Available online at www.sciencedirect.com
ScienceDirect
Bioresource Technology 99 (2008) 3233–3243
BIORESOURCE TECHNOLOGY

Evolution of microorganisms in thermophilic-dry anaerobic digestion

B. Montero *, J.L. Garcia-Morales, D. Sales, R. Solera

Department of Chemical Engineering, Food and Environmental Technologies, Faculty of Marine and Environmental Sciences, University of Cadiz, Campus Rio San Pedro s/n, 11510 Puerto Real, Cadiz, Spain

Received 11 January 2007; received in revised form 21 May 2007; accepted 21 May 2007
Available online 26 July 2007

Abstract

Microbial population dynamics were studied during the start-up and stabilization periods in thermophilic-dry anaerobic digestion at lab-scale. The experimental protocol was defined to quantify *Eubacteria* and *Archaea* using Fluorescent *in situ* hybridization (FISH) in a continuously stirred tank reactor (CSTR), without recycling solids. The reactor was subjected to a programme of steady-state operation over a range of the retention times from 40 to 25 days, with an organic loading rate between 4.42 and 7.50 kg volatile solid/m^3/day. Changes in microbial concentrations were linked to traditional performance parameters such as biogas production and VS removal. The relations of *Eubacteria*:*Archaea* and H_2-utilising methanogens:acetate-utilising methanogens were 88:12 and 11:1, respectively, during start-up stage. Hydrogenotrophic methanogens, although important in the initial phase of the reactor start-up, were displaced by acetoclastic methanogens at steady-state, thus their relation were 7:32, respectively. The methane yield coefficient, the methane content in the biogas and VS removal were stabilized around 0.30 LCH_4/gCOD, 50% and 80%, respectively. Methanogenic population correlated well with performance measurements.
© 2007 Elsevier Ltd. All rights reserved.

Keywords: Anaerobic digestion; Municipal solid waste; Fluorescent *in situ* hybridization; Population dynamics

1. Introduction

Anaerobic digestion has been widely used as a suitable treatment for organic waste, including the organic fraction of municipal solid waste (OFMSW) (Akao et al., 1992; Moorhead and Nordstedt, 1993). This process has many advantages and these include a low sludge generation, reduced energy consumption and high methane production. The main disadvantage of anaerobic digestion is its slowness (Chanakya et al., 1992). Anaerobic processes operating under thermophilic (55 °C) conditions have attracted a great deal of attention in recent years due to their apparent advantages, which include high pathogen destruction, enhanced hydrolysis of complex organic/biological materials, and foaming reduction (Hartmann and Ahring, 2005). Besides, with this technology two residual effluents are produced: biogas (mainly methane and carbon dioxide) which can be used as an energy source, and a liquid effluent which could be used as a soil conditioner due to its physicochemical properties (Flotats et al., 1997).

Anaerobic digestion of organic fraction of municipal solid waste (OFMSW), which is ultimately converted into methane and carbon dioxide, is carried out by the coordinated action of various groups of microorganisms and goes through several intermediate stages. The intermediary products are volatile fatty acids, acetic, propionic, and butyric acids. Two-thirds or more of the methane produced in anaerobic bioreactors is derived from acetate (Zinder, 1993). The conversion of acetate to methane by methanogenic populations becomes the rate-limiting step in biogas production, as methanogens are known for their slow growth, resulting in a relatively small population size (Zinder, 1993). The methanogens occupy the terminal position in the anaerobic food chain and are normally divided into two main groups based on their substrate conversion capabilities. Acetoclastic methanogens are capable of converting acetate to methane and carbon dioxide and

* Corresponding author. Tel.: +34 956016157.
E-mail address: blanca.montero@uca.es (B. Montero).

0960-8524/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2007.05.063

are regarded as playing a dominant role in methane production since ca. 70% of the methane produced in digesters comes from acetate (Zinder, 1993). Hydrogenotrophic methanogens convert H_2/CO_2 to methane. These species also play a key role in the overall process by maintaining the very low partial pressures of H_2 (<10 Pa) necessary for the functioning of the intermediate trophic group, the syntrophic bacteria, which are responsible for the conversion of acids organic and alcohol intermediates to direct methane precursors (Pauss et al., 1990).

The parameters normally employed in the control of anaerobic digestion, such as the percentage of COD removal, the concentration of volatile fatty acids and the amount and composition of biogas generated in the process, are not always representative of the composition and physiological state of biomass contained within the system. From a practical standpoint, given the importance of methanogens in anaerobic treatment processes, the ability to monitor methanogens and understand their ecology is essential to make effective controls of the start-up and operation of anaerobic bioreactors possible.

Consequently, and in order to acquire more detailed information in respect of this biomass, other parameters have also been used in the characterization of the microorganisms responsible for the anaerobic processes. Direct count procedures by microscopic methods yield the highest estimates of members of microorganisms and are occasionally used for indirect calculation of biomass. Epifluorescence microscopy with fluorometric stains are widely used for direct counting of microorganisms, since it does not require culturing (Kepner and Pratt, 1994).

There are, however, several drawbacks to direct observational methods, including the inability to distinguish living from dead microorganisms and the inability to perform further studies on the observed microorganisms. Because of this, an important avenue of research has been the development and utilization of molecular techniques. Molecular techniques have successfully been applied for the direct detection and identification *in situ* of individual microbial cells and have therefore been used to monitor the spatial distribution of microorganisms in environmental samples and treat systems. However, it must be kept in mind that the physiological state (living or non-living) is operationally defined and based on the general properties of a particular stain, and dormant or extremely slow-growing cells cannot be detected (Williams et al., 1998).

Whole cell fluorescence *in situ* hybridization (FISH) is a technique that uses fluorescently labelled phylogenetic oligonucleotide probes to detect specific whole cells/organisms in biological samples. DeLong et al. (1989) first demonstrated its use with bacteria. It can be a valuable tool for the study of microbial dynamics in natural environments (Hugenholtz et al., 2001; Davenport and Curtis, 2004). For instance, it is possible to carry out a hierarchical phylogenetic analysis on a particular environment to identify the dominant groups of microorganisms present, after which temporal and spatial changes in the diversity and abundance of specific microbial population can be monitored in relation to environmental effects (Amann et al., 2001; Head et al., 1998; Zheng et al., 2006). Two good examples can be found in Sekiguchi et al. (1999), who used FISH to study the morphology of the flocs in an UASB reactor, and Santegoeds et al. (1999), who studied the morphology of aggregates present in three UASB lab-scale reactors. Raskin et al. (1994a,b) have used to identify and quantify species and genus of methanogens present in anaerobic reactors. The microorganisms in anaerobic reactors belongs to three domains: *Bacteria*, *Archaea* and *Eucarya*. *Bacteria* is the majority of the microorganisms in the reactors, *Archaea* are present in smaller amounts and *Eucarya* is present at very low levels (below 1% in most cases) which it indicates that anaerobic protozoa likely is not abundant in anaerobic digesters (Griffin et al., 1998).

The main objective of this work was to estimate the concentration of principal groups of microorganisms involved in anaerobic depuration during start-up and stabilization of a thermophilic-dry anaerobic reactor. This was accomplished by Fluorescent *in situ* Hybridization (FISH), employing different oligonucleotide probes.

2. Methods

2.1. Experimental system

The experimental protocol was designed to quantify the main microbial population contained in a laboratory-scale continuously stirred tank reactor (CSTR), without recycling solids (see Fig. 1). In this reactor, the solids and hydraulic retention times are equal. The reactor was operated from 40 to 25 days of retention time (RT). The organic loading rate added to the system was modified, but a constant organic loading rate (expressed as kg volatile solids/m^3/day) was maintained in each RT. It was used four organic loading rates: 4.42, 5.07, 5.92 and 7.50 kg volatile solids/m^3/day.

Fig. 1. Diagram of CSTR used in the experimental protocol.

The stirred tank reactor consisted of vertical cylinder tank. The active liquid volume was 4.5 L. Reactor temperature was maintained at 55 °C and the biogas generated was collected in a Tedlar bag (40 L). The feed was added in a semi-continuous mode: one dose per day. Shaking stick was used to mix and homogenise the liquid in the system (13 rpm). pH was controlled by a controller on/off, using dissolutions NaOH 5 N and H_3PO_4 1 N, that they were added 0.2 L/h by peristaltic pump when it was strictly necessary. Initial pH of OFMSW–inoculum was 7.2. The range of operation of pH controller is around 6.5–8. These values are appropriate for methanogenic population (Vavilin et al., 1998).

2.2. Inoculum and feed solution

The reactor was loaded with 1.5 kg of milled dry synthetic OFMSW (90% TS). The moisture was adjusted using an inoculum from SEBAC that consisted of a 1:1 v/v mixture of thermophilic sludge and leachate. In this sense, 4 L of inoculum (2 L of sludge + 2 L of leachate) were required to add moisture to the synthetic OFMSW. The compositions of the different inoculum and wastes used are given in Table 1 (Fernández-Güelfo et al., 2005).

A synthetic feed based on the nutritional requirements of the main populations of microorganisms involved was prepared (Martin et al., 1999). This type of feed avoids the problem of high variations in the composition of the source selected OFMSW.

The inoculum used was a 1:1 v/v mixture of leachate and sludge from the SEBAC (Sequential Batch Anaerobic Composting) reactors A and B, respectively. The reactor A was constituted of OFMSW from restaurant of Faculty of Marine and Environmental Sciences in University of Cadiz and anaerobically digested swine wastes, and the reactor B was mesophilic anaerobic sludge from "*Guadalete*" municipal wastewater treatment plant in Jerez.

The control of the total solid concentration of the feed is necessary to obtain a suitable level of performance for the dry anaerobic digestion. Therefore, pre-treatment of the OFMSW samples was necessary to adjust them to the required optimum values. The samples were dried at 55 °C for 48 h and then at ambient temperature for 72 h until final moisture content of 10% was achieved. The dried OFMSW was milled until a particle size of approximately 1 cm was obtained and, finally, the moisture was adjusted to 70–75% (25–30% TS, which is characteristic of dry anaerobic digestion) with water, leachate from garbage, sludge or combinations of these (De Baere, 2000).

2.3. Start-up strategy

The protocol employed for the start-up phase was presented by Fernández-Güelfo et al. (2005). This approach allows a reduction in the time necessary for the start-up and stabilization stages of a thermophilic anaerobic reactor operating with a high solid concentration (30% TS). The new procedure uses a mixture of two thermophilic inocula (anaerobic sludge and leachate) from SEBAC (Sequencing Batch Anaerobic Composting) technology to adapt the inoculum to the solid waste and the operational conditions prior to seeding the semi-continuous reactor. The level of inoculum employed was 88% of the final digester volume and started the daily feeding schedule immediately. The start-up of the thermophilic anaerobic reactor operating with a high solids concentration (30% TS) was reached to 14 days approximately and the stabilization phase to 30 days of operation.

2.4. Analytical methods

The analytical determinations made in this study can be grouped in two categories: those utilised to monitor and control the anaerobic digestion process and those utilised to count the microbial population in the reactors.

Table 1
Composition of inoculum and wastes (Fernández-Güelfo et al., 2005)

Parameter	Units	Inoculum		Synthetic OFMSW	OFMSW/inoculum mixture
		Leachate	Sludge		
pH	SU	8.62	8.35	7.78	8.70
Density	kg/m^3	980	985	750	1116
Alkalinity	$gCaCO_3/L$	21.78	16.54	4.29	5.14
Ammonium	gNH_3-N/L	26.88	14.56	1.68	2.80
Total nitrogen	gNH_3-N/L	25.66	21.46	207.20	72.80
Total suspend solid	gTSS/L	14.46	20.46	–	–
Volatile suspend solid	gVSS/L	10.73	9.16	–	–
Mineral suspend solid	gMSS/L	3.73	11.30	–	–
Total solid	gTS/L	–	–	0.90	0.31
Total volatile solid	gTVS/L	–	–	0.71	0.25
Total mineral solid	gTMS/L	–	–	0.19	0.07
Total carbon	mg/g	80.78	35.27	112.60	65.07
Total inorganic carbon	mg/g	2.07	0.96	0.29	0.30
Total organic carbon	mg/g	78.41	34.31	112.30	64.75
Acidity	mgAcH/L	12403	17353	1440	356

2.4.1. Analytical determinations utilised to monitor and control the anaerobic digestion process

For the control of the reactors the following parameters were determined: the volume and composition of the biogas (H_2, O_2, N_2, CH_4 and CO_2), volatile fatty acids (VFA), total solids (TS), volatile solids (VS), pH and chemical oxygen demand (COD). The analytical techniques were measured according to Standard Methods (APHA; AWWA; WPCF (1990)) and Alvarez-Gallego (2005).

2.4.2. Analytical determinations utilised to count microbial population

The main steps of fluorescence *in situ* hybridization (FISH) of whole cells using 16S rRNA-targeted oligonucleotide probes are cell fixation, consequent permeabilization and hybridization with the desired probe(s).

The samples were collected from thermophilic-dry anaerobic reactor into sterile universal bottles. Absolute ethanol was added to the bottles in a volume ratio of 1 sample:1 ethanol. The samples were stored at −20 °C until they were fixed as described in the following section (generally within two weeks).

The technique used for fixation and permeabilization of cells was based on the one used by Amann et al. (1990a). Previously, the samples were pre-treated. The most appropriate pre-treatment applied for microbiological count of high solids content samples was the addition of Tween 80 and 120 seconds of shaking. 1 mL of pre-treated OFMSW sample was transferred to a 1.5 mL eppendorf tube and centrifuged at 13,000*g* for 3 min. The supernatant was removed and the sample was washed using 1 mL of phosphate buffer saline (PBS). The sample was vortexed and centrifuged at 13,000*g* for 3 min. The supernatant was removed and the pellet was resuspended in 0.25 mL of PBS and 0.75 mL of 4% paraformaldehyde fixative solution. The sample was then incubated at 4 °C over night. Before hydridization, the fixed samples were diluted with PBS (pH 7.2). Cell samples for *in situ* hybridization with a family *Methanobacteriacea* specific probe (MB1174) were treated by a freeze thawing method to improve probe permeability into the cells (Sekiguchi et al., 1999).

Fixed cells (10 μL of sample/well) were spotted on to gelatin coated slides, air-dried at 37 °C for 20 min, and dehydrated by serial immersion of the slides in 50%, 80% and 96% (v/v) aqueous ethanol solutions for 3 min each (Amann et al., 1992). For each hybridization, 8 μL hybridization solution (4.5 M NaCl, 200 mM Tris–HCl [pH 7.2], 10% SDS and formamide [theoretical concentrations for each probe, see Table 2]) and 2 μL of probe solution (50 ng/μL) were applied to each well of the slides. The following 16S rRNA-targeted oligonucleotide probes were used in this study: *Bacteria*-universal probe EUB338 (Amann et al., 1990a,b), *Archaea*-universal probe ARC915 (Stahl and Amann, 1991), H_2-utilising methanogens probe MB1174 (specifically *Methanobacteriaceae*) (Raskin et al., 1994a,b; Sekiguchi et al., 1999) that are the main hydrogen scavengers during start-up of thermo-

Table 2
Oligonucleotide probes used in this study, classified according to the oligonucleotide probe database (Alm et al., 1996)

Probes	Position	Probe sequences (from 5′ to 3′)	Target	Formamide (%)	Temperature (°C)	Reference
S-D-Bact-0338-a-A18	338–355	GCTGCCTCCCGTAGGAGT	*Eubacteria*	20	46	Amann et al. (1990b)
S-D-bact-0338-a-S-18	338–355	ACTCCTACGGGAGGCAGC	None (negative control)	20	46	Amann et al. (1990a)
S-D-Arch-0915-a-A-20	915–934	GTGCTCCCCCGCCAATTCCT	*Archaebacteria*	35	46	Stahl and Amann (1991)
S-F-Mbac-1174-a-A-22	1174–1195	TACCGTCGTCCACTCCTTCCTC	*Methanobacteriaceae* (H_2-utilising methanogens)	35	46	Raskin et al. (1994a,b), Sekiguchi et al. (1999)
S-F-Msae-0825-a-A-23	825–847	TCGCACCGTGGCCGACACCTAGC	*Methanosaeta* (acetate-utilising methanogens)	20	46	Raskin et al. (1994a,b)

philic-dry anaerobic reactors (Griffin et al., 1998) and acetate-utilising methanogens probe MX825 (specifically *Methanosaeta*) (Raskin et al., 1994a,b). The genus *Methanosaeta* use only acetate like characteristic substrate (Griffin et al., 1998; Raskin et al., 1994a,b). All probes were labelled with 6-FAM at 5′ terminal, except S-D-bact-0338-a-S-18 labelled with Cy3. The slides were incubated at the appropriate temperature for the probe (usually 46 °C) for 1.5 h in an isotonic moisture chamber.

After hybridization, the slides were flushed with 2 mL of wash solution (4.5 M NaCl, 200 mM Tris–HCl [pH 7.2], 10% SDS and 0.5 M EDTA [pH 8.0]) and immediately were immersed in enough volume of the same wash solution as to cover the wells of interest (e.g. approx. 50 mL in an universal tube) at 48 °C for 15 min. This step was repeated. Finally, the slides were rinsed thoroughly with milliQ water and were air-dried for 5 min. These slides may be stored in the dark at −20 °C, or viewed immediately.

For viewing, the slides were mounted in a small drop of the antifadant Citifluor (AF1, Canterbury, UK) prior to the addition of a cover glass. The edges of the cover glass should be coated in colourless nail varnish, to prevent movement of the slide and evaporation of the Citifluor, when an oil-immersion lens is to be used during microscopy. The slides prepared in this manner can be stored in the dark at 4 °C. If the slide is not viewed using an oil-immersion lens, the cover glass may be removed after viewing, the Citifluor can be rinsed away with milliQ water, and the slide air-dried prior to storage in the dark at −20 °C. The samples were examined visually and cells counted using a Nikon Labophot-2A/2 epifluorescence microscope fitted with a 100 W mercury lamp and an x100 oil objective. According of labelled probe, if the fluorochrome was 6-FAM, the filter was used B-2A (DM 510, Excitation 450–490 and Barrer 520) and Cy3, the filter was G-2A (DM 580, Excitation 510–560 and Barrer 590). Digital images of the slides were taken with a digital camera. For each sample, 20 fields of view in randomly located were counted.

2.4.3. Statistical analysis

The relation between microbial populations and performance and operating parameters in reactor were studied by Pearson correlation. The results that present a Pearson's correlation coefficient higher than 0.8 were checked for linear regression with Spss v11.5 program.

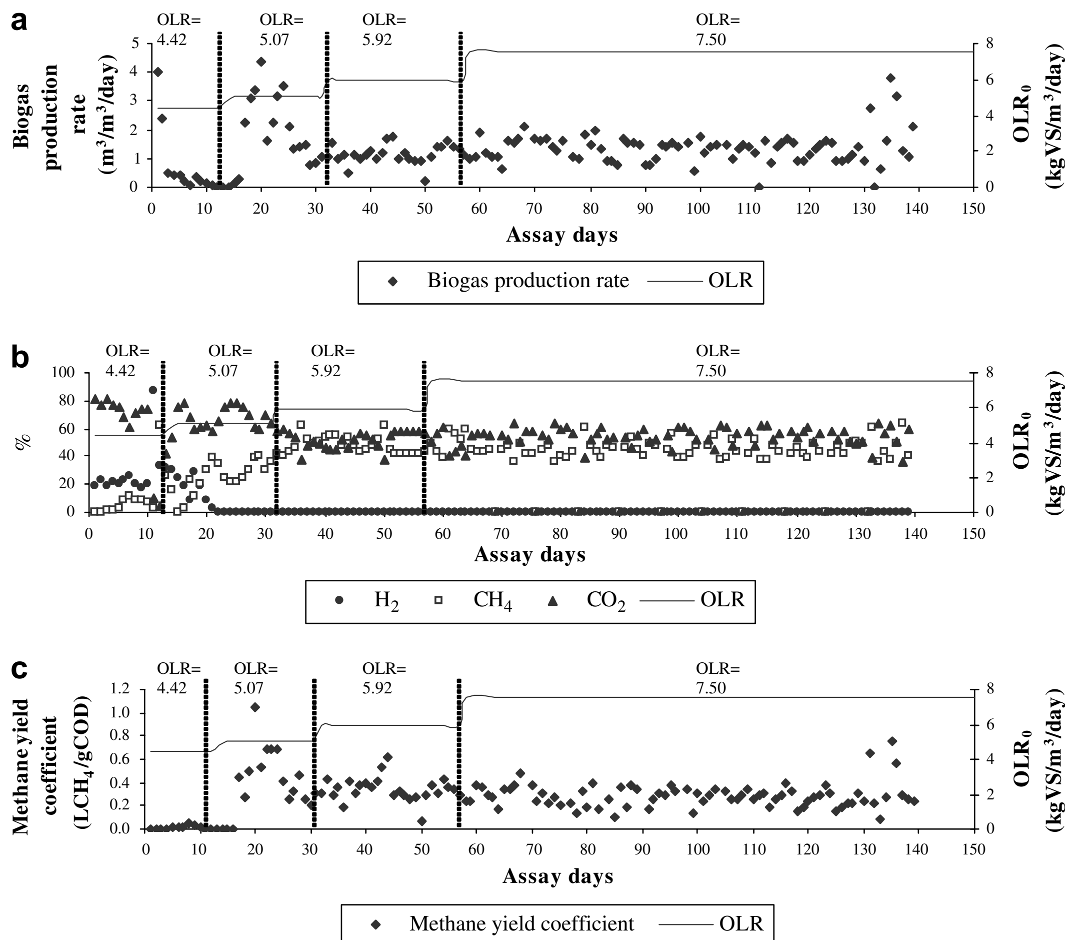

Fig. 2. Performance and operating parameters for the control of the anaerobic process I (Fernández-Güelfo et al., 2005): biogas production rate (a); percentage of hydrogen, carbon dioxide and methane (b) and methane yield coefficient (c).

3. Results and discussion

3.1. Microbial characterization of the inoculum

Since the methanogenesis is critically important during start-up, we determined methanogens levels in the inoculum using oligonucleotide hybridization probes (Table 2). The inoculum contained a percentage of *Archaea*, approximately 23%. This value is higher than those obtained by Griffin et al. (1998) (12.1%) and McMahon et al. (2001, 2004) (4.52%). Therefore, high content methanogens in our inoculum could be due to its previous acclimation and the utilization of anaerobic sludge and leachate as source of inoculum. In fact, the protocol used allows a reduction in the time necessary for the start-up and stabilization stages of a thermophilic anaerobic reactor operating with a high solid concentration (30% TS). This procedure uses SEBAC (Sequencing Batch Anaerobic Composting) technology to adapt an inoculum to the solid waste and the operational conditions prior to seeding the reactor.

The relation of main methanogenic group (H_2-utilising methanogens and acetoclastic methanogens) present in the inoculum was 15:8, respectively. Previous work (Griffin et al., 1998) showed levels of H_2-utilising methanogens lower than obtained in our case. The high percentage of H_2-utilising methanogens in the inoculum used could explain the very fast start-up of our digester due to the key role played for this microbial group as H_2-consumers during hydrolytic/acidogenic stage.

3.2. Digester performance and microbial population dynamics

The reactor was started under thermophilic conditions (55 °C) and four organic loading rates (OLR_0) were assayed in order to study the dynamics of microbial population during the start-up and stabilization phase of anaerobic process. The organic loading rate added to the system was modified, but a constant organic loading rate (expressed as kg VS/m^3/day) was maintained in each period.

The microbial community structure was evaluated in combination with physicochemical parameters to assess digester performance during start-up and stabilization periods. A selection of rRNA-oligonucluotide probes (Table 2)

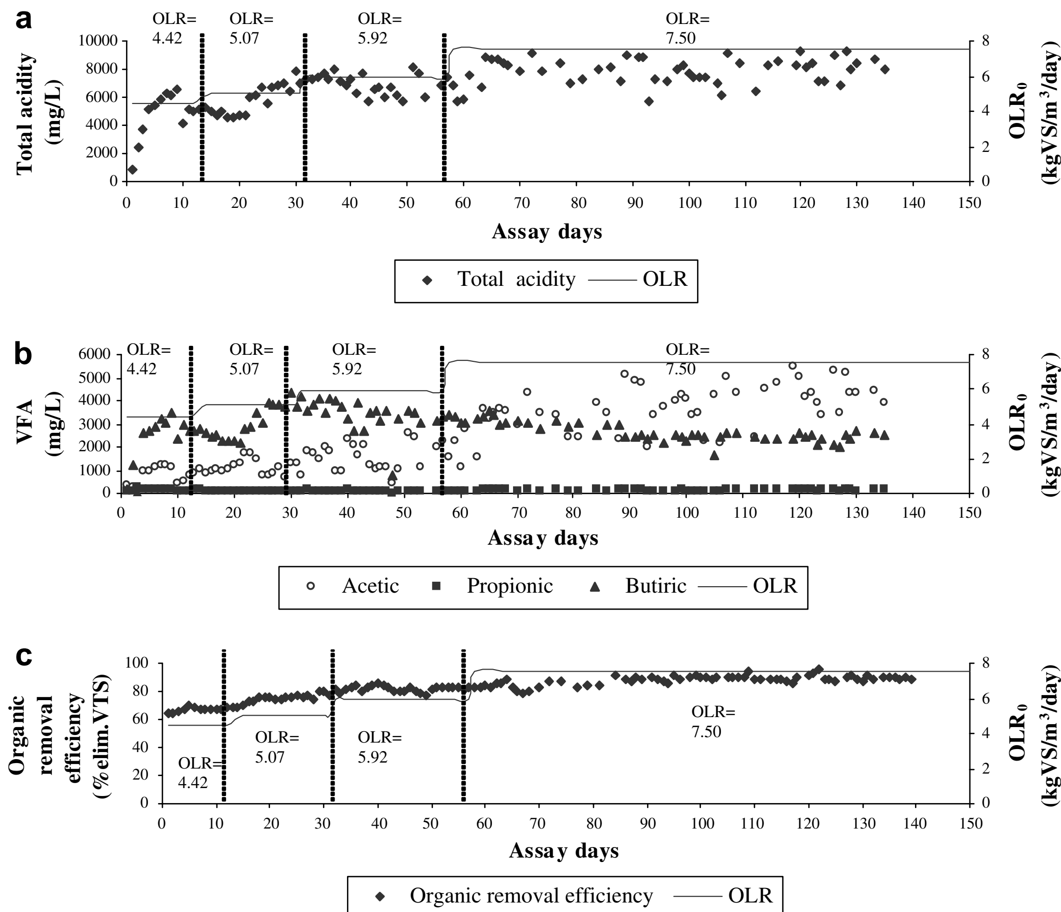

Fig. 3. Performance and operating parameters for the control of the anaerobic process II (Fernández-Güelfo et al., 2005): total acidity (a); volatile fatty acids (b) and organic removal efficiency (c).

Table 3
Microbial community structure in thermophilic-dry anaerobic reactor (% ±SD)

Probe	Target group	OLR = 4.42	OLR = 5.07	OLR = 5.92	OLR = 7.50
S-D-Bact-0338-a-A-18	Most *Bacteria*	87.58 ± 32.01	67.43 ± 16.74	60.66 ± 7.89	60.41 ± 15.10
S-D-Arch-0915-a-A-20	Most *Archaea*	12.42 ± 7.10	32.57 ± 17.31	39.34 ± 8.61	39.59 ± 8.57
S-F-Mbac-1174-a-A-22	*Methanobacteriaceae* (H_2-utilising methanogens)	11.11 ± 5.56	8.58 ± 3.34	6.78 ± 2.12	7.19 ± 1.39
S-F-Msae-0825-a-A-23	*Methanosaeta* (acetate-utilising methanogens)	1.32 ± 5.28	23.99 ± 16.91	32.56 ± 7.01	32.40 ± 7.81

All the results shown are the average values for each OLR is organic loading rate expressed as kg volatile solids/m^3/day.

was used to determine the concentrations of the main domains (*Eubacteria* and *Archaea*) and H_2-utilising and acetoclastics methanogens in samples collected from the reactor during the course of the experiment.

Performance and operating parameters for the control of the anaerobic process are shown in Figs. 2 and 3 (Fernández-Güelfo et al., 2005). Results of microorganims obtained by FISH are shown in Table 3. All the results shown are the average values for each OLR assayed. The dynamics of microbial populations are represented in Fig. 4. The sum of the relative amounts of *Eubacteria* and *Archaea* was estimated as 100% because the main anaerobic groups in the anaerobic reactors are contained within these two domains (Griffin et al., 1998).

In the first stage, the OLR_0 imposed was relatively slow (4.42 kg volatile solids/m^3/day) in order to check if the system evolved appropriately. This value reported in the literature. Bolzonella et al. (2003) carried out start-up phase in the mesophilic range with an extremely low organic loading rate – less than 1 kg volatile solids/m^3/day – for approximately 40 days, and continuing subsequently with an increase in the OLR and temperature. As consequence, the start-up period described in the literature are around 250 days (Bolzonella et al., 2003; Sebastien et al., 2002), whereas the start-up of the reactor studied was reached to 14 days approximately as described in Section 2.3. This can be explain by considering that, in our case, the start-up phase was carried out using inoculum adapted to the waste and operational conditions: i.e., thermophilic range and dry condition, with a high content of H_2-utilising methanogens.

During the hydrolysis phase complex molecules are transforming into others simpler products, without methane production. In the first days, fementers can acclimate more quickly to new conditions because of their relatively high growth rates, while methanogens grow much slower. Hydrogen, carbon dioxide and butyrate were the main products of the fermentative pathways of hydrolytic and fermentative bacteria. Because of the metabolic capacity of methanogens was initially not sufficient to balance increasing activity of the fementers, acetate and hydrogen were not consumed at the same rate at which they are produced. In this sense, even though significant levels of methanogens were present in the digester, they were apparently not able to adjust within 1 day to operation conditions, as demonstrate by the low methane levels in the biogas on the first stage. Under these conditions, the electron flux through reduced intermediates (butyrate) increased (Fig. 3a and b). In fact, *Eubacteria* were the main group in the reactor (88%) while *Archaea* were maintained constant 12%, except on the first day. Thus, the gas production rate was low and its composition was usual for hydrolytic phase: H_2 (20%) and CO_2 (80%) (see Fig. 2b).

The total methanogen concentrations remained relatively constant during the first few days of operation, because the loss in acetate-utilising methanogens was compensated by an increase in the H_2-utilising methanogens levels. These microorganisms are the most important H_2-consumer in the acidogenic phase. The latter apparently served as the main hydrogen scavengers during this period of rapidly increasing activity, reflected by rapid increases in the gas production rate and the level of methane in the

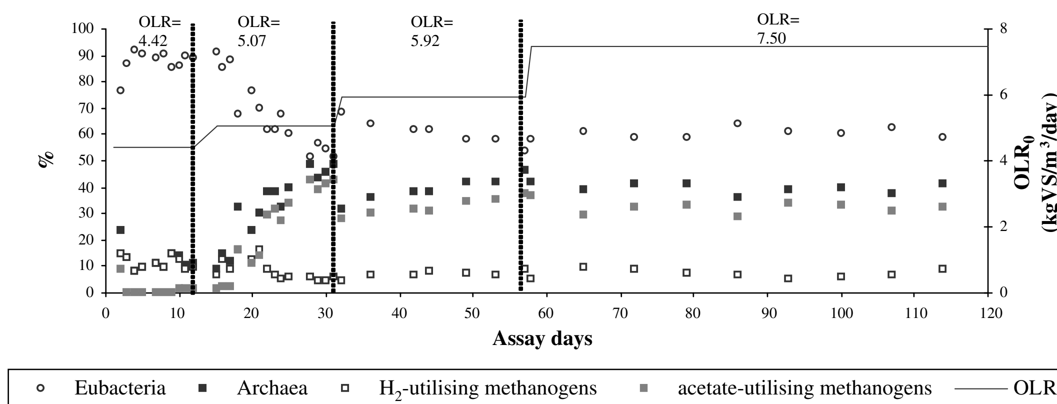

Fig. 4. Evolution of *Eubacteria*, *Archaea,* H_2-utilising methanogens and acetate-utilising methanogens in reactor of OFMSW.

biogas during the next stage (Fig. 2a and b). When the levels of H_2-utilising methanogens increased, butyrate levels also decreased after 10 days of operation in the digester. However, acetate levels increased continuously during all studied period (Fig. 3b). The relation between H_2-utilising methanogens and acetate-utilising methanogens was 11:1 during start-up stage (Fig. 4).

The results obtained in the first 14 days were favourable and therefore the organic loading rate was increased to 5.07 kg VS/m^3/day. During this stage, methane content in the biogas had increased until reached 45% at 30 days. This can be explained by the VFA biodegradation by acetogens and subsequent methane generation by acetate-utilising methanogens. In fact, during this period, the decrease of hydrogen content was accompanied by an increased of acetate-utilising methanogens until these became a 24% of *Archaea*. After a short start-up period (30 days) stable performance was observed with high organic removal efficiency (89% VS), high gas production rates (1.94 m^3/m^3/day) and substantial levels of methane in the biogas (45%) (see Fig. 2a and b and Fig. 3c). The increase of *Archaea* was higher than *Eubacteria*, thus the relation between them was 33:67, respectively (Fig 4). The rise of stable performance was paralleled by an increase in acetoclastic methanogens.

In the OLR_0 5.92 kg VS/m^3/day period, after a slight decrease in the *Archaea*, these was recovered, no accumulating great amount of VFA because of control of pH by a controller on/off connected system. Nevertheless, the volume of biogas generated and methane yield coefficient decreased from 1.94 to 1.16 m^3/m^3/day and from 0.42 to 0.34 LCH_4/gCODdegraded, respectively (Fig. 2a and c). This was because most of the initial residue which the reactor was loaded had been degraded. However, the methane content in the biogas increased from 25% to 48% (Fig. 2b). At the end of this stage, the composition of biogas was stabilized with values of CO_2 and CH_4 at around 50% which indicated that the balance between the different microbial populations involved in the digestion was reached. The relative abundance between *Eubacteria* and *Archaea*, was 61:39, while acetoclastic constituted 33% of those all of methanogens (see Fig. 4).

In the last period, the microorganisms concentrations increased since there was an increased of OLR_0 (7.5 kg VS/m^3/day). The relation *Eubacteria* and *Archaea* was 60:40, with 32% of acetoclastic methanogens (see Fig 4). These values were practically similar to those obtained in previous stage and according to the physicochemical parameters. Thus, the organic removal efficiency and methane yield coefficient were maintained constant around 80% VTS and 0.30 LCH_4/gCODdegraded, respectively (see Fig. 2c and Fig. 3c).

The gas production rate increased to 1.36 m^3/m^3/day due to the higher organic loading rate (Fig. 2a). This result was compared favourably to those obtained in other studies where gas production rates varied between 1.6 and 1.7 m^3/m^3/day (Stroot et al., 2001). The VS removal obtained to our study (88%) was better compared to data provided in the literature (67–68%) (Stroot et al., 2001). However, an increase of organic loading rate is accompanied by an increased of acetate concentration. Thus, acetatoclastic methanogens were nor able to consume acetate quickly in the digester to prevent acid accumulation. A history of high acetate concentrations appears to select for a population of methanogens capable of more rapid acetate turnover. This effect was observed previously in similar system (Griffin et al., 1998; McMahon et al., 2001), confirming previous hypothesis (Zinder, 1993): *Methanosarcina* (generalist with high growth rates at elevated acetate concentrations) should be favoured in systems with significant acetate accumulation, while *Methanosaeta* (specialist with a higher affinity for acetate) should have a competitive advantage in much more stable habitats, in which acetate levels are low. In fact, it had been shown that digesters that started up successfully contained high levels *Methanosaeta* (McHugh et al., 2003; McMahon et al., 2004; Pender et al., 2004).

Since the propionate level persisted at relatively low (between 110 and 150 mg/L) during all assay, we suggest that propionate-degrading syntrophs (e.g., *Syntropher wolinii*) could be present in high numbers in our inoculum. These syntrophs only can use a very limited range of substrate (Schink, 1992) and have very low specific growth rates, so they need an extensive amount of time to reduce propionate concentrations. On the other hand, butyrate-degrading syntrophs (e.g., *Syntrophomonas wolfei*) could be not present in high numbers in our inoculum. Thus, while propionate was consumed rapid in the digester, the accumulated butyrate was removed very slowly by washout and/or conversion to acetate by butyrate-degrading syntrophs. To further investigate this hypothesis, our studies of population dynamics in digester systems need to be complemented with studies of population dynamics of propionate-degrading syntrophs and others syntrophic fatty acid oxidizing bacteria.

3.3. Correlations between microorganisms and physicochemical parameters

Some correlations were obtained between physicochemical parameters and microbial concentrations (see Fig. 5).

These results have shown that changes in microbial population, mainly methanogens, can be linked to the traditional performance parameters.

The results of total methanogenic population (*Archaea*) and specifically acetate-utilising methanogens were positive linear correlation with the volume of methane generated in the digester ($R^2 = 0.76$ and 0.72, respectively). These results are in accordance to obtain in mesophilic anaerobic digestion treating others kind of feedstock (Stroot et al., 2001).

Finally, *Archaea* and acetoclastic methanogens were positive linear correlation with organic loading rate removal ($R^2_{\text{Archaea}} = 0.79$ and $R^2_{\text{acetoclastic methanogens}} = 0.76$) and VS removal ($R^2_{\text{Archaea}} = 0.80$ and $R^2_{\text{acetoclastic methanogens}} = 0.80$).

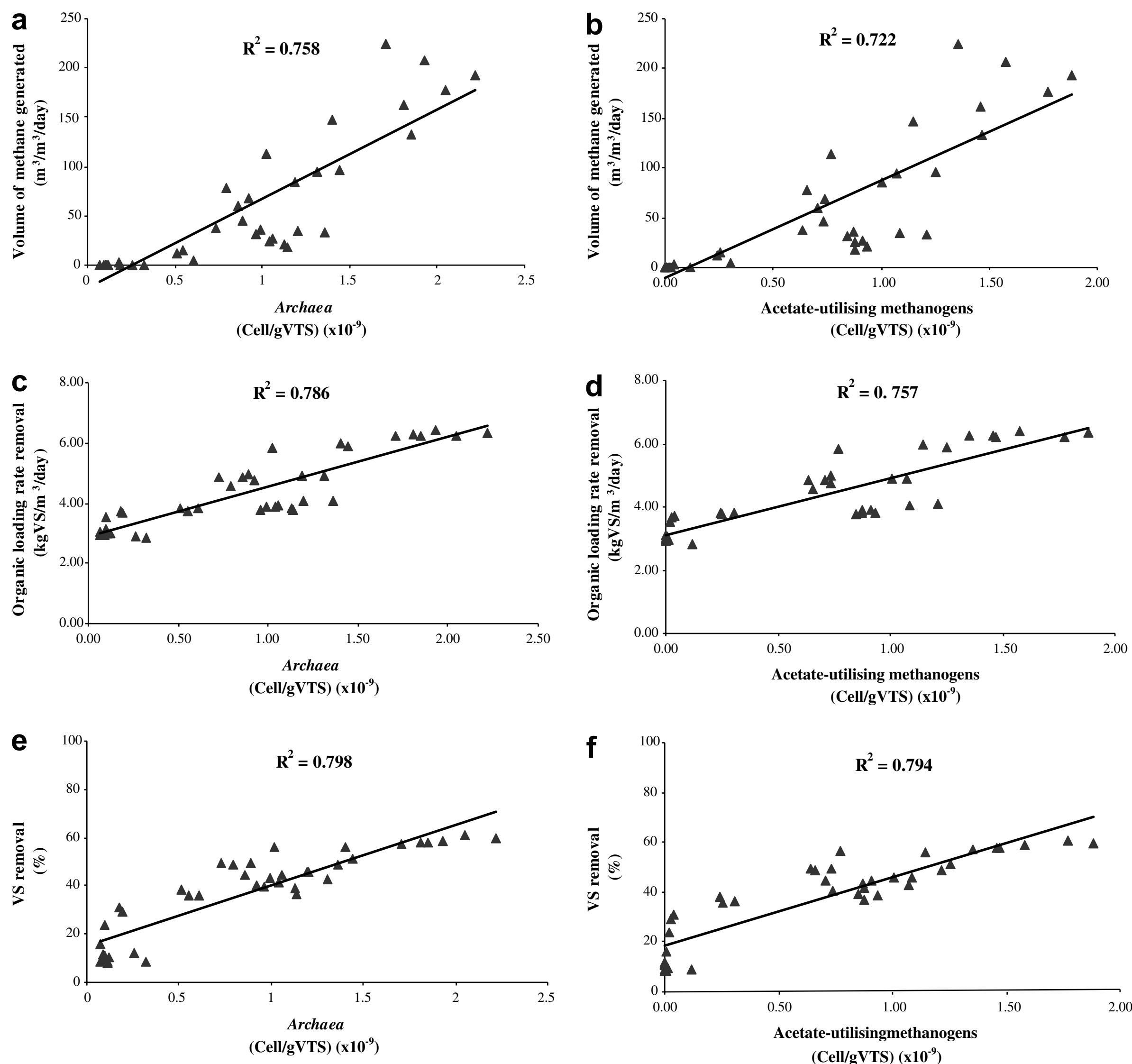

Fig. 5. Linear correlations between physicochemical parameters and microbial concentrations. *Archaea* with volume of methane generated, organic loading rate removal and VS removal (a, c, e), respectively and acetate-utilising methanogens with volume of methane generated, organic loading rate removal and VS removal (b, d, f).

During this stage, these physicochemical parameters reached their maximum values.

4. Conclusions

It has been shown that the application of 88% inoculum from thermophilic SEBAC with 23% of methanogens (15% H_2-utilising methanogens: 8% acetoclastic methanogens) has been successful to reach rapid start-up of reactor.

The development of a stable microbial community, *Eubacteria* and *Archaea*, during start-up of the reactor has been shown with a ratio 88:12, respectively. It was clearly indicated that hydrogenotrophic methanogens, although important in the initial phase of the reactor start-up (11% of total methanogens), were displaced by acetoclastic methanogens at steady-state (32% of total methanogens). In the stable conditions, the percentages were maintained at 60:40 for *Eubacteria* and *Archaea*.

We demonstrated links between digester operating conditions, physical and chemical performance parameters, and microbial population dynamics. The results have clearly indicated that the relative abundance of *Archaea* and acetoclastic methanogens was directly correlated with organic loading rate, volatile solids removals and methane production by anaerobic reactor.

Acknowledgements

This work was supported by the Spanish Ministry of Science and Technology (MCyT) and Education and Science (MEC), projects PPQ2001-4032 and CMT2004-01655.

B. Montero thanks funding from Council of Education and Science of Junta de Andalucia.

The authors thank Dr. T. Curtis and Dr. R. Davenport (Department of Civil Engineering and Geosciences. Newcastle University) for teaching molecular techniques using in this work.

References

Akao, T., Mizokl, E., Saito, H., Okumura, S., 1992. The methane fermentation of Citrus unshu peel pretread with fungus enzymes. Bioresour. Technol. 41, 35–39.

Alm, E.W., Oerther, D.B., Larsen, N., Stahl, D.A., Raskin, L., 1996. The oligonucleotide probe database. Appl. Environ. Microbiol. 62, 3557–3559.

Alvarez-Gallego, C.J., 2005. Testing different procedures for the start up of a dry anaerobic codigestión process of OFMSW and sewage sludge at thermophilic range. ISBN-84-7786-356-3. University of Cadiz.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., Stahl, D.A., 1990a. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.

Amann, R.I., Krumholz, L., Stahl, D.A., 1990b. Fluorescent-oligonucleotide probing of whole cells for determinative phylogenetic and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Amann, R.I., Ludwig, W., Schleifer, K.-H., 1992. Identification and *in situ* detection of individual bacterial cells. FEMS Microbiol. Lett. 100, 45–50.

Amann, R.I., Fuchs, B.M., Behrens, S., 2001. The identification of microorganisms by fluorescence *in situ* hybridisation. Curr. Opin. Biotechnol. 12, 231–236.

APHA; AWWA; WPCF, 1990. Métodos normalizados. Para análisis de aguas potables y residuales. Ed. Díaz de Santos, S.A.

Bolzonella, D., Innocenti, L., Pavan, P., Traversa, P., Cecchi, F., 2003. Semi-dry thermophilic anaerobic digestion of the organic fraction of municipal solid waste: focusing on the start-up phase. Bioresour. Technol. 86, 123–129.

Chanakya, H.N., Borgaonkar, S., Rajan, M.G.C., Wahi, M., 1992. Two-phase anaerobic digestion of water hyacinth or urban garbage. Bioresour. Technol. 42, 123–131.

Davenport, R., Curtis, T., 2004. Quantitative fluorescence *in situ* hybridisation (FISH): statistical methods for valid cell counting. Molecular Microbial Ecology Manual, second ed. 7.07, pp. 1487–1516.

De Baere, L., 2000. Anaerobic digestion of solid waste: state of art. Water. Sci. Technol. 41 (3), 283–290.

DeLong, E.F., Wickham, G.S., Pace, N.R., 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science 243, 1360–1363.

Fernández-Güelfo, L.A., Alvarez-Gallego, C.J., Sales, D., Romero, L.I., 2005. Start-up and stabilization of a semicontinuous reactor for the thermophilic-dry anaerobic digestión of the organic fraction of municipal solid waste. 4th International symposium Anaerobic Digestion of Solid Waste, Copenhagen.

Flotats, X., Campos, E., Bonmati, A., 1997. Aprovechamiento energético de residuos ganaderos. Curs d'Enginyeria Ambiental: Aprofitament energétic de residus orgánics (3°: 1997: Lleida). Memorias. pp. 1–21. [Energetic profit from livestock farming, 3°: 1997: Lleida. Memorias: pp. 1–21].

Griffin, M.E., McMahon, K.D., Mackie, R.I., Raskin, L., 1998. Methanogenic population dynamics during start-up of anaerobic digesters treating municipal solid waste and biosolids. Biotechnol. Bioeng. 57, 342–355.

Hartmann, H., Ahring, B.K., 2005. A novel process configuration for anaerobic digestion of source-sorted household waste using hyper-thermophilic post-treatment. Biotechnol. Bioeng. 90, 830–837.

Head, I.M., Saunders, J.R., Pickup, R.W., 1998. Microbial evolution, diversity and ecology: a decade of ribosomal RNAs analysis of uncultivated microorganisms. Microbial Ecol. 35, 1–21.

Hugenholtz, P., Tyson, G.W., Blackall, L.L., 2001. Design and evaluation of 16S rRNA-targeted oligonucleotide probes for fluorescence *in situ* hybridization. Methods in Molecular Biology, vol. 176: Steroid Receptor Methods: Protocols and Assays, pp. 29–236 (Chapter 6).

Kepner, R.L., Pratt, J.R., 1994. Use of fluorochromes for direct enumeration of total bacteria in environmental samples: past and present. Microbial Rev. 58, 603–615.

Martin, D.J., Potts, L.G.A., Reeves, A., 1999. Small-Scale simulation of waste degradation in landfills. Biotechnol. Lett. 19, 683–686.

McHugh, S., Carton, M., Mahony, T., O'Flaherty, V., 2003. Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol. Lett. 219, 297–304.

McMahon, K.D., Stroot, P.G., Mackie, R.I., Raskin, L., 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions – II. Microbial population dynamics. Water Res. 35, 1817–1827.

McMahon, K.D., Zheng, D., Stams, A.J.M., Mackie, R.I., Raskin, L., 2004. Microbial population dynamics during start-up and overloading conditions of anaerobic digesters treating municipal solid waste and sewage sludge. Biotechnol. Bioeng. 87, 823–834.

Moorhead, K.K., Nordstedt, R.A., 1993. Batch anaerobic digestion of water hyacinth: effects of particle size, plant nitrogen content and inoculum volume. Bioresour. Technol. 44, 71–76.

Pauss, A., Samson, R., Guiot, S., Beauchemin, C., 1990. Continuous measurement of dissolved H2 in an anaerobic reactor using a new hydrogen/air fuel cell detector. Biotechnol. Bioeng. 35, 492–501.

Pender, S., Toomey, M., Carton, M., Eardly, D., Patching, J.W., Colleran, E., O'Flaherty, V., 2004. Long-term effects of operating temperature and sulphate addition on the methanogenic community structure of anaerobic hybrid reactors. Water Res. 38, 619–630.

Raskin, L., Stromley, J.M., Rittmann, B.E., Stahl, D.A., 1994a. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. Appl. Environ. Microbiol. 60, 1232–1240.

Raskin, L., Poulsen, L.K., Noguera, D.R., Rittmann, B.E., Stahl, D.A., 1994b. Quantification of methanogenic groups in anaerobic biological reactors by olignucleotide probe hybridization. Appl. Environ. Microbiol. 60, 1241–1248.

Santegoeds, C.M., Damgaard, L.R., Hesselink, G., Zopfi, J., Lens, P., Muyzer, G., de Beer, D., 1999. Distribution of sulphate-reducing and methanogenic bacteria in anaerobic aggregates determined by microsensor and molecular analyses. Appl. Environ. Microbiol. 65, 4618–4629.

Schink, B., 1992. Syntrophismamong prokaryotes. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.H. (Eds.), The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Springer, New York, pp. 276–299.

Sebastien, M., Nicolas, B., Pierre, B., Michel, R., René, M., 2002. Methane yield as a monitoring parameter for the start-up of anaerobic fixed film reactors. Water Res. 36, 1385–1391.

Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A., Harada, H., 1999. Fluorescence *in situ* hybridization using 16S rRNA-targeted oligonucleotides reveals localization of methanogens and selected uncultured bacteria in mesophilic and thermophilic sludge granules. Appl. Environ. Microbiol. 65, 1280–1288.

Stahl, D.A., Amann, R., 1991. Development and application of nucleic acid probes. In: Stackebrandt, E., Goodfellow, M. (Eds.), Nucleic Acid Techniques in Bacterial Systematics. John Wiley & Sons, Chichester, U.K, pp. 205–248.

Stroot, P.G., McMahon, K.D., Mackie, R.I., Raskin, L., 2001. Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions – I. Digester performance. Water Res. 35, 1804–1816.

Vavilin, V., Ya Lokshina, L., Rytov, S., Kotsyurbenko, O., Nozhevnikova, A., 1998. Modelling low-temperature methane production from

cattle manure by acclimated microbial community. Bioresour. Technol. 63 (2), 159–171.

Williams, S.C., Hong, Y., Danavall, D., Howard-Jones, H., Gibson, D., Firscher, M., Verity, P., 1998. Distinguishing between living and nonliving bacteria: evaluation of the vital satin propidium iodide and its combined use within molecular probes in aquatic samples. J. Microbiol. Methods 32, 225–236.

Zheng, D., Angenent, L.T., Raskin, L., 2006. Monitoring granule formation in anaerobic upflow bioreactors using oligonucleotide hybridization probes. Biotechnol. Bioeng. 94, 458–472.

Zinder, S.H., 1993. Physiological ecology of methanogens. In: Ferry, J.G. (Ed.), Methanogenesis: Ecology, Physiology, Biochemistry and Genetic. Chapman & Hall, New York, pp. 128–206.