

# Microbial community dynamics in the two-stage anaerobic digestion process of two-phase olive mill residue

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**Abstract** The microbial communities in a two-stage anaerobic digestion process treating olive mill “solid” residues were studied by molecular identification techniques. The microbial species identification in the hydrolytic-acidogenic step and in the methanogenic step was carried out by polymerase chain reaction amplification of 16S ribosomal RNA genes, denaturing gradient gel electrophoresis, cloning, and sequencing. This study revealed that Firmicutes (from 31.1 to 61.1 %, average 42.1 %) mainly represented by Clostridiales, and the Chloroflexi (from 29.4 to 53.3 %, average 47.35 %) were the most abundant species for the hydrolytic-acidogenic reactor. Other microorganisms such as Gamma-Proteobacteria (*Pseudomonas* species as the major representative; from 3.9 to 9.7 %, average 5.7 %), Actinobacteria (from 1.0 to 10.2 %, average 4.6 %) and Bacteroidetes (from 1.1 to 3.1 %, average 1.7 %) were also detected. The methanogenic communities detected in the methanogenic reactor were mainly represented by members of the obligate acetotrophic methanogenic genus *Methanosaeta*. *Methanosaeta* was the crucial Archaea to obtain a high methane yield in the methanogenic stage.

**Keywords** Bacterial and Archaeal populations · Biomethanization · Olive mill solid waste · Poly-phenolic compounds

## Introduction

Anaerobic digestion (AD) processes are an attractive alternative for high organic load substrate treatments. AD allows a high degradation of the organic components through the action of different groups of microorganisms (hydrolytics, acetogens and methanogens) producing biogas [mixture of methane (60–70 %) and carbon dioxide (30–40 %)] (Amani et al. 2011) with high calorific value (20,833–25,000 kJ m<sup>-3</sup>). A low percentage of the microorganisms present in anaerobic digestion processes has been cultured and isolated. This lack of knowledge might lead to malfunction and unexplainable failure of biogas fermenters. Therefore, the influence of the microbial communities on biogas yield and system stability must be analyzed in more detail (Weiland 2010). The two-phase olive mill “solid” residue (OMSR) generated in the “ecologic” two-phase centrifugation olive oil manufacturing process is produced in high quantities (0.8 kg OMSR kg<sup>-1</sup> olives milled). Although named “solid”, it has a high water content (60–85 %) because it retains all the water from the olive. The high organic content (total chemical oxygen demand (COD) of 162 g L<sup>-1</sup> after stone removal) of the OMSR and its high concentration of poly-phenolic compounds (15 g L<sup>-1</sup>) place this waste as a very phytotoxic waste. The disposal and management of the OMSR constitute important environmental problems (Borja et al. 2006). AD processes are attractive solutions for OMSR treatments.

The microorganisms participating in the AD processes have different levels of sensitivity to the environmental conditions, different growing kinetics and show differences in physiological and nutritional requirements (Anderson et al. 1994). The separation of the process in two independent steps, hydrolytic-acidogenic (H-A) and methanogenic (M),

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is very convenient in some cases. Most of the studies concerning stage separation are related to inhibition and toxicity (Demirel and Yenigun 2002). In addition, two-stage processes reduce the risk of accumulating toxic intermediates such as volatile fatty acids that can inhibit the development of methanogenic communities (Veeken and Hamelers 2000). The inhibition of the anaerobic processes by phenols and poly-phenolic compounds has been studied at different temperatures and substrates. Most of these studies point out the importance of acclimation periods to obtain good results (Chen et al. 2008). Wang et al. (1991) studied the inhibition of acetate-utilizing methanogens by phenols. They found how the rate of methane production decreased progressively with increasing concentration of phenolic compounds. A noncompetitive inhibition model was found to best fit the experimental data with inhibition constants of 1,000–2,000 mg L<sup>-1</sup> for hydroxyphenols, and 900 mg L<sup>-1</sup> for phenol. Bacterial genera like *Desulfotomaculum* and *Clostridium*, and Archaea within the *Methanosaetaceae*, *Methanomicrobiales* and *Methanobacteriaceae*, have been described for the anaerobic transformation of different phenols (Fang et al. 2004; Zhang et al. 2005).

The current studies on the microbial communities involved in the OMSR degradation were carried out in one-stage, in a unique reactor with all the microorganisms participating in the process working together (Rincón et al. 2008a). No microbiological study has been carried out in two stages (H-A and M steps) for the OMSR until now. The aim of this work was to study the microbial communities participating in the two-stage anaerobic digestion process treating OMSR and their evolution. The knowledge of the communities best adapted to treat OMSR and the poly-phenolic compounds degradation to avoid toxic inhibitions and to obtain the highest methane production could be an important tool for the optimization of the anaerobic treatment of this waste for future industrial purpose.

These experiments were carried out in the labs of the “Instituto de la Grasa” (CSIC) and “Recursos Naturales” (CSIC) in Sevilla, Spain during 2009.

## Materials and methods

### Two-phase olive mill solid residue used

The waste used in this study was olive mill “solid” residue collected from a two-phase olive oil factory processing olives of the ‘Picual’ variety (“Instituto de la Grasa” (CSIC), Seville, Spain). The olives were harvested at the beginning of the olive season with a low ripening level: 2.5 (Garcia and Yousfi 2005). Pieces of husk and stones in the OMSR were removed before characterization and utilization in the experiments. The main characteristics and

**Table 1** Characteristics of the two-phase OMSR used in the experiments

Parameters	OMSR
pH	5.3
TALK (as CaCO <sub>3</sub> )	1.1
TVFA (as acetic acid)	1.4
COD	162.0
S-COD	57.5
TS	143.0
MS	17.0
VS	126.0
TSS	106.0
MSS	11.0
VSS	95.0
Phosphorous	0.0035
Oils and fats	2.2 %
Moisture	86.7 %
Total phenols (as caffeic acid)	15.0

All units are expressed in g L<sup>-1</sup> except the moisture, oils, fats and pH. Values are averages of six determinations; there was virtually no variation (<5 %) between analyses

COD total chemical oxygen demand, S-COD soluble chemical oxygen demand, TVFA total volatile fatty acids, TALK total alkalinity, TS total solids, MS total mineral solids, VS total volatile solids, TSS total suspended solids, MSS mineral suspended solids, VSS volatile suspended solids

composition of the OMSR used in the experiments are shown in Table 1.

### Equipment

A two-stage anaerobic digestion process was carried out in two different reactors at mesophilic temperature (35 ± 2 °C). The first reactor or H-A reactor was a 1.5 L digester; it was initially loaded with 0.9 L of active biomass, 0.3 L of a nutrient-trace solution and 0.3 L of distilled water. The M reactor was loaded with 1 L of sludge, 0.4 L of a nutrient-trace element solution and 0.4 L of distilled water, keeping the effective reactor volume at 1.8 L. The nutrient-trace element solution has been previously described (Borja et al. 2001). Trace metals are known to stimulate the activity of anaerobic microorganisms involved in the degradation of organic matter (Krongthamchat et al. 2006). To prevent the loss of microorganisms with the reactor effluents, the mixture contained in the M reactor was supplemented with saponite (0.8 g mL<sup>-1</sup>), a low density magnesium silicate support media [(Mg,Fe)<sub>3</sub>(Si,Al)<sub>4</sub>O<sub>10</sub>(OH)<sub>2</sub>·4H<sub>2</sub>O]. An inoculum/support (saponite) ratio of 1:1 was kept. The M reactor was also equipped with a 0.5 L settler located on its upper part (Rincón et al. 2009). Biogas was collected by a water



displacement system fitted to the reactor (8 L Boyle-Marriott reservoir). The CO<sub>2</sub> produced in the process was scrubbed by bubbling the gas mixture through a 3 M NaOH solution. The volume of methane produced during the process was estimated by the volume of water displaced by this gas. Both reactors were continuously stirred at 260 rpm keeping an adequate inoculum-substrate mass transfer.

### Inoculum

Both reactors (H-A and M) were inoculated with the same inoculum, which was an anaerobic sludge obtained from an industrial upflow anaerobic sludge blanket (UASB) reactor placed at a local brewery. Some of the characteristics of the inoculum used were: pH 8.1, TSS: 34.9, MSS: 8.9, VSS: 26.0, TS: 37.4, MS: 11.0 and VS: 26.4 g L<sup>-1</sup> (TS: total solids, MS: total mineral solids, VS: total volatile solids, TSS: total suspended solids, MSS: mineral suspended solids, VSS: volatile suspended solids). All values were averages of triplicate samples with standard deviations lower than 5 %.

### Experimental procedure

Before starting the experiments, the inoculum into the H-A and M reactors became acclimatized to the respective substrates OMSR and acidified OMSR. For the H-A reactor, this adaptation period had a double objective: an inoculum-substrate adaptation stage and an enrichment of the hydrolytic-acidogenic populations in the inoculum. The high susceptibility of the Archaea to high acidic concentrations prevented the coexistence of methanogens in the hydrolytic-acidogenic reactor. Therefore, the high duplication times of the methanogenic microorganisms and the short hydraulic retention times studied caused an enrichment of hydrolytic-acidogenic microorganisms in the H-A reactor.

The H-A reactor was adapted to the OMSR during 45 days, in this period the organic loading rate (OLR) was increased from 0.5 to 3 g COD L<sup>-1</sup> d<sup>-1</sup>. After that eight OLRs were studied. The study of these growing OLRs (OLRs of 3.2, 5.6, 7.4, 9.6, 11.0, 12.9, 14.0, and 15.1 g COD L<sup>-1</sup> d<sup>-1</sup>) in the H-A reactor enabled to determine the OLR of 12.9 g COD L<sup>-1</sup> d<sup>-1</sup> (equivalent to an hydraulic retention time (HRT) of 12.4 days) as the optimum OLR to lead the highest total volatile fatty acid (TVFA) concentration (Rincón et al. 2008b). The effluents obtained at this OLR were used to feed the methanogenic reactor.

For the M reactor, an adaptation of the inoculum to the acidified substrate coming from the H-A reactor was carried out with three different dilutions of acidified OMSR (25, 50 and 75 %) for 45 days. The first dilution was used

to keep the OLR between 0.5 and 1.5 g COD L<sup>-1</sup> d<sup>-1</sup>, the second to increase the OLR between 1.5 and 2.2 g COD L<sup>-1</sup> d<sup>-1</sup> and, finally, the third to increase the OLR to 3 g COD L<sup>-1</sup> d<sup>-1</sup>. After the inoculum became acclimatized to the acidified OMSR, OLRs of 0.8, 2.0, 3.5, 5.0, 6.5, 8.6, 10.5, 12.8, 14.0, 15.5, 17.0, 18.5, 20.0 and 22.0 g COD L<sup>-1</sup> d<sup>-1</sup> were assessed in the M reactor (HRTs between 142.9 and 4.6 days). Each OLR was kept during 2–3 hydraulic retention times, once the steady-state conditions were achieved for each run, and when the deviations between the observed values for consecutive measurements of some specific parameters (e.g., methane production, effluent CODs, etc.) were <5 %, the samples were collected for analysis. Five different samples were taken over five consecutive days to ensure the representativeness of the data obtained for chemical analysis. 2 mL of these samples were collected to analyze the microbial community and were preserved at -80 °C until they were processed for DNA extraction.

### Chemical analyses

The chemical analyses were performed according to the recommendations of the standard methods of APHA (APHA 1989). COD and S-COD were determined according to the method number 5220 C. TS, MS, VS, TSS, MSS and VSS were analyzed according to the method numbers 2540B and 2540E. TALK was determined using the 2320B method. The pH was determined with a Crison, model basic 20 pH-meter. Phosphorous was measured by spectrophotometry (880 nm) using the normalized methods 4500-P, B and E. Oils and fats were analyzed by Soxhlet extraction with n-hexane using the official method of the EEC N°2568/91 (European Community Official Diary, L248/1 of 05.09.1991). Poly-phenolic compounds were measured by spectrophotometry at 725 nm using the Folin-Ciocalteu method (Rincón et al. 2007) after extraction following the procedure by Romero et al. (2002). Finally, TVFA were measured by distillation using the method number 5560C modified and titrating with sodium hydroxide.

### Molecular characterization of microbial communities

The diversity of the microorganisms involved in the OMSR anaerobic treatment was assessed through culture-independent molecular techniques. DNA extraction was performed using the Nucleospin Food DNA extraction kit (Macherey-Nagel, Düren, Germany) from steady-state samples. The microbial communities, both bacterial and archaeal, were analyzed through molecular fingerprinting and cloning of the 16S ribosomal RNA (16S rRNA) genes.

Each sample was represented by its microbial community fingerprint obtained by Denaturing Gradient Gel



Electrophoresis (DGGE). DGGE was performed according to Muyzer et al. (1993) with the modifications incorporated by Portillo and González (2008) to obtain relative quantitative fingerprints from the analyzed samples. Polymerase Chain Reaction (PCR) amplification aimed for DGGE analysis was carried out with the primer pairs 341F-GC (5'-CCT ACG GGA GGC AGC AG containing a GC-rich tail at its 5' end) and 518R (5'-ATT ACC GCG GCT GCT GG) for bacterial sequences, and 344F-GC (5'-ACG GGG CGC AGC AGG CGC GA containing a GC-rich tail at the 5' end) and 518R for archaeal sequences (González and Sainz-Jimenez 2004). The size of PCR products for DGGE analyses was approximately 160–190 bp. Amplification thermal conditions were: 95 °C for 2 min followed by 30 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 30 s; and a final step of 72 °C for 10 min. The GC-rich tail incorporated to the 5' end of the 341F-GC and 344F-GC primers was 5'- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G (Muyzer et al. 1993).

For PCR amplification and cloning, the primer pairs 27F (5'-AGA GTT TGA TYM TGG CTC AG) and 1522R (5'-AAG GAG GTG ATC CAG CCG CA), for bacterial sequences, and 20bF (5'-YTC CSG TTG ATC CYG CSR GA) and 1492bR (5'-GGY TAC CTT GTK WCG ACT T), for archaeal sequences, were used (Orphan et al. 2001). PCR thermal conditions were: 95 °C for 2 min followed by 30 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 2 min; and a final step of 72 °C for 10 min. The amplification products (approximately 1,470–1,500 bp) were used to construct 16S rRNA gene libraries aiming to identify the major components of the microbial communities. The 16S rRNA gene library construction was carried out with the TA-TOPO cloning kit (Invitrogen, Carlsbad, California) following the manufacturer recommendations. The screening procedure of library clones was carried out by DGGE and selected clones were sequenced. The major components of the microbial communities under analysis were selected by comparison of community fingerprints with the migration of the band corresponding to each clone (González et al. 2003).

Homology searches based on the Blast algorithm (Altschul et al. 1990) were performed online (<http://www.ncbi.nlm.nih.gov/blast>) to identify the closest relatives to the sequences retrieved during this study. Sequences were inspected for the presence of chimeras using the method described by González et al. (2005).

## Results and discussion

After the acclimation period, OLRs of between 3.2 and 15.1 g COD L<sup>-1</sup> d<sup>-1</sup> were studied in the H-A reactor. The maximum TVFA concentration, 14.5 g CH<sub>3</sub>COOH L<sup>-1</sup>

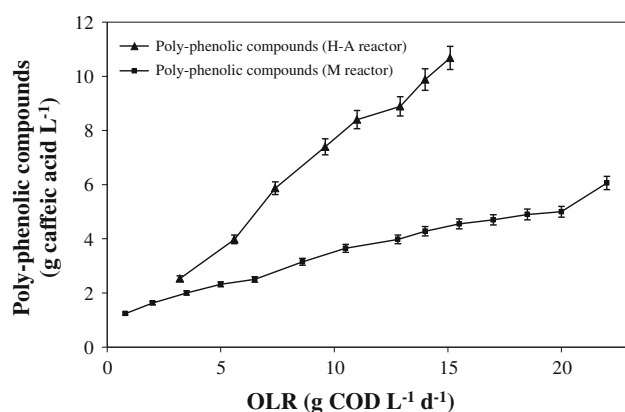
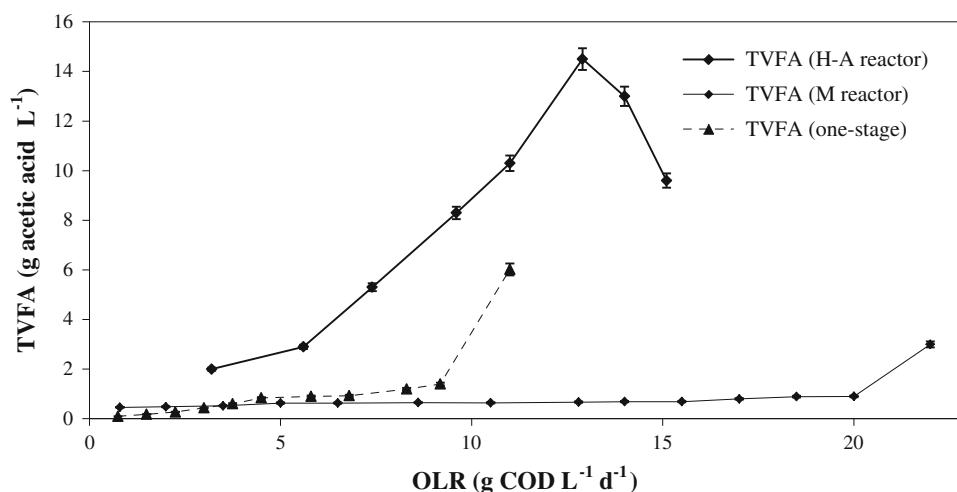
was searched for an OLR of 12.9 g COD L<sup>-1</sup> d<sup>-1</sup>. The microbial communities analyzed in the H-A step were corresponding to OLRs of 11.0, 12.9 and 14.0 g COD L<sup>-1</sup> d<sup>-1</sup>, OLR values for which the highest TVFA concentrations were achieved. Figure 1 shows the variation of the TVFA concentration with OLR in the H-A reactor and the M reactor. The variation of the TVFA with the OLR in a one-stage anaerobic digestion process, previously studied by Rincón et al. (2007) is also shown. The TVFA concentration for all the OLRs studied in the M stage of the two-stage process was very low, under 1 g L<sup>-1</sup> (as acetic acid), as shown in Fig. 1. Only for OLR higher than 20 g COD L<sup>-1</sup> d<sup>-1</sup>, the concentration increased until 3 g L<sup>-1</sup> (as acetic acid). For the one-stage process, in a unique reactor (2007), TVFA concentrations over 1 g L<sup>-1</sup> (as acetic acid) were achieved at lower OLR than in the two-stage process. For an OLR of 11 g COD L<sup>-1</sup> d<sup>-1</sup>, the TVFA concentration was 1.4 g L<sup>-1</sup>, causing a decrease in methane production. It seems that the acidification in a previous H-A reactor helps the consumption of the volatile fatty acids in the M reactor allowing to achieve higher OLR of work than in the digestion in one unique step.

For the M step, the microbial communities at OLRs of 2.0, 3.5, 6.5, 8.6, 10.5, 12.8, 15.5, 17.0, 18.5, 20.0 and 22.0 g COD L<sup>-1</sup> d<sup>-1</sup> were studied. The highest methane production rate, 3.24 L CH<sub>4</sub> L<sub>reactor</sub><sup>-1</sup> d<sup>-1</sup>, was achieved in the M reactor for an OLR of 20.0 g COD L<sup>-1</sup> d<sup>-1</sup> and 5 days of HRT. The methane yield obtained was 0.268 ± 0.003 L CH<sub>4</sub> STP g<sup>-1</sup> COD removed (Rincón et al. 2009). This high and stable OLR achieved in the M reactor could be explained because the previous H-A stage behaved as an initial treatment of the OMSR, where the most complex compounds were hydrolyzed and solubilized. Among them a reduction of the poly-phenol compounds could be very beneficial before a second step as reported by other authors (Borja et al. 1997).

The biotoxicity and inhibitory activity of phenols on the kinetics of acetoclastic methanogenesis is well known (Obied et al. 2007). In the H-A reactor, working at an OLR of 12.9 g COD L<sup>-1</sup> d<sup>-1</sup>, a reduction of 40.7 % in the poly-phenolic concentration was achieved, when the highest TVFA production was achieved. Figure 2 shows the poly-phenolic concentration as a function of the OLR in the H-A step and in the M step. When the concentration of poly-phenolic compounds reached values higher than 5 g L<sup>-1</sup> (OLR > 20 g COD L<sup>-1</sup> d<sup>-1</sup>) in the M reactor, the process started to fail and a reduction in the methane production yield was observed. For the study in one-stage (Rincón et al. 2007), poly-phenolic concentrations higher than 4.4 g L<sup>-1</sup> did fail the system but this concentration was obtained for an OLR of only 9.2 g COD L<sup>-1</sup> d<sup>-1</sup>. It is possible to conclude that the two-stage anaerobic treatment allows higher OLRs with improved stability than one-stage



**Fig. 1** Variation of the total volatile fatty acids concentrations in the hydrolytic-acidogenic step and in the methanogenic step of the two-stage anaerobic digestion process with the OLR. The variation of the total volatile fatty acids concentrations with the OLR in the one-stage OMSR anaerobic digestion obtained by Rincón et al. (2007) is also shown



**Fig. 2** Variation of the poly-phenolic compounds in the hydrolytic-acidogenic step and in the methanogenic step of the two-stage anaerobic digestion process with the OLR

process. Similar results were reported by Hernandez and Edyvean (2004), a reduction of phenol concentration in a first acidogenic reactor, decreasing the toxic effect of phenols. In both cases, one-stage and two-stage anaerobic digestion processes with concentrations over 4.4–5.0 g L<sup>-1</sup> of poly-phenolic compounds, resulted in the inhibition of the methanogenic Archaea and the methane produced in the M reactor was considerably reduced.

During this study, a total of 253 clones (200 bacterial and 53 archaeal) from 16S rRNA gene libraries were analyzed for the identification of the major components of the microbial communities developed in the reactors at different OLRs (Table 2). Figure 3 shows the bacterial community fingerprints obtained by DGGE in the H-A reactor. This figure indicates the presence of highly dynamic microbial communities and intense microbial interactions dependent on the organic load existing in this reactor. Table 2 shows the correspondence between the identification letters (ID) used in Fig. 3, the classification of these microorganisms, their accession numbers, their

closest homologues accession numbers, and the percentages of similarity between the retrieved sequences and their closest relatives. The fractions represented by the major phylotypes detected during this analysis were obtained from quantitative DGGE analysis and they are shown in Tables 3 and 4 for Bacteria and Archaea, respectively. These results show a dynamic composition of the microbial communities at different OLR, although the major components remained in the communities. In the present study, the dominant microorganisms in the H-A reactor, at OLRs of 11.0, 12.9 and 14.0 g COD L<sup>-1</sup> d<sup>-1</sup> where the best acidification conditions were achieved, were the Firmicutes (from 31.1 to 61.1 %, average 42.1 %) mainly represented by Clostridiales, and the Chloroflexi (from 29.4 to 53.3 %, average 47.35 %) (Table 3). These two groups have been reported as being able to degrade a large number of organic compounds under a variety of conditions (Nelson et al. 2011). Chloroflexi has been found in a wide range of anaerobic environments, including anaerobic bioreactors operating at both mesophilic and thermophilic temperatures (Chouari et al. 2005). Cirne et al. (2007) found Firmicutes as the major group in the leachate fraction of two-stage anaerobic digestion reactors of energy crops. Firmicutes has been also reported as an important phylum into two-stage anaerobic digestion of food waste-recycling wastewater (Shin et al. 2010). This bacterial phylum carries out a major role in OMSR degradation and it is able to outcompete with other microorganisms during the process. The degradation of OMSR might experience further improvements if additional studies are performed on these two bacterial groups which represent preferred targets for future optimizations of the studied two-stage processes by selected enrichments of members of these two groups. Other microorganisms such as Gamma-Proteobacteria (*Pseudomonas* species as the major representative; from 3.9 to 9.7 %; average 5.7 %), Actinobacteria (from 1.0 to 10.2 %; average 4.6 %) and Bacteroidetes (from 1.1 to





**Table 2** Bacteria and Archaea identified during this study and the number of clones obtained during the analysis of 16S rDNA gene libraries

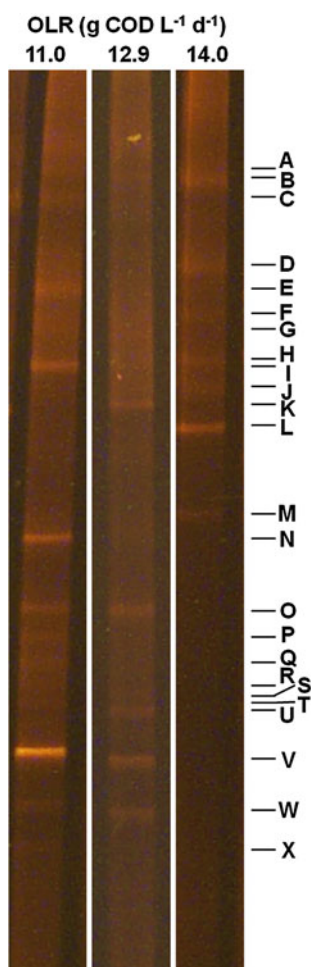
ID	Identified group/genus	GenBank accession no.	Closest homologue acc. no.	Similarity (%)	Number of clones
<b>Bacteria</b>					
A	Chloroflexi	AY935682	AJ249111	98	5
B	Chloroflexi	AY935680	AJ249111	99	1
C	Chloroflexi	AY935673	AF323767	98	2
D	<i>Clostridium</i> (Firmicutes)	AY935677	AY386136	99	2
E	<i>Bacillus</i> (Firmicutes)	AY935679	AJ277983	99	1
F	Chloroflexi	AY935664	AJ249111	99	25
G	Chloroflexi	AY935665	AJ249111	99	9
H	<i>Clostridium</i> (Firmicutes)	AY935683	AJ009459	93	6
I	Chloroflexi	AY935659	AJ249111	98	35
J	<i>Clostridium</i> (Firmicutes)	AY935661	AY386236	98	29
K	Chloroflexi	AY935666	AJ249111	97	7
L	Chloroflexi	AY935667	AJ009488	98	8
M	<i>Pseudomonas</i> (Gammaproteobacteria)	AY935658	AJ278174	91	1
N	<i>Paenibacillus</i> (Firmicutes)	AY935675	U81668	99	1
O	Chloroflexi	AY935653	AF424402	96	11
P	<i>Pseudomonas</i> (Gammaproteobacteria)	AY935662	AF289505	99	18
Q	Chloroflexi	AY935654	AJ249193	99	1
R	<i>Clostridium</i> (Firmicutes)	AY935681	AB088977	92	2
S	Chloroflexi	AY935668	AJ306792	97	1
T	Gammaproteobacteria	AY935671	AF332298	96	3
U	<i>Clostridium</i> (Firmicutes)	AY935678	AY360624	99	1
V	<i>Clostridium</i> (Firmicutes)	AY935674	AJ506119	98	28
W	<i>Rubrobacter</i> (Actinobacteria)	AY935656	AB116447	97	2
X	<i>Bacteroides</i> (Bacteroidetes)	AY935672	AB186816	97	1
				Total	200
<b>Archaea</b>					
A'	<i>Methanosaeta</i>	AY935685	AY692054	99	18
B'	<i>Methanosaeta</i>	AY935687	AY570662	99	15
C'	<i>Methanosaeta</i>	AY935686	AY692054	99	7
D'	<i>Methanosaeta</i>	AY935688	AY835796	99	13
				Total	53

ID letters correspond to the nomenclature of Figs. 3 and 4 for Bacteria and Archaea, respectively

3.1 %; average 1.7 %) were detected, although represented lower fractions of the studied microbial communities (Table 3). Figure 3 shows that for an OLR of 11 g COD L<sup>-1</sup> d<sup>-1</sup>, the main microorganisms found were *Clostridium* (Firmicutes) (H and V), Chloroflexi (I and O) and *Paenibacillus* (N) (Table 2). But for the OLR showing the maximum generation of volatile fatty acids (12.9 g COD L<sup>-1</sup> d<sup>-1</sup>), Chloroflexi (K and O) and *Clostridium* (V) were the main bacteria and other members of the community drastically reduced their significance (i.e., H and N). These results suggest a progressive higher significance of Chloroflexi at increasing organic loads which represents an important finding from this study. The dominance of distinct phylotypes at different OLR values suggests that the organic matter added to the reactor per

volume unit and time is an essential factor shaping microbial communities under development in OMSR treating reactors. The dominance of different microorganisms under distinct OLR suggests that the organic load and composition, as well as the initial inoculum composition (Moreno-Andrade and Buitrón 2004; Nyholm et al. 1984) could be decisive factors for optimum treatment results during the anaerobic digestion of OMSR. The TVFA concentrations achieved in the first stage [10.3, 14.5 and 13.0 g L<sup>-1</sup> (as acetic acid)] led to a practical disappearance of methanogenic Archaea which was undetectable in the H-A reactor for the OLRs and HRTs studied. Other authors have not found a disappearance of the methanogenic microorganisms, although significant shifts after 13 days of operation have been reported (Liu et al. 2002). These





**Fig. 3** DGGE fingerprints showing the major components of the bacterial communities developed in the H-A reactor at OLRs of 11.0, 12.9 and 14.0 g COD L<sup>-1</sup> d<sup>-1</sup>. The identified bacteria indicated on the right side with letters correspond to the listing and Accession no. provided in Table 2

changes in the microbial community were accompanied with the increase in TVFA production and a decrease in methane formation. Variations in the morphology of autofluorescent methanogens depending on volatile fatty acid concentrations in the acid reactor have been described reporting that methanogens remained between 0.01 and 3 % of the total population (Ince and Ince 2000).

The methanogenic communities detected in the M process were mainly represented by members of the archaeal genus *Methanosaeta* as shown in Fig. 4 and Table 2 (A', B', C' and D'). These results are in agreement to those obtained in single-stage anaerobic processes for OMSR degradation (Rincón et al. 2008a) and in two-stage anaerobic processes treating residues of cassava meal industry (Paixao et al. 2000). *Methanosaeta* has been suggested as the most tolerant methanogen to the inhibitory/toxic substances present in wastewaters such as olive oil mill wastewaters (García-García et al. 2000; Tabatabaei et al. 2010). The archaeal

**Table 3** Fractions (%) of the bacterial OTUs identified in this study from quantitative community fingerprinting analysis

ID	OLR (g COD L <sup>-1</sup> d <sup>-1</sup> )			Avg (sd) <sup>a</sup>
	11.0	12.9	14.0	
A	6.02	12.21	3.21	7.14 (4.60)
B	1.98	2.34	16.67	6.99 (8.38)
C	3.13	2.61	0.87	2.20 (1.18)
D	1.44	0.25	16.00	5.90 (8.77)
E	9.86	0.65	0.14	3.55 (5.47)
F	1.75	5.92	3.90	3.85 (2.08)
G	1.86	3.51	0.07	1.81 (1.71)
H	8.89	2.63	9.58	7.03 (3.83)
I				
J	0.16	6.79	8.43	5.12 (4.38)
K	0.06	9.20	nd	4.60 (6.50)
L	0.82	2.23	20.40	7.82 (10.93)
M	0.43	1.82	7.67	3.30 (3.84)
N	15.28	4.36	0.86	6.83 (7.50)
O	4.98	11.61	2.14	6.25 (4.85)
P	2.80	0.62	0.73	1.38 (1.22)
Q	3.63	0.67	2.44	2.25 (0.98)
R	0.85	nd	1.89	0.91 (0.95)
S	0.74	1.67	0.40	0.93 (0.66)
T	0.68	2.15	0.32	1.05 (0.97)
U	nd	6.44	1.09	2.51 (3.44)
V	29.06	11.25	1.04	13.78 (14.17)
W	2.51	10.22	1.05	4.60 (4.93)
X	3.06	0.85	1.10	1.67 (1.20)
Total	100	100	100	

ID letters correspond to the nomenclature of Fig. 3 and Table 2

nd not detected by DGGE fingerprinting

<sup>a</sup> Average (Standard deviation)

community fingerprints obtained by DGGE showed relatively constant profiles at most OLRs (2.0–18.5 g COD L<sup>-1</sup> d<sup>-1</sup>) (Fig. 4), where the reactor operation parameters were stable and the methane production was increasing. At the OLR with maximum methane production (20.0 g COD L<sup>-1</sup> d<sup>-1</sup>), the methanogenic phylotype C' was barely detected (Table 4). At higher OLRs, when the methane production decreased, there was a decrease in the proportion of the methanogenic phylotype D' (Table 4). This suggests its active role in reaching a maximum methane production and likely an inhibition of the growth at the high OLR tested. The possibility of these phylotypes being washed out is low because a support media (saponite) was used for microorganism immobilization with the aim of avoiding these losses. This dominant obligate acetotrophic methanogenic genus, *Methanosaeta*, was also found in a two-phase leach-bed system operating exclusively with triticale silage as the sole substrate. The detected Archaea

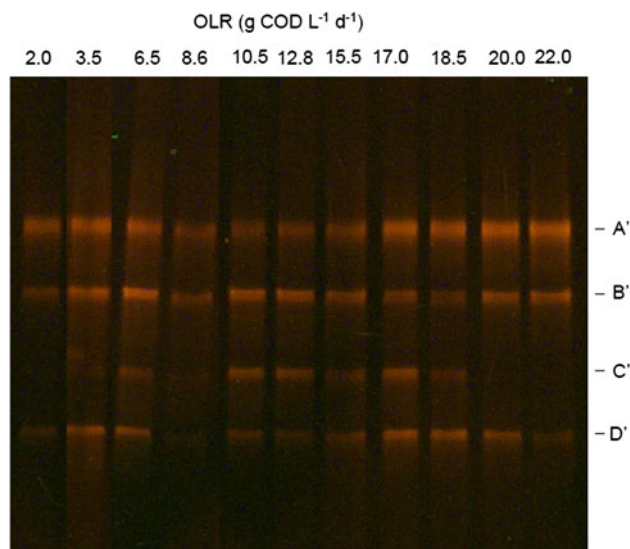


**Table 4** Fractions (%) of the archaeal OTUs identified in this study from quantitative community fingerprinting analysis

ID	OLR (g COD L <sup>-1</sup> d <sup>-1</sup> )											Avg (sd) <sup>a</sup>
	2.0	3.5	6.5	8.6	10.5	12.8	15.5	17.0	18.5	20.0	22.0	
A'	47.63	40.54	34.55	54.71	23.73	28.61	33.81	37.92	46.29	51.30	58.23	41.57 (11.02)
B'	36.70	30.03	37.02	25.26	35.93	36.76	34.94	17.34	16.06	26.61	31.13	29.80 (7.68)
C'	8.80	3.07	9.89	1.38	26.02	23.40	15.39	21.68	10.74	1.02	1.87	11.21 (6.74)
D'	6.87	26.36	18.54	18.65	14.32	11.23	15.86	23.06	26.91	21.07	8.77	17.42 (6.74)
Total	100	100	100	100	100	100	100	100	100	100	100	

ID letters correspond to the nomenclature of Fig. 4 and Table 2

<sup>a</sup> Average (Standard deviation)



**Fig. 4** DGGE fingerprints showing the major components of the Archaea developed in the M reactor at OLR of 2.0, 3.5, 6.5, 8.6, 10.5, 12.8, 15.5, 17.0, 18.5, 20.0 and 22.0 g COD L<sup>-1</sup> d<sup>-1</sup> (listing and Accession numbers provided in Table 2)

was closely related to *M. concilii* (with 0.2–0.5 % of divergence) (Klocke et al. 2008). Similar results were found by another study using 44 bioreactors with single-strand conformation polymorphism analyses. These reactors treated effluents from different origins such as agriculture, food processing, petro-chemical industries, pulp and paper plants, breweries, slaughterhouses and municipal wastes, and a vast number of existing reactor configurations were represented (fixed film, fluidized bed, stirred-tank, etc.). In these cases, the most frequent (84 % of digesters) archaeal sequences were close relatives of *Methanosaeta concilii* (Leclerc et al. 2004). *Methanosaeta* dominate in stable habitats, where the acetate levels are low, as they are specialist with a high affinity for acetate (McMahon et al. 2001; Ma et al. 2006). McHugh et al. (2003) found a dominance of *Methanosaeta* spp. in six different sludges, treating a variety of low- and high-strength, simple and complex wastewaters at psychrophilic (10–14 °C), mesophilic (37 °C) and thermophilic (55 °C) temperatures irrespective of wastewater type or

operating temperature, indicating the importance of this organism in the stable and efficient operation of an anaerobic bioreactor (McHugh et al. 2003).

## Conclusion

The microbial populations involved in the two-stage OMSR anaerobic digestion were represented by dynamic communities which adapt to the degradable substrate and to the reactors conditions. Two major bacterial groups dominated in the H-A stage: the Clostridiales (Firmicutes) and the Chloroflexi. The *Methanosaeta* genus among the Archaea was the main methanogen during the M stage. Understanding the behavior of microorganisms during the OMSR anaerobic treatment is important to obtain optimum results during the processing of these wastes. The knowledge of how the system works is decisive to optimize specific treatments and organic loads of anaerobic digesters for industrial application development.

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