into the reactor. Moreover, the high TSS concentration, which ranged from 19% to 24% (Table 1), diluted the ammonia concentration, which was also observed by Jewell et al. (1999).

3.2. Bacterial and archaeal 16S rRNA gene analysis

To determine the biodiversity of the prokaryotic communities in the MSW digester, the total DNA from the DMSW1, DMSW2 and DMSW4 samples were used to generate six clone libraries. To avoid sequencing clones with identical 16S rRNA genes, the insert-containing plasmids were digested with the restriction enzyme *BfuCl* to generate ARDRA patterns. OTUs were defined as a unique ARDRA pattern. A total of 89 different patterns were generated and sequenced. The chimeras were removed and 64 (48 of bacteria and 16 of archaea) OTUs were phylogenetically analyzed.

The library coverages (82–91% and 94–98% for the bacteria and archaea, respectively) suggest that the number of analyzed OTUs was satisfactory and that most bacteria and archaea present in the samples were detected. The majority of the OTUs displayed relationships with environmental sequences from various uncultured bacterial clones from municipal wastewater, swine waste and solid waste anaerobic digesters (Tables 2–4). The OTU sequences displayed similarity to sequences deposited in the RDPII and NCBI databases, and few sequences were identified at the species level (>97% similarity). These results are in agreement with Martín-González et al. (2011), who also reported that most of bacterial 16S rRNA gene sequences were similar to those of uncultured clones.

In this study, the number of clones selected by ARDRA and analyzed in each clone library was not sufficient to describe the total microbial diversity in detail. However, these results were confirmed by DGGE analysis and combined with qPCR, FISH and CARD-FISH, which accurately reflects the diversity and dynamics of the microbial communities during the operation of this reactor.

Table 1
Physical–chemical analysis of municipal solid waste (MSW) and their digested samples (DMSW) from anaerobic reactor in different periods.

Parameters*	MSW (Average)	Samples			
		DMSW1 05/2009	DMSW2 10/2009	DMSW3 02/2010	DMSW4 05/2011
TSS (%)	48.1	23.6	21.3	18.0	23.7
VSS (%)	28.8	9.0	8.4	9.5	8.7
pH	NA	7.7	7.7	7.9	7.5
Temperature (°C)	NA	35	31	37	35
Ammonium (mg/l)	NA	NA	4139	4760	4460
Conductivity (μS/cm)	NA	NA	49.0	49.3	48.0
VFAs (mg/l)	NA	449	628	961	7184
Acetate (mg/l)	NA	415	555	759	4100
Propionate (mg/l)	NA	34	73	202	2636
Butyrate (mg/l)	NA	NA	NA	NA	448
Alcalinity (mg-$CaCO_3$/l)	NA	14.2	17.1	17.6	17.6
CH_4 (% in biogas)	NA	57	60	60	58

* Municipal solid waste to be treated (MSW); Digested of municipal solid waste treated in different periods of the treatment: start-up (DMSW1; DMSW2; and DMSW3) and stable (DMSW4) conditions, collected inside of the reactor; Total suspended solids (TSS), volatile suspended solids (VSS), Volatile fatty acids (VFAs), NA, not analyzed.

Table 2
Phylogenetic filiations and distribution of bacterial and archaeal clones analyzed from digested municipal solid waste 1 (DMSW1).

Phylogenetic group	OTU	Clon No.	Closest sequence/microorganism	Order/Family	Acession No.	Identity (%)	Habitat of closest relative
Bacteroidetes	BL1-1	1	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919517	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	2	25	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919514	98	Thermophilic biogas reactor fed with renewable biomass
	3	2	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	FN436026	98	Thermophilic biogas reactor fed with renewable biomass
	4	5	Uncultured bacterium clone 01a03	*Porphyromonadaceae*	GQ138680	99	ASBR reactor treating swine waste
	5	1	Uncultured bacterium clone HAW-R60-B-609d-C	*Porphyromonadaceae*	FN436026	95	Thermophilic biogas reactor fed with renewable biomass
	6	1	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919067	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	7	1	Uncultured Bacteroidetes bacterium	*Porphyromonadaceae*	CU919667	97	Mesophilic anaerobic digester which treats municipal wastewater sludge
Firmicutes-Clostridia	8	3	Uncultured bacterium clone 02f07	*Peptostreptococcaceae*	GQ138525	92	ASBR reactor treating swine waste
	9	1	Uncultured bacterium clone PISD-AIB	*Incertae Sedis*	AM982570	99	Pig saw dust spent bedding
	10	1	Uncultured bacterium clone G35-D8-L-F	*Clostridiales*	EF559144	99	Mesophilic anaerobic digester at 35 degrees Celsius
	11	1	Uncultured *Symbiobacterium* sp.	*Incertae Sedis*	EU639305	99	Thermophilic microbial fuel cell time zero control
	12	1	Uncultured Firmicutes bacterium	*Clostridiales*	CU921622	90	Mesophilic anaerobic digester which treats municipal Wastewater sludge
Firmicutes-Bacilli	13	1	Uncultured bacterium clone		AM982570	99	Pig saw dust spent bedding
Gammaproteobacteria	14	6	*Pseudomonas* sp.	*Pseudomonadaceae*	AY954288	99	Anaerobic digestive reactor of waste water reatment plant
Actinobacteria	15	1	*Actinomyces europaeus*	*Actinomycineae*	NR026363	98	Strain CCUG 32789A
	16	2	*Actinomyces europaeus*	*Actinomycineae*	AM084230	97	Isolate C Strain CUG 32789AT
Euryarchaeota							
Methanosarcinales	AL1-1	4	Uncultured *Methanosarcina barkeri*	*Methanosarcinaceae*	EU857627	97	Nisargruna Biogas Plant
	2	5	*Methanosarcina barkeri*	*Methanosarcinaceae*	AF028692	97	Ricefield soils
Methanomicrobiales	3	38	*Methanoculleus* sp.	*Methanomicrobiaceae*	AJ550158	99	Rumen
	4	1	*Methanoculleus bourgensis*	*Methanomicrobiaceae*	AF095269	99	Strain: MS2
	5	27	*Methanoculleus bourgensis*	*Methanomicrobiaceae*	AB065298	99	Strain:DSM 6216

3.3. Bacterial diversity

A phylogenetic analysis of the bacterial 16S rDNA sequences from the clone libraries revealed that the bacterial composition changed during the study period. The following bacterial phyla were identified in the reactor: *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Actinobacteria*, *Tenericutes* and the candidate division WWE1 (Fig. 2 and Tables 2–4). According to Nelson et al. (2011), using a meta-analysis approach, the majority of the bacterial communities in anaerobic digesters were classified within four phyla (*Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Chloroflexi*), and *Actinobacteria* was identified as a 'minor' phylum.

The *Bacteroidetes* and *Firmicutes* phyla contained most of the OTUs (85–95%) identified in all of the samples (Fig. 2). *Bacteroidetes* was represented by the *Porphyromonadaceae* family and accounted for 68% of the sequences in DMSW1, 63% in DMSW2 and 84% in DMSW4. Species from the *Bacteroidetes* phylum are acidogenic, sugar-fermenting, saccharolytic and proteolytic bacteria that produce propionate, acetate and succinate as their primary products. The *Firmicutes* phylum was represented by the *Clostridia* and *Bacilli* classes. The OTUs affiliated with the *Clostridia* class were represented in all of the samples and accounted for 13.5% of the DMSW1 clones, 32% of DMSW2 and 10% of DMSW4 (Fig.2). The sequences from the *Peptostreptococcaceae*, *Clostridiaceae*, and *Ruminococcaceae* families, as well as Incertae sedis, were present, being some of these sequences similar at the genus level (>95%) to *Symbiobacterium* sp., *Clostridium* sp. and *Tissierella* sp. (Tables 2–4). *Ruminococcaceae* and *Clostridiaceae* families are represented by

Table 3
Phylogenetic filiations and distribution of bacterial and archaeal clones analyzed from from digested municipal solid waste 2 (DMSW2).

Phylogenetic group	OTU	Clon No.	Closest sequence/microorganism	Order/Family	Acession No.	Identity (%)	Habitat of closest relative
Bacteroidetes	BL2-1	2	Uncultured *Alkaliflexus* sp.	*Marinilabiaceae*	EU887836	84	Aerobic predigester
	2	6	Uncultured *Bacteroidetes* bacterium	*Porphyromonadaceae*	CU919914	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	3	2	Uncultured bacterium clone PeH15		AJ576333	95	Hindgut homogenate of Pachnoda ephippiata larva
	4	1	Uncultured eubacterium clone LKB108		AJ746506	98	Landfill leachate
	5	26	Uncultured bacterium clone TE-3-E11		JQ337397	99	Composting sample at 12 days
	6	1	Uncultured eubacterium clone LKB106		AJ746505	99	Landfill leachate
	7	2	Uncultured eubacterium clone LKB104		AJ746504	99	Landfill leachate
	8	13	Uncultured *Bacteroidetes* bacterium		CU919517	98	Mesophilic anaerobic digester which treats municipal wastewater sludge
Firmicutes-Clostridia	9	1	Uncultured bacterium clone 03d07	*Incertae Sedis*	GQ134018	98	ASBR reactor treating swine waste
	10	1	Uncultured *Tissierella* sp.	*Incertae Sedis*	GU112188	95	Biogas slurry derived from anaerobic fermentation of pig manure
	11	2	Uncultured bacterium clone LL143-7E10		FJ671370	94	MARC beef cattle feedlot
	12	1	*Clostridium colinum* DSM6011	*Clostridiaceae*	NR026151	91	Strain DSM:6011
	13	1	Uncultured *Clostridium* sp.	*Clostridiaceae*	AB231801	97	Cellulose enrichment culture
	14	2	Uncultured prokaryote clone 08031003-Z7EU_2TH_2_2_A06	*Clostridiales*	HQ156029	97	Biogas Z7 sample
	15	17	Clostridiales oral clone P4PB_12	*Ruminococcaceae*	AF538854	93	Periodontal microflora
	16	2	Uncultured bacterium clone D53	*Ruminococcaceae*	AM500759	93	Composting sample
Firmicutes-Bacilli	17	1	*Bacillus dipsosauri*	*Bacillaceae*	AB101591	99	Strain DSM11125T
	18	1	Uncultured bacterium clone M35-D20-H-B-B		EF586027	98	Solid waste digester fed with methanol
	19	1	*Vagococcus* sp.	*Enterococcaceae*	FJ211190	99	Wastewater treatment factory
Gammaproteobacteria	20	2	*Pseudomonas* sp.	*Pseudomonadaceae*	DQ337603	99	Swine effluent applied soil
Euryarchaeota							
Methanomicrobiales	AL2-1	8	*Methanoculleus bourgensis*	*Methanomicrobiaceae*	AB065298	97	Strain:DSM 6216
Methanobacteriales	2	2	Uncultured *Euryarchaeote* clone 1C		GQ365371	98	Labscale digester Inoculated with anaerobic digester sludge
	3	4	Uncultured archaeote clone T8		EU662689	97	Sludge from a manure pit
Methanosarcinales	4	1	*Methanosarcina* sp.	*Methanosarcinaceae*	EU857627	98	Nisargruna Biogas Plant

cellulolytic and amylolytic bacteria, which have been isolated from several AD reactors (Yu et al., 2010). The *Bacilli* class was a minor component of the reactor community, detected only during the start-up of the reactor, and was represented by the *Bacillaceae* (OTU BL2-17, 99% similarity with *Bacillus dipsosauri*) and *Enterococcaceae* (OTU BL2-19, 99% similarity with *Vagococcus* sp.) families (Table 2). Perhaps the presence of *Bacillus* sp. can be associated with the presence of *Symbiobacterium* sp. According to Ueda and Beppu (2007), ammonium, peptidic substances and amino acids generated by the metabolic activity of *Bacillus* sp. enhance the growth rate of *Symbiobacterium thermophilum* under an atmosphere of mostly CO_2, which is the case in an anaerobic digester.

Pseudomonas sp. was the only OTUs affiliated with *Proteobacteria*. This group decreased with time, eventually disappearing from the reactor once it reached steady-state conditions. The predominance of γ-*Proteobacteria* has been reported in a laboratory-scale anaerobic digester for the treatment of household solid waste (Cardinali-Rezende et al., 2009).

The sequences assigned to *Actinobacteria* were represented by different strains of *Actinomyces europaeus* (Tables 2 and 4). Member of this phylum was previously observed in anaerobic reactors at mesophilic temperatures (Chouari et al., 2005).

Interestingly, the emergence of some groups affiliated with the *Tenericutes* phylum, previously isolated from swine wasters (Table 4) and the candidate division WWE1 (Table 4), also identified in a wastewater reactor (Chouari et al., 2005), were detected in the reactor at steady-state conditions (DMSW4).

The predominance of fermentative acidogenic and hydrolytic bacteria was responsible for the increased VFA content (acetate, propionate and butyrate) in the reactor. The increase of the VFAs and the accumulation of ammonium (Table 1) are typical responses for a reactor during organic overloading. The increase of VFAs was accompanied by an increase of the bacterial and archaeal communities in the reactor (Table 5), particularly in the DMSW4 sample, which was removed when the reactor was completely full and had reached steady-state conditions. VFAs are produced during acidogenesis, and their high concentration could affect the MSW digestion and biogas production. However, the reactor stability was maintained and the digestion occurred normally because a constant pH was maintained (Table 1).

3.4. Archaeal diversity

A phylogenetic analysis of the OTU sequences from the archaeal libraries revealed that most of the OTU sequences (15 of 16) were affiliated with the *Euryarchaeota* phylum (Fig. 3 and Tables 2–4), which was represented by methanogenic archaea from the orders *Methanobacteriales*, *Methanomicrobiales* and *Methanosarcinales*.

The hydrogenotrophic methanogens dominated all of the samples, most of which included the *Methanomicrobiales* order (these OTU sequences were >97% similar to *Methanoculleus bourgensis* and *Methanoculleus* sp.). During the start-up phase and until the steady-state conditions were reached, the production of methane in the reactor was dominated by the syntrophic relationship between the hydrogenotrophic methanogenic archaea, particularly from the *Methanomicrobiales* order (*Methanoculleus bourgensis*) (Fig. 3 and Tables 2–4), and the acetate-reducing and propionate-oxidizing bacteria, which were previously observed by Shigematsu et al. (2006). In an another study, a tracer experiment using 13C-labeled acetate revealed that approximately 80% of the acetate was decomposed via a non-aceticlastic oxidative pathway, whereas the remainder was converted to methane via an aceticlastic path-

Table 4
Phylogenetic filiations and distribution of bacterial and archaeal clones analyzed from from digested municipal solid waste 4 (DMSW4).

Phylogenetic group	OTU	Clon No.	Closest sequence/microorganism	Order/Family	Acession No.	Identity (%)	Habitat of closest relative
Bacteroidetes	BL4-1	22	Uncultured bacterium clone TE-3-E11		JQ337397	97	Composting sample at 12 days
	2	11	Uncultured bacterium clone A35_D28_L_B_A07		EF559196	99	Mesophilic anaerobic solid waste digestor
	3	5	Uncultured *Bacteroidetes* bacterium	*Porphyromonadaceae*		97	Anaerobic digester which treats municipal wastewater sludge
	4	1	Uncultured bacterium clone HAW-R60-B-609d-C	*Porphyromonadaceae*	FN436026	97	Thermophilic biogas reactor fed with renewable biomass
	5	14	Uncultured *Bacteroidetes* bacterium clone De2105		HQ183932	99	Leachate sediment
Firmicutes Clostridia	6	2	Uncultured bacterium partial 16S rRNA gene, clone MS14623-B032	*Ruminococcaceae*	FN994085	99	Biogas completely stirred tank reactor
	7	2	Uncultured bacterium clone A55_D21_L_B_	*Ruminococcaceae*	EF559050	99	Thermophilic anaerobic digester at 55 °C
	8	1	*Clostridiales* oral clone P4PB_122 P3	*Ruminococcaceae*	AF538854	97	Periodontal microflora
	9	1	Uncultured bacterium clone E94		FJ205856	99	Biogas plant
Tenericutes	10	1	Uncultured bacterium clone 04g04	*Acholeplasmataceae*	GQ136883	99	ASBR reactor treating swine waste
Actinobacteria	11	1	*Actinomyces europaeus*	*Actinomycineae*	AM084230	97	Isolate CCUG 32789AT
Candidate Division WWE1	12	1	Uncultured WWE1 bacterium		CU917955	95	Mesophilic anaerobic digester which treats municipal wastewater sludge
	13						
Crenarchaeote	AL4-1	26	Uncultured crenarchaeote TREC89-34	Thermoproteales	AY487102	99	Tomato rhizosphere
Euryarchaeota-				Methanosarcinaceae			Genomic DNA strain C2J
Methanosarcinalea	2	18	Methnosarcina siciliae		MSU89773	97	
	3	1	Uncultured Methnosarcina sp.	Methanosarcinaceae	EU857628	98	Nisargruna Biogas Plant
				Methanosarcinaceae			Food soil of Cubitermes
	4	1	Uncultured Methanimicrococcus sp.		AY487186	95	fungifaber
			Uncultured Methanimicrococcus sp.	Methanosarcinaceae			Low temperature anaeobic
	5	5			JN173199	97	bioreactor
Euryarchaeota-				Methanomicrobiaceae			
Methanomicrobiales	6	25	Methanoculleus bourgensis		AB065298	99	Strain DSM 6216
Euryarchaeota-				Methanobacteriaceae			Rumen
Methanobacteriales	7	2	Uncultured Methanobrevibacter sp.		FJ919272	95	

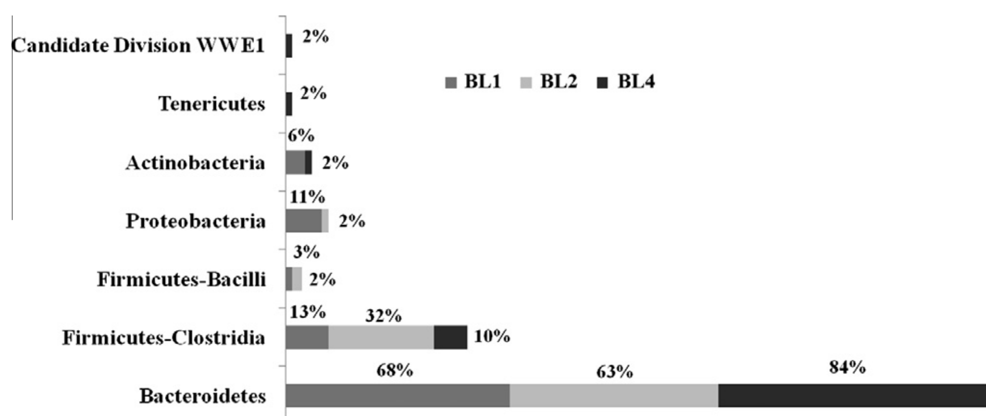

Fig. 2. Distribution of the bacterial clones in the DMSW libraries. BL1, BL2 and BL4 represent the 16S rDNA bacterial clone libraries from DMSW1, DMSW2 and DMSW4, respectively.

way (Sasaki et al., 2011). In the present study, archaeal 16S rRNA analyses demonstrated that the hydrogenotrophic methanogens *Methanoculleus* sp. accounted for >90% of detected methanogens, and the aceticlastic methanogens *Methanosarcina* sp. were the minor constituents. *Methanoculleus bourgensis* also predominated in other anaerobic reactors for the treatment of MSW (Weiss et al., 2008; Cardinali-Rezende et al., 2009; Nayak et al., 2009). *Methanoculleus* sp. requires H_2/CO_2, formate and some secondary alcohols as methanogenic substrates and acetate as a growth factor (Shigematsu et al., 2006).

Over time, some of the CO_2 that was required by the hydrogenotrophic methanogens may have been formed by the acetate-utilizing anaerobic bacteria through the cleavage of acetyl coenzyme A and the oxidation of the methyl and carbonyl groups of acetate, rather than via methanogenic acetate cleavage (Ferry, 1992). This hypothesis may explain the predominance of hydrogenotrophic methanogens in the MSW reactor. A loss of biodiversity in this group from the start-up to the steady-state conditions (88% of the OTUs in DMSW1, 54% in DMSW2 and 32% in DMSW4) was observed (Fig.3). This decrease was accompanied by the emergence of the *Methanobacteriales* order in the DMSW2 and DMSW4 samples (OTU ALA4-7, 95% similarity with *Methanobrevibacter* sp.). The members of this order are bacilli that utilize either H_2/CO_2 or formate as substrates for methanogenesis. The hydrogenotrophic *Methanobrevibacter* sp. (Fig. 3 and Tables 3 and 4) was also identified in a MSW laboratory-scale anaerobic reactor (Cardinali-

Table 5
Number of cell of bacteria and archaea using qPCR, FISH and CARD-FISH techniques.

Sample	Technique	Taxa					
		Bacteria	Archaea	Methanosaeta	Methanobacteriales	Methanomicrobiales	Methanosarcinaceae
DMSW1	qPCR	*2.3×10^{10} (12)	$1.3x\ 10^{8}$ (21.5)	9×10^{6} (25.7)	(23)	(20)	
	FISH	1.52×10^{9}	$3.15x\ 10^{8}$	3.52×10^{7}			
DMSW2	qPCR	1.9×10^{10} (12.3)	4.4×10^{7} (23)	3.6×10^{7} (30)	1.4×10^{9} (21)	(21)	(25)
DMSW4	qPCR	1×10^{11} (10)	2.3×10^{10} (17.7)	$1.4x10^{6}$ (28)	3.5×10^{7} (27)	(19)	(23)
	CARD-FISH	2.27×10^{12}	3.72×10^{11}				

Number in parentheses corresponds to the cycle where the maximum fluorescence crosses the log phase of amplification and the amount of amplicon is detected.
* Cells gr^{-1} of DMSW samples.

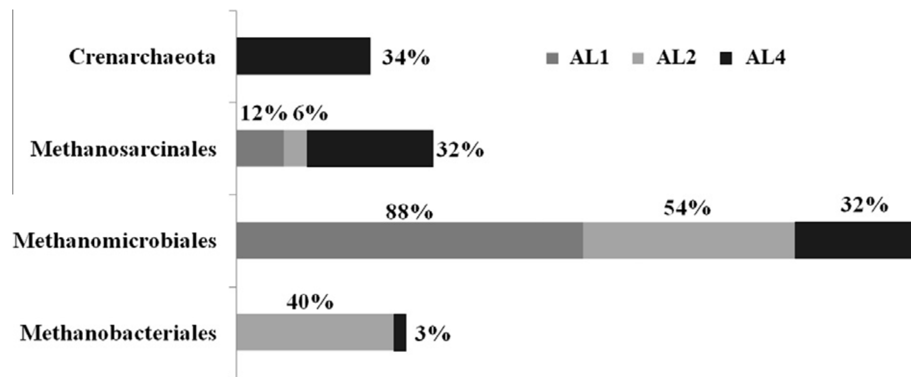

Fig. 3. Distribution of the archaeal clones in the DMSW libraries. AL1, AL2 and AL4 represent the 16S rDNA archaeal clone libraries from DMSW1, DMSW2 and DMSW4, respectively.

Rezende et al., 2009) and in a thermophilic MSW anaerobic reactor (Weiss et al., 2008).

Among the acetoclastic methanogens, the *Methanosarcinales* order was represented in the reactor by sequences from the *Methanosarcinaceae* family. According to Tables 2–4, a shift from *Methanosarcina barkeri* to *Methanosarcina siciliaea* was evident. Additionally, the emergence of OTUs with sequences >95% similar to uncultured *Methanimicrococcus* sp. in the DMSW4 sample was also observed. The genera *Methanosarcina* and *Methanimicrococcus*, both of which were identified in the DMSW samples and previously identified in a thermophilic MSW reactor (Weiss et al., 2008), are methanol consumers and may be competing for this substrate in the reactor. The *Crenarchaeota*-affiliated OTUs were found only in the DMSW4 sample and contained 33.5% of the sequences associated with the *Thermoproteales* order (Fig. 3 and Table 4).

In steady-state conditions and after the methanogenic community had matured and adapted to the MSW reactor, the production of methane was predominantly via the hydrogenotrophic pathway and only marginally through the acetilastic and methanol-degrading pathways.

3.5. Bacterial and archaeal 16S rRNA gene analysis using DGGE

The microbial diversity and the shifts in the bacterial and archaeal communities present in the DMSW1, DMSW2 and DMSW3 samples were observed using DGGE patterns of the partial 16S rRNA gene amplicons. Although the DGGE band patterns shared many of the same bands, some changes in the microbial communities are evident (Fig. 4). A total of 54 bands were excised from the DGGE fingerprints. However, only 17 DGGE bands from bacteria (DB) and four from archaea (DA) were successfully analyzed and phylogenetically identified.

The sequences chosen for analysis were affiliated with the *Firmicutes* and *Bacteroidetes* phyla. Although DGGE is not a quantitative technique, the greater fluorescence intensity of the *Bacteroidetes* bands indicated that this group was more prevalent, an observation that was also confirmed by the clone library analysis. The *Firmicutes* phylum was represented by bands only affiliated with the *Clostridia* class. Some sequence bands exhibited similarities to OTU sequences from the DMSW libraries, such as: DB1-8 (bacterial band 8 from the DMSW1 sample) and DB2-6 (bacterial band 6 from the DMSW2 sample), which displayed 92% identity with an uncultured *Symbiobacterium* sp. (OTU BL1-11, Table 2); and DB2-3 and DB3-9, which displayed 95% identity with the *Clostridiales* oral clone P4PB_12 identified in the OTU BL2-15 (Table 3).

Representatives of other phyla detected in the clone libraries were not identified using the DGGE technique. Some of the sequences from the fragments that migrated to different positions on the DGGE gel (Fig. 4), such as bands DB3-1 to DB3-7, exhibited the same phylogenetic affiliation.

The partial sequence bands of the archaeal communities were affiliated with the order *Methanosarcinales*. The bands DA1-3, DA2-5, and DA3-5 exhibited 99% identity with the uncultured *Methanosarcina barkeri* (Fig. 4), which was also identified in the OTU AL1-1 (Table 2).

A UPGMA cluster analysis of the bacterial and archaeal band sequences from the DMSW 1, 2 and 3 samples was performed to compare the communities in these samples. This analysis demonstrated that the prokaryote communities in DMSW1 were the most dissimilar observed in the dendrograms (Fig. S1).

3.6. Quantitative analysis of the microbial community using qPCR

The abundance of bacteria, archaea and methanogenic archaea in the digester during start-up (DMSW1 and 2) and steady-state conditions (DMSW4) is listed in Table 5. The amplification efficiencies were more than 95% with $r^2 > 0.99$. The bacterial and archaeal communities increased over time, and their cell numbers were one and two orders of magnitude higher, respectively, in DMSW4 than in DMSW1 (Table 5). The increase in these communities accompa-

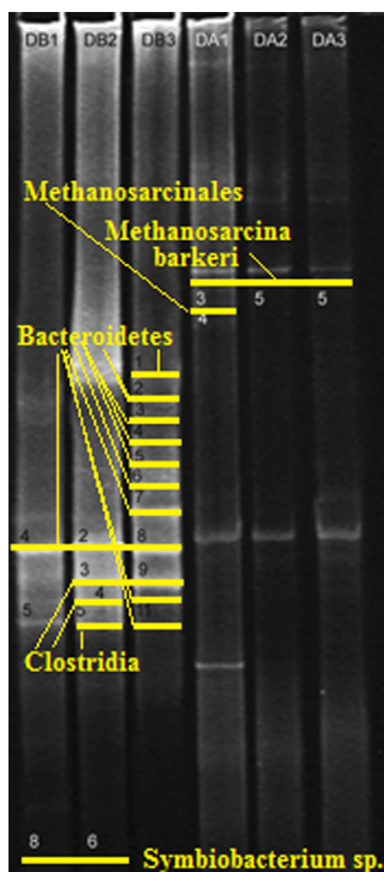

Fig. 4. DGGE temporal analysis of the Bacterial (DB) and Archaeal (DA) communities from the DMSW1, DMSW2 and DMSW3 samples (i.e., DB1 and DA1 are from the DMSW1 sample). The band fragments excised and their phylogenetic identities are represented by the numbers in each lane.

nied the daily increase of the reactor feed material up to complete capacity and the commencement of steady-state conditions in DMSW4.

The changes in the methanogenic archaeal cell counts (Table 5 and Fig. 5) and the substitution of these groups in the community were also monitored (Tables 2–4). The concentrations of hydrogenotrophic *Methanobacteriales*, and particularly *Methanomicrobiales*, were higher than the acetoclastic *Methanosarcina* sp. and *Methanosaeta* sp. The predominance of *Methanomicrobiales* was observed in the community throughout the operation of the anaerobic reactor (Fig. 3 and Tables 2–5). qPCR analysis revealed the increase of the *Methanobacteriales* community in DMSW2 and its decrease in the DMSW4 samples (Fig. 5 and Table 5) and the same phenomenon was observed in the AL libraries of these samples.

The composition of the aceticlastic methanogen community also shifted. The number of *Methanosarcina* sp. cells increased from DMSW2 to DMSW4 and coincided with the emergence of the methanogenic *Methanomicrococcus* sp. in the DMSW4 sample and with the increase of acetate concentration from the start-up phase (415 mg/L) to the steady-state phase (4100 mg/L) (Fig. 3 and Tables 1, 3 and 4). These genera compete when the acetate concentration is <500 mg/L (Jetten et al., 1992). *Methanosaeta* sp. exhibited low concentrations throughout the MSW treatment (Table 5). According to qPCR analysis and the library analysis of archaeal community, the hydrogenotrophic methanogens dominated in the reactor (Fig.3 and Table 5). In contrast, the dominance of *Methanosaetaceae* was previously demonstrated in wastewater sludge reactors (Diaz et al., 2006).

In this work, qPCR analysis of the *Bacteria* and *Archaea* domains uncovered and quantified the diversity of the communities in an environmental sample and incorporated a standard curve generated from a robust control: the PCR products from the pooled DNA of the three DMSW samples. In environmental samples, differences in the G + C content of the 16S rRNA gene in different prokaryotes may lead to the formation of more than one peak in the melting curve (Sharma et al., 2007), which occurred in the samples analyzed here. However, both the standard and the samples present the same melting curve profiles, which confirm the reliability of the results. In this work, shifts occurred in the archaeal community from DMSW1 to DMSW4 (Tables 2–4), being these changes also reflected in the melting curves generated by qPCR analysis. Additionally, the quantification of specific methanogenic archaea, which typically exhibit low biodiversity in anaerobic reactors, resulted in only one peak in the melting curve, as expected. An example was the *Methanobacteriales* order, whose sequences were dominated by *Methanobrevibacter* sp. (Fig. 5).

3.7. Quantitative analysis of the microbial community using FISH and CARD-FISH

FISH was successfully obtained and used to identify the relative abundances of *Bacteria*, *Archaea* and the methanogenic archaean *Methanosaeta* sp. in the DMSW1 sample. *Methanosaeta* sp. was detected only with the use of the more sensitive techniques in this study (qPCR and FISH); particularly in the DMSW1 sample (Table 5).

Several problems occurred during hybridization with the specific probes for other bacterial and archaeal groups in the DMSW1 sample. FISH was also performed with the DMSW2, DMSW3 and DMSW4 samples, as well as the positive and negative controls. However, only the positive controls were successfully hybridized. After several attempts and negative results, the CARD-FISH technique was used to hybridize the DMSW4 sample. The sample and positive control filters were treated with lysozyme and proteinase K to ensure sufficient permeabilization in any individual experiment and to interpret a negative result correctly. The positive controls hybridized with success, which suggests that the permeabilization procedure was sufficient for the bacterial and archaeal groups. However, only the probes specific to *Bacteria* (EUB338) and *Archaea* (Arch915) were successfully hybridized to the cells from the DMSW4 sample. Hybridization was not obtained for other microorganism groups. The negative controls with probe NON338 consistently yielded no fluorescently labeled cells. The MSW samples are characterized by the presence of humic acids, metals, colloids, and organic and inorganic substances, which could have prevented the penetration of the probes into the cells or hybridization with the probes or led to the loss of cell viability in the DMSW samples. These results confirm that pretreatment of the sample is a critical step that may greatly affect the measurement of bacterial and archaeal quantities.

A total of 1.8×10^9 cells g^{-1} was stained with DAPI in the DMSW1 sample. A total of 84% of the cells corresponded to the *Bacteria* (1.52×10^9 cells g^{-1}) and 16% to the *Archaea* domains (3.15×10^8 cells g^{-1}). The *Methanosaeta* sp. was comparatively rare (3.52×10^7 cells g^{-1}), which represented only 11% of the total archaeal cells. Using the CARD-FISH technique, a total of 3×10^{12} cells g^{-1} were stained with DAPI. A total of 86% of the cells that hybridized belonged to the *Bacteria* (2.27×10^{12} cells g^{-1}) and 14% to the *Archaea* (3.72×10^{11} cells g^{-1}). Several cell morphologies, such as rods, long-bowed rods and cocci that occurred singly, in pairs or in chains, were visible in the DMSW1 and DMSW4 samples. According to FISH and CARD-FISH, the total bacterial and archaeal cell numbers increased from DMSW1 to DMSW4, and a similar increase was detected using qPCR (Table 5).

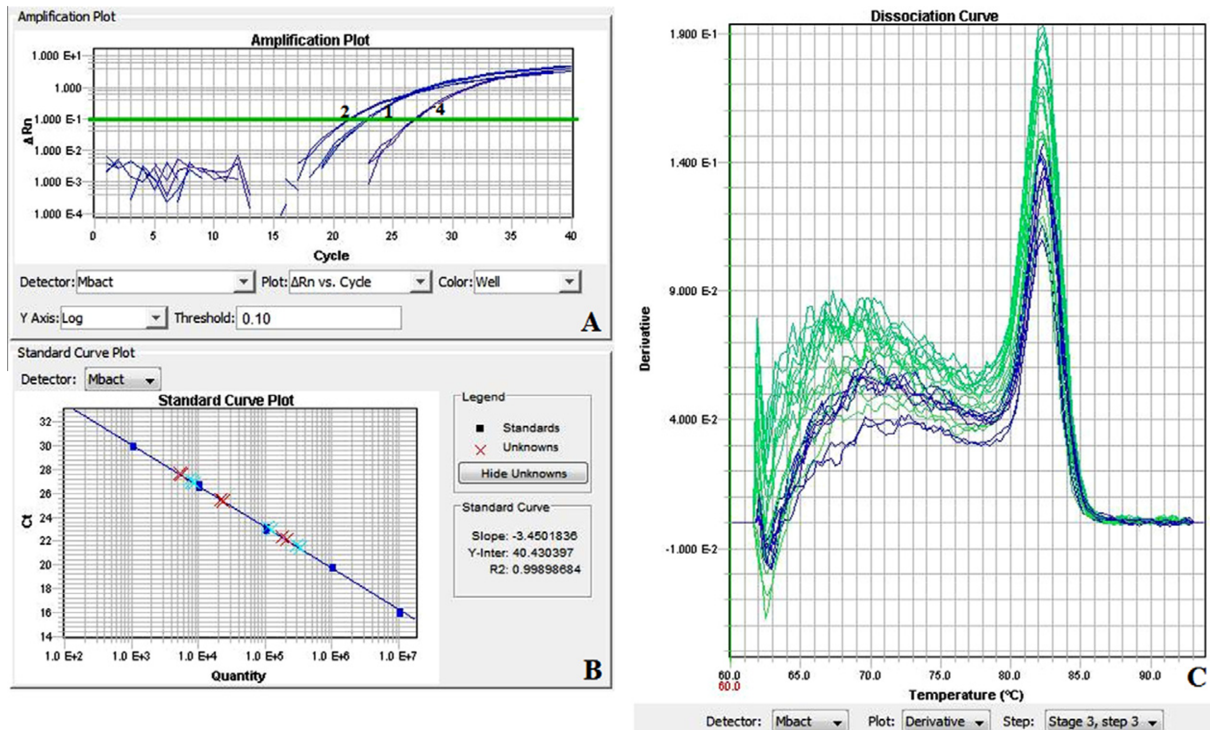

Fig. 5. qPCR analysis of the methanogenic archaeal *Methanobacteriales* order from the DMSW samples. (A) The amplification curves produced by the amplicons from DMSW1 (1), DMSW2 (2) and DMSW4 (4); (B) the standard curve produced from the standards and the sample amplicons; and (C) the melting curves obtained for the Methanobacteriales amplicons and the standards generated during the qPCR analysis of the DMSW samples (1, 2 and 4).

3.8. qPCR versus FISH and CARD-FISH

Variation in the relative abundances of the microbial cells in the digester was evaluated using several quantitative techniques. A comparison of FISH with qPCR (Table 5) revealed that in the DMSW1 sample the number of cells enumerated by FISH from the *Archaea* domain and *Methanosaeta* sp. was 2.4 and 4 times higher, respectively, than by qPCR. In contrast, for the *Bacteria* domain, the number of cells enumerated was one order of magnitude lower by FISH. In the DMSW4 sample, the bacterial and archaeal cell numbers were both one order of magnitude higher using CARD-FISH.

DNA extraction and purification may lead to a significant loss of DNA and considerable change in the quantification data obtained from qPCR. Overall, the hybridization by FISH was proportional to the intracellular level of rRNA, which was also proportional to the metabolic cellular activity in the sample (Wagner et al., 1994). In contrast, this observation was not true for CARD-FISH, which could explain the different results obtained from the use of both techniques.

The analysis of this microbial community revealed temporal shifts in the archaeal and bacterial populations during the operation of a reactor, a phenomenon that was observed earlier in pilot-scale solid waste reactors (Cardinali-Rezende et al., 2009).

4. Conclusions

Shifts in the prokaryotic community took place in a full-scale OF-MSW anaerobic reactor from start-up to steady-state conditions, increasing both bacterial and archaeal cell number over the time. The fermentatives *Bacteroidetes* and *Firmicutes* and the H_2-consumers methanogens *Methanomicrobiales* predominated. Aceticlastic methanogens *Methanosarcina* and *Methanimicrococcus* were identified mainly with the reactor working in steady-state conditions. *Methanosaeta* could be only detected by qPCR and FISH, revealing the sensitivity of these quantitative techniques. The use of several molecular tools to determine the microorganisms that performer the anaerobic digestion is a first effort at understanding and improving performance of anaerobic MSW digesters.

Acknowledgements

We are grateful to the Área de Gobierno de Medio Ambiente of Madrid and the Dirección General Parque Tecnológico Valdemingómez for allowing the collection of the MSW samples from the anaerobic digesters, to J. Antonio Fernández Rodríguez of the Planta de Biometanización Las Dehesas for his assistance, and to Fernando Carrasco of the Centro de Biología Molecular Severo Ochoa de la Universidad Autónoma de Madrid (CBMSO-UAM) for qPCR analysis support. We appreciate the financial support provided by Methanum Environmental and Energy Solutions, the Financiadora de Estudos e Projetos (FINEP), the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), the Pró-reitoria de Pesquisa da Universidade Federal de Minas Gerais (PRPq-UFMG) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) in the form of a scholarship to Juliana Cardinali Rezende.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2012.05.136.

References

Amann, R.I., Binder, B.J., Olson, B.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990. Combination of 16S rRNA-target oligonucleotide probes with flow cytometry

for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

APHA-AWWA-WEF, 2005. Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association-American Water Works Association-Water Environment Federation, Washington, DC.

Bertin, L., Bettini, C., Zanaroli, G., Frascari, D., Fava, F., 2012. A continuous-flow approach for the development of an anaerobic consortium capable of an effective biomethanization of a mechanically sorted organic fraction of municipal solid waste as the sole substrate. Water Res. 46, 413–426.

Cardinali-Rezende, J., Debarry, R.B., Colturato, L.F.D.B., Carneiro, E.V., Chartone-Souza, E., Nascimento, A.M.A., 2009. Molecular identification and dynamics of microbial communities in digester tresting organic household waste. Appl. Microbiol. Biotechnol. 84, 777–789.

Chouari, R., Le Paslier, D., Dauga, C., Daegelen, P., Weissenbach, J., Sghir, A., 2005. Novel major bacterial candidate division within a municipal anaerobic sludge digester. Appl. Environ. Microbiol. 71, 2145–2153.

Diaz, E.E., Stams, F., Amils, R., Sanz, J.L., 2006. Phenotypic properties and microbial diversity of methanogenic granules from a full-scale UASB reactor treating brewery wastewater. Appl. Environ. Microbiol. 72, 4942–4949.

Ferry, J.G., 1992. Methane from acetate. J. Bacteriol. 174, 5489–5495.

Hammer, Ø., Harper, D.A.T., Ryan, P.D., 2001. PAST: Paleontological statistics software package for education and data analysis. Palaeontol. Electron. 4, 1–9, http://www.aebiom.org/IMG/pdf/Brochure_BiogasRoadmap_WEB.pdf/30/04/2012, http://europa.eu/rapid/pressReleasesAction.do?reference=STAT/05/113&format=HTML&aged=0&language=EN&guiLanguage=en/29/02/2012, http://www.madridiario.es/2008/Noviembre/medioambiente/gasnatural/118868/plants biometanizacion-Valdemingómez-empiezan-rodaje.html/29/02/2012.

IEA., International Energy Agency. 2003. Bio-energy anaerobic digestion activity. Biogas from municipal solid waste: an overview of systems and markets for anaerobic digestion of MWS (booklet). Copenhagen. Minister of Energy Danish Energy Agency.

INE, Instituto Nacional de Estadística, 2009. Encuesta sobre recogida y tratamiento de residuos 2008. Madrid. Librería Del INE – Servicios Centrales de Madrid.

Jetten, M.S.M., Stams, A.J.M., Zehnder, A.J.B., 1992. Methanogenesis from acetate: a comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina* spp. FEMS Microbiol. Rev. 88, 181–198.

Jewell, W.J., Kim, T., Alvarez, C.J., Montserrat, P.G., 1999. Anaerobic composting of animal waste: diary system. In: Mata-Alvarez, J., Tilche, A., Cecchi, F. (Eds.), Proceedings of the Second International Symposium on Anaerobic Digestion of Solid Wastes, Barcelona, vol. 1. Graques 92, 107–112.

Klamppenbach, J.A., Saxman, P.R., Cole, J.R., Schidmidt, T.M., 2001. Rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. 29, 181–184.

Martín-González, L., Castro, R., Pereira, M.A., Alves, M.M., Font, X., Vicent, T., 2011. Thermophilic co-digestion of organic fraction of municipal solid wastes with FOG wastes from a sewage treatment plant: Reactor performance and microbial community monitoring. Bioresource Technol. 102, 4734–4741.

Mata-Alvarez, J., Macé, S., Llabrés, P., 2000. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresource Technol. 74, 3–16.

Nayak, B.S., Levine, A.D., Cardoso, A., Harwood, V.J., 2009. Microbial population dynamics in laboratory-scale solid waste bioreactors in the presence or absence of biosolids. J. Appl. Microbiol. 107, 1330–1339.

Nelson, M.C., Morrison, M., Yu, Z., 2011. A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresource Technol. 102, 3730–3739.

Pernthaler, A., Pernthaler, J., Amann, R., 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria Appl. Environ. Microbiol. 68, 3094–3101.

Ryan, P.D., Harper, D.A.T., Whalley, J.S., 1995. PALSTAT, Statistics for palaeontologists. Chapman & Hall (now Kluwer Academic Publishers).

Sánchez-Andrea, I., Rodríguez, N., Amils, R., Sanz, J.L., 2011. Microbial diversity of anaerobic sediments of Rio Tinto: a natural acid and high heavy metal content environment. Appl. Environ. Microbiol. 77, 6085–6093.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning. A laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, New York, N.Y.USA.

Sasaki, D., Hori, T., Haruta, S., Ueno, Y., Ishii, M., Igarashi, Y., 2011. Methanogenic pathway and community structure in a thermophilic anaerobic digestion process of organic solid waste. J. Biosci. Bioeng. 111, 41–46.

Sharma, S., Radl, V., Kloos, K., Fuka, M.M., Engel, M., Schauss, K., Schloter, M., 2007. Quantification of functional genes from prokaryotes in soil by PCR. J. Microbiol. Meth. 68, 445–452.

Shigematsu, T., Era, S., Mizuno, Y., Ninomiya, K., Kamegawa, Y., Morimura, S., Kida, K., 2006. Microbial community of a mesophilic propionate-degrading methanogenic consortium in chemostat cultivation analyzed based on 16S rRNA and acetate kinase genes. Appl. Microbiol. Biotechnol. 72, 401–415.

Supaphol, S., Jenkins, S.N., Intomo, P., Waite, I.S., O'Donnell, A.G., 2011. Microbial community dynamics in mesophilic anaerobic co-digestion of mixed waste. Bioresource Technol. 102, 4021–4027.

Tang, Y.Q., Shigematsu, T., Ikbal, Morimura, S., Kida, K., 2004. The effects of micro-aeration on the phylogenetic diversity of microorganisms in the thermophilic anaerobic municipal solid-waste digester. Water Res. 38, 2537–2550.

Ueda, K., Beppu, T., 2007. Lessons from studies of *Symbiobacterium thermophilum*, a unique syntrophic bacterium. Biosci. Biotechnol. Biochem. 71, 1115–1121.

Yu, Z., Morrison, M., Schanbacher, F.L., 2010. Production and utilization of methane biogas as renewable fuel. In: Alain Vertes, N.Q., Yukawa, Hideaki., Blaschek, Hans. (Eds.), Biomass to Biofuels: Strategies for Global Industries. Wiley, New York.

Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D., et al., 1994. Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. Appl. Environ. Microbiol. 60, 792–800.

Weiss, A., Jérôme, V., Freitag, R., Mayer, H.K., 2008. Diversity of the resident microbiota in a thermophilic municipal biogas plant. Appl. Microbiol. Biotechnol. 81, 63–73.