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EFFECTIVE MANAGEMENT OF SEWAGE SLUDGE

Microbial diversity in innovative mesophilic/thermophilic temperature-phased anaerobic digestion of sludge

M. C. Gagliano · C. M. Braguglia · A. Gallipoli · A. Gianico · S. Rossetti

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Abstract Anaerobic digestion (AD) is one of the few sustainable technologies that both produce energy and treat waste streams. Driven by a complex and diverse community of microbes, AD may be affected by different factors, many of which also influence the composition and activity of the microbial community. In this study, the biodiversity of microbial populations in innovative mesophilic/thermophilic temperature-phased AD of sludge was evaluated by means of fluorescence in situ hybridization (FISH). The increase of digestion temperature drastically affected the microbial composition and selected specialized biomass. Hydrogenotrophic *Methanobacteriales* and the protein fermentative bacterium *Coprothermobacter* spp. were identified in the thermophilic anaerobic biomass. Shannon–Weaver diversity (*H'*) and evenness (*E*) indices were calculated using FISH data. Species richness was lower under thermophilic conditions compared with the values estimated in mesophilic samples, and it was flanked by similar trend of the evenness indicating that thermophilic communities may be therefore more susceptible to sudden changes and less prompt to adapting to operative variations.

Keywords Methanogenic archaea · Syntrophic association · Ultrasounds pretreatment · Anaerobic digestion · Mesophilic/thermophilic dual stage · Waste-activated sludge · Microbial diversity · Fluorescence in situ hybridization

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M. C. Gagliano · C. M. Braguglia · A. Gallipoli · A. Gianico · S. Rossetti (✉)
Water Research Institute, IRSA-CNR, Via Salaria km 29,300, Monterotondo, RM 00015, Italy
e-mail: rossetti@irsa.cnr.it

Introduction

Anaerobic digestion (AD) is a robust and efficient technology for the energetic valorization of various types of biomass (including organic wastes and sewage sludge), and it is expected to play a crucial role in the future of renewable energy production (Lauwers et al. 2013). AD of organic matter occurs through the sequential cooperation of different microbial groups in order to achieve degradation of a variety of polymeric and monomeric substrates (O'Flaherty et al. 2006). The anaerobic degradation of organic matter proceeds in a series of four metabolic steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. A diverse number of bacteria take part in the first three steps, and methane formation is mainly derived from acetate and hydrogen/CO_2 conversion by methanogenic archaea. A balanced interaction between the microorganisms in this chain is crucial for an efficient biogas production. Thus, a better understanding of the structure and function of microbial communities during AD may help to improve the reactor performances. However, organic particulate matter degradation efficiency of AD of waste-activated sludge (WAS) remains limited, because of the hydrolysis, considered the rate-limiting step of the entire process (Pavlostathis and Giraldo-Gomez 1991; Bougrier et al. 2006). In order to increase or accelerate the biodegradation rates, various pretreatments (thermal, enzymatic, chemical, or mechanical) have been widely investigated (Carrère et al. 2010; Carlsson et al. 2012). Ultrasounds are currently applied as pretreatment also at full scale on sewage treatment plants. The action of the ultrasonic treatment is to disrupt mechanically the sludge floc matrix and the cell structure by means of shear forces due to cavitation phenomena. Moreover, phased AD in which two or more digesters are used in series is a promising technology for the treatment of wastewater sludges (Zamanzadeh et al. 2013). In the last years, a temperature-phased anaerobic process (TPAD) with a short thermophilic stage acting as thermal

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pretreatment, followed by a longer mesophilic one was investigated with the aim to separate microbial groups into two phases (Coelho et al. 2011; Ge et al. 2010). In the first thermophilic with short hydraulic retention time, hydrolytic and acidogenic/fermentative bacteria degrade polymers to monomers and produce organic acids. In the second stage (with a much longer retention time), these products are then converted into methane by strict cooperation between bacteria and archaea during acetogenesis and methanogenesis (Merlino et al. 2013). However, detailed knowledge of the microbial community structure and their function is lacking (Pervin et al. 2013a).TPAD is particularly applicable to AD of activated sludge, as it allows increased performance regarding solids removal and methane production at a moderate energy input and moderate pathogen removal (Paul et al. 2012). Studies of these microbial communities are limited, and knowledge of the ecology and how that may be related to the system operation is just beginning to develop (Pervin et al. 2013b). Nevertheless, the microbial population is likely to be competent in anaerobic hydrolytic and acidogenic functions, and hence likely to be bacterial rather than archaeal or eukaryotic (Amani et al. 2010). The new approach of this study was based on the idea to subdivide the AD process into three different stages: (1) an ultrasounds pretreatment to improve hydrolysis, (2) a short mesophilic stage to improve volatile fatty acids (VFA) formation, and (3) a final thermophilic stage to convert these intermediates into methane and contemporarily assure the complete hygienization of the digested sludge. Focus of this study was the characterization of the microbial communities growing in this innovative system, fed by real WAS, either untreated or ultrasounds pretreated. The study was specifically aimed at understanding the microbial composition of the mesophilic and thermophilic anaerobic biomass under different conditions of temperature, organic loading rate, and substrate solubilization and availability. Investigation was performed by fluorescence in situ hybridization (FISH) using a wide range of oligonucleotide probes with broad and narrow specificity.

Material and methods

Sludge

WAS samples were obtained from the municipal "Roma-Nord" wastewater treatment plant, characterized by an organic load of about 700,000 p.e., high sludge age (20 days) and a chemical oxygen demand (COD) average value of incoming sewage of 200 mg/L. The activated sludge was sampled directly from the oxidation tank. The anaerobic inoculum utilized for the startup of the mesophilic stage was sampled from the full-scale digester of the plant-treating mixed sludge, while the anaerobic biomass for the thermophilic reactors originated from a previous experimentation with semicontinuous thermophilic system (Gianico et al. 2013).

Sludge pretreatment

The disintegration by ultrasound was performed with an ultrasonic processor UP400S (dr. Hielscher, Germany) operating at 300 W and 24 kHz. Sonication energy input was set at 0.4–0.5 kWh kg^{-1} dry solid on 500 mL of WAS (2.9–4.7 % total solid (TS)) placed in 1 L beaker with the probe allocated at 3 cm above the beaker bottom.

Mesophilic/thermophilic dual-stage AD

Sludge digestion was carried out using four anaerobic digesters operated in semicontinuous mode. Two reactors, as control line, were used to carry out the mesophilic/thermophilic digestion of untreated WAS; the other two reactors, as experimental line, were selected to treat the same sludge, but after sonication (Fig. 1). All jacketed reactors (V=7 L) were completely mixed: the first mesophilic digester was maintained at the constant temperature of 37 °C, while the thermophilic reactor was maintained at 55 °C. In the first test, the organic loading rate (OLR) to the first mesophilic reactor was fixed at 3.9 kg volatile solid (VS) m^{-3} day^{-1} and to the successive thermophilic at 1.2 kg VS m^{-3} day^{-1}; in the second test, the OLR was increased up to 10 for the mesophilic and 2.5 kg VS m^{-3} day^{-1} for the thermophilic reactor, by decreasing the hydraulic retention time (HRT) in the mesophilic reactor and by prethickening the incoming feed sludge. Table 2 lists the operating conditions of the AD tests.

Biogas collection and analysis

The produced biogas was collected by water displacement in a biogas collection unit. The gas meter consisted of a volumetric cell for gas–liquid displacement, a sensor device for liquid level detection, and an electronic control circuit for data processing and display. The methane content in the biogas was measured using a PerkinElmer Auto System Gas Chromatographer equipped with a thermal conductivity detector (TCD) as described in Gianico et al. (2013).

Matter composition

TS and VS were determined in triplicates according to standard methods (APHA 1998). The pH was measured by a portable pH meter (WTW, pH 330/SET-1). To analyze sludge organic content, the particulate sludge matter was removed by centrifugation (10 min at 5,000 rpm), and the resulting concentrate was filtrated through 0.2, 0.45, and 1.2 μm pore size membrane filters.

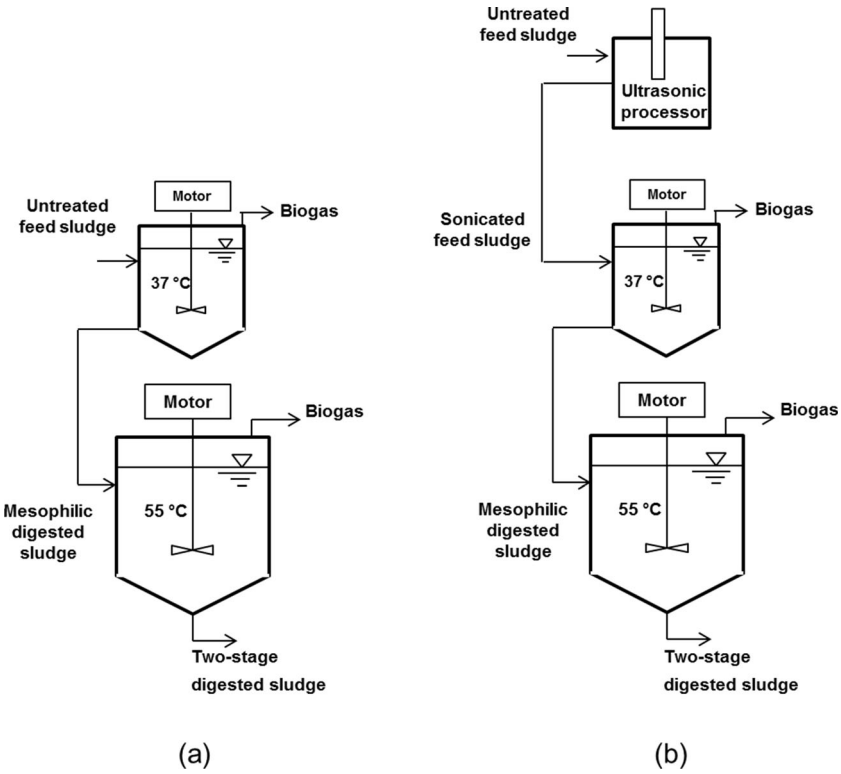

Fig. 1 Schematic diagram of the mesophilic/thermophilic process on untreated (**a**) and sonicated (**b**) WAS

VFA were quantified from 0.2 μm filtrate (soluble phase) by gas chromatography using PerkinElmer Auto System Gas Chromatographer with flame ionization detector (FID). The GC analyses were performed on a stainless steel column packed with 60/80 mesh Carboxen C, 0.3 % Carbowax (Supelco, USA), under the following conditions: injector, 200 °C; oven, 175 °C; and detector, 250 °C. Nitrogen was used as a carrier gas at a flow rate of 30 mL/min. Soluble COD (sCOD), was determined on 0.45 μm filtrate (soluble phase) by Cell Test Spectroquant (Merck) as described in Gianico et al. (2013). Protein content was determined on 1.2 μm filtrate (colloidal phase) by BCA colorimetric method as described in Braguglia et al. (2012).

Microbial community analysis

Sample collection

Effluent sludge samples were collected from reactors during the steady-state operation of the systems. These samples were fixed with paraformaldehyde and ethanol for FISH analysis as described in Amann and Binder (1990).

Fluorescence in situ hybridization

FISH on fixed sludge samples was performed as previously described (Braguglia et al. 2012). Details of the employed oligonucleotide probes are available at probeBase (Loy et al. 2007). Probes were labelled with Cy3 or FITC fluorophores. To identify thermophilic archaea of the order *Methanobacteriales,* the protocol was modified as described in Nakamura et al. (2006), by applying the enzyme pseudomurein endopeptidase (rPeiW) to improve probe penetration inside cells. The lyophilized rPeiW was provided from Dr. Kohei Nakamura (Laboratory of Environmental Microbiology and Engineering, Faculty of Applied and Life Sciences, Gifu University).

Microscopy and fluorescence signal quantification

Samples were examined by epifluorescence microscopy (Olympus BX51) using filters for FITC (excitation, 470–490 nm; emission, 520 nm) and for CY3 (exCitation, 546 nm; emission, 590 nm). Autofluorescence was tested by performing a control test on unstained samples observed with both filters used for FISH analysis. No autofluorescence was retrieved in all screened samples. Fluorescence signal was quantified on microscopic images taken from the samples with a digital camera (Olympus XM-10) and the software Cell F. All the hybridizations with specific probes were carried out in combination with DAPI staining to estimate the portion of cells targeted by group specific probes out of the total cells. Area measurements of the hybridised cells were reported as a portion of the area covered by total DAPI stained cells in each field. Area measurements were performed on at least ten JPEG images (or other image format with 8 bit size of 1,388×

1,040 pixels) using ImageJ software package (version1.37v, Wayne Rasband, National Institute of Health, Bethesda, MD, USA, available in the public domain at http://rsb.info.nih.gov/ij/index.html) as described in Braguglia et al. (2012).

Microbial diversity

The relative abundance of each microbial component calculated by FISH out of total cells (estimated by DAPI staining) was utilized to calculate the biodiversity of the mixed microbial communities by means of Shannon–Weaver index of diversity (*H'*) (Shannon and Weaver 1963) and Pielou's evenness index (Heip 1974).

Results and discussion

Reactor performances and microbial population dynamics

The innovative process described here is based on the integration of a mechanical pretreatment before a dual-stage mesophilic–thermophilic digestion process (Gianico et al. 2014). The scope of this layout was to improve the hydrolysis and the fermentation steps during the mesophilic digestion of sonicated sludge, while the successive long thermophilic stage could additionally improve organic matter biodegradation obtaining higher methane yield and sludge hygenization. The composition and the structure of the anaerobic biomass selected during both mesophilic and thermophilic stages were investigated to better understand the effect of the operating conditions on microbial community in order to achieve better process performances.

Mesophilic microbial community

As shown in Fig. 2, the bacterial population identified by FISH ranged between 55 and 65 % of total DAPI-stained cells and was mainly composed of *Proteobacteria*, commonly found as main components of activated sludge (Wilén et al. 2008). Changes in HRT did not affect the amount of bacteria retrieved in the anaerobic biomass in the reactor fed with ultrasound-pretreated sludge (Fig. 2b), despite the higher organic loading rate (Table 1). Overall, in the reactor fed with ultrasound-pretreated sludge, the impact of operative changes was negligible with respect to the control reactor, although slight variations in the relative abundance of individual components were observed. The relative abundance of archaeal population decreased with the increase of OLR in both sonicated and untreated biomass. (Fig. 2).This is in agreement with the biogas production of the mesophilic reactors that was higher in test 1 with respect to test 2 (Fig. 3). Shortening the HRT of the first mesophilic stage reduced the extent of conversion of substrates in methane, benefiting the biogas production of following thermophilic stage in test 2 (Fig. 3). The latter was confirmed by VFA measurements in mesophilic samples during the two tests (Fig. 4). In test 1 (Fig. 4a, b), only acetate and propionate were detected, but the concentration was very low (<30 mg/L). Diversely, during test 2, VFA were found at higher concentrations, ranging from 0.5 to 1 g/L in the control reactor and from 0.7 to about 2.5 g/L in the reactor fed with sonicated sludge (Fig. 4c, d). Additionally, VFA were present also in the form of butyrate and isobutyrate. These VFA, conveyed as feed into the thermophilic reactor, enhanced consequently the biogas yield with respect to test 1. This indicated that in the mesophilic stage of test 1 the conversion rate of organic substrates into methane was higher than in test 2, with a consequent loss of the methanogenic potential incoming in the thermophilic reactor. Indeed, in test 1 the mesophilic stage produced the majority of the total biogas during the two-stage digestion (Fig. 3). Shortening the HRT and increasing the OLR of the mesophilic reactor led to the improvement of the performance of the following thermophilic stage in test 2, encountering the original aim of the mesophilic stage. The application of specific FISH probes for archaea showed the presence of long filamentous and rods of *Methanosaeta* spp. The latter was found in all digestion phases, highlighting the occurrence of acetotrophic methanogenesis as expected in a mesophilic anaerobic system. The occurrence of these microorganisms is strictly related to acetate concentration, because *Methanosaeta* is a specialist in using acetate and grows only at low acetate concentrations. Fluctuations in relative abundance (Fig. 5) of this microorganism during the two tests (a decrease during test 1 and an increase during test 2 in both reactors) are mainly due to rapid changes in acetate concentration (which constitutes the largest portion of VFA present in the system) observed during the fermentative step of AD, as shown in Fig. 4. The remaining part of archaeal population was not identified by applying the available FISH probes.

Thermophilic microbial community

As shown in Fig. 6, proteolitic fermentative bacteria *Coprothermobacter* and hydrogenotrophic *Methanobacteriales* were found as component in the microbial community. Microbial composition of the inoculum is reported in Fig. 6a. *Methanobacteriales* cells were further identified as *Methanothermobacter* sp. by generating an archaeal 16S rRNA gene clone library from total community DNA isolated from sludge samples (data not shown). The latter finding is in line with many previous studies where *Coprothermobacter* and *Methanothermobacte*r were found to live in strict syntrophic associations (Sasaki et al. 2011; Ge et al. 2012). Proteolytic activity of *Coprothermobacter* is well documented (Etchebehere et al. 1998; Tandishabo et al. 2012; Majeed

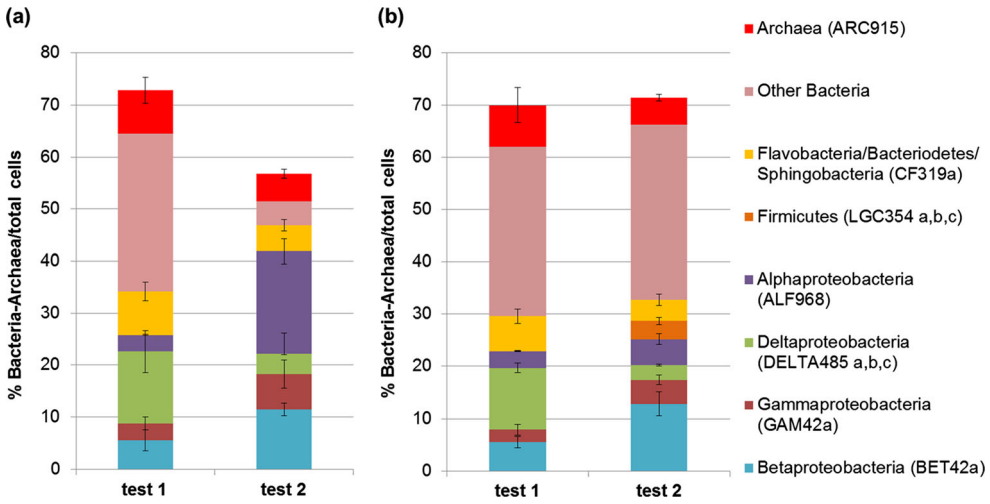

Fig. 2 Relative abundance of bacteria and archaea, out of total cells in mesophilic reactors fed with untreated (**a**) and sonicated (**b**) sludge operating at steady-state conditions. FISH oligonucleotide probes applied for the analysis are reported in *brackets*

et al. 2013; Lü et al. 2014), and in particular this microorganism is capable to ferment proteins, growing well in presence of peptides (Ollivier et al. 1985). Therefore, the solubilization of proteins into small molecules can promote *Coprothermobacter* establishment, especially at high temperatures (Lee et al. 2009). In this mesophilic/thermophilic digestion system, *Coprothermobacter* decreased with increased OLR indicating a failure in acclimation under the new reactor conditions (Fig. 6). Sasaki et al. (2011) reported that the growth of *Coprothermobacter* was proportional to protein consumption. Tandishabo et al. (2012) highlighted that *Coprothermobacter* population size was controlled by the type of substrates in the feed. Evidently, fermentative pathways were different with respect to the original thermophilic reactor from which the inoculum was sampled. In particular, sonication pretreatment and/or mesophilic stage generated substrates in which proteic components were not degraded into small molecules. Sonication pretreatment generally seems to affect *Coprothermobacter* relative abundance in test 1 (Fig. 6b, c), with the increase of other bacteria in the system fed with sonicated sludge (Fig. 6c). In test 1, after sonication and mesophilic stage, proteins concentration was about 260 mg/L and the sCOD value increased up to 250 mg/L (Table 2). Regarding the second test, the OLR increase of the mesophilic stage highly improved sCOD and proteins release and accumulation in the reactor, but this had no positive effect on the maintenance of *Coprothermobacter* population in the thermophilic reactor. Indeed, as described in Bougrier et al. (2005), after low-frequency ultrasounds pretreatment, proteins were made soluble but not completely degraded, as very little organic nitrogen was transformed into ammonium.

An additional factor influencing *Coprothermobacter* population was likely the HRT of the mesophilic stage: the shortening of HRT from tests 1 to 2 may have decreased the proportion of protein degradation during mesophilic hydrolysis. The percentage of acetate degradation in thermophilic

Table 1 Operating conditions of mesophilic (1° stage) and thermophilic (2° stage) digestion tests

	Test 1		Test 2	
	1° stage	2° stage	1° stage	2° stage
T (°C)	37 °C	55 °C	37 °C	55 °C
OLR (g VS L^{-1} day^{-1})	3.9	1.2	10	2.5
HRT (day)	5	10	3	10
Test duration (day)	97	97	81	81

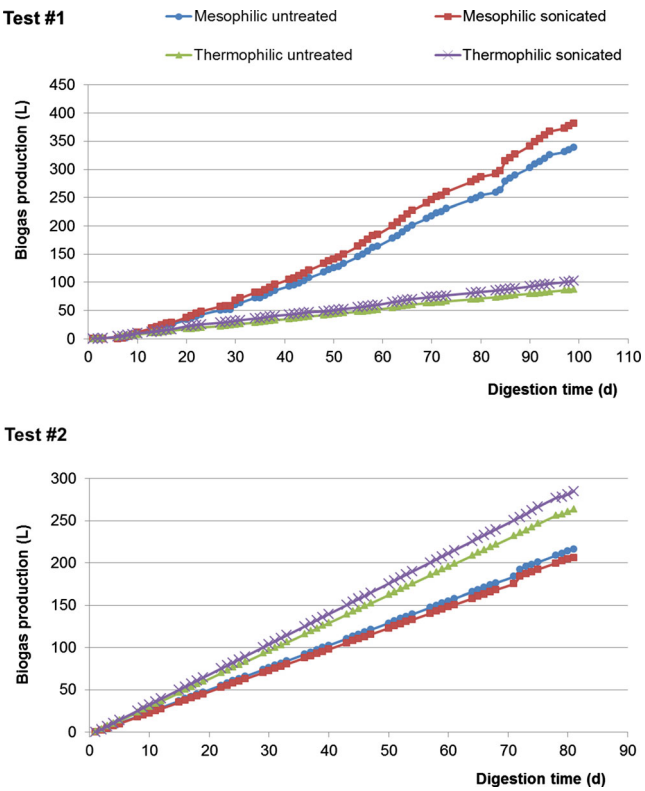

Fig. 3 Mesophilic and thermophilic cumulative biogas production during the anaerobic tests

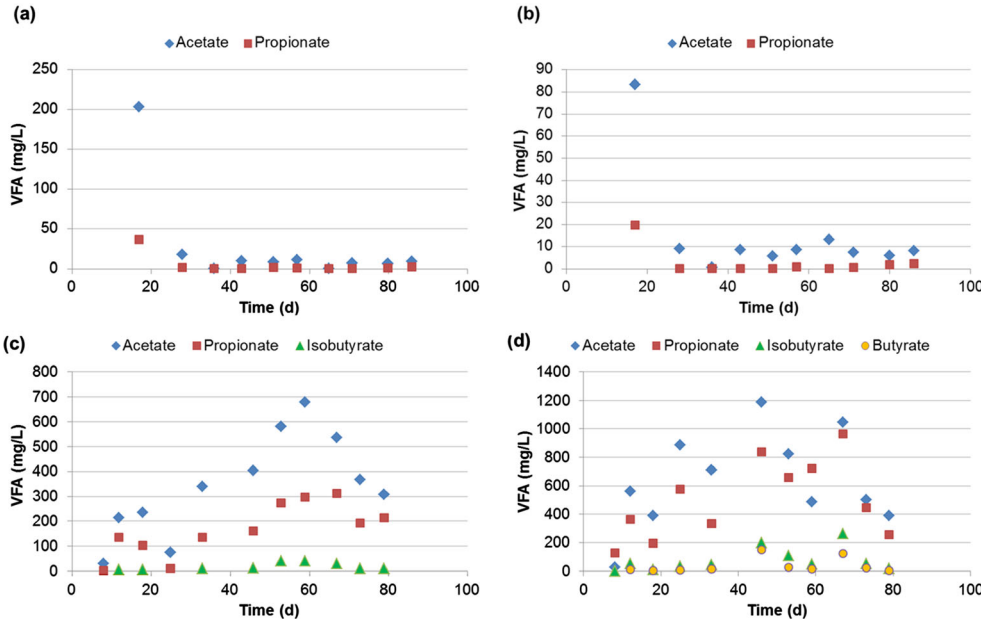

Fig. 4 VFA trends in mesophilic reactors fed with untreated (**a**, **c**) and sonicated (**b**, **d**) sludge during tests 1 (**a** and **b**) and 2 (**c** and **d**)

reactors during test 2, with respect to the incoming sludge from mesophilic reactors is shown in Fig. 7. Acetate degradation occurred in both reactors, but was higher in the reactor fed with sonicated sludge. Thus, the high concentration of VFA transferred from mesophilic to thermophilic stage in test 2, definitely changed the pathway of methanogenesis. All these observations can easily explain the decrease in relative abundance of *Coprothermobacte*r in the end-stage compared with the startup population. Conversely, *Methanothermobacter* was the only methanogen retrieved in the anaerobic sludge (Fig. 6). This was mainly due to the VFA accumulation from mesophilic stage, because *Methanothermobacter* is less sensitive than acetoclastic methanogens to increases in VFA concentration (Hori et al. 2006). This means that hydrogenotrophic methanogenesis was the main way of methane production, but the hydrogen production pathway was not driven by *Coprothermobacter* population, and likely other kind of syntrophic associations take place. The hydrogen supply seemed to be the key parameter affecting biogas production, rather than the population size of *Methanothermobacter*, as described in Morgan et al. (1997). The lack of aceticlastic methanogens indicated that acetate was neither cleaved nor oxidized by archaea, and therefore, under these conditions, at high acetate concentration, the most probable way of methane production is the syntrophic oxidation of acetate (SAO) to hydrogen by syntrophic acetate-oxidizing bacteria (not identified in this study), followed by hydrogen removal by *Methanothermobacter* (Karakashev et al. 2006; Ge et al. 2012). SAO is a key pathway at elevated temperatures (Ho et al. 2013). However, some studies have recently found that SAO became predominant in thermophilic or stressed environmental conditions (Hao et al. 2011), but microorganisms involved in this pathway are widely under investigations. As reported by Lü et al. (2014), *Coprothermobacter* may also operate SAO with consequent production of hydrogen, but cooperation with several bacterial species is required. Thus, population composition of startup thermophilic biomass progressively changed, but strong

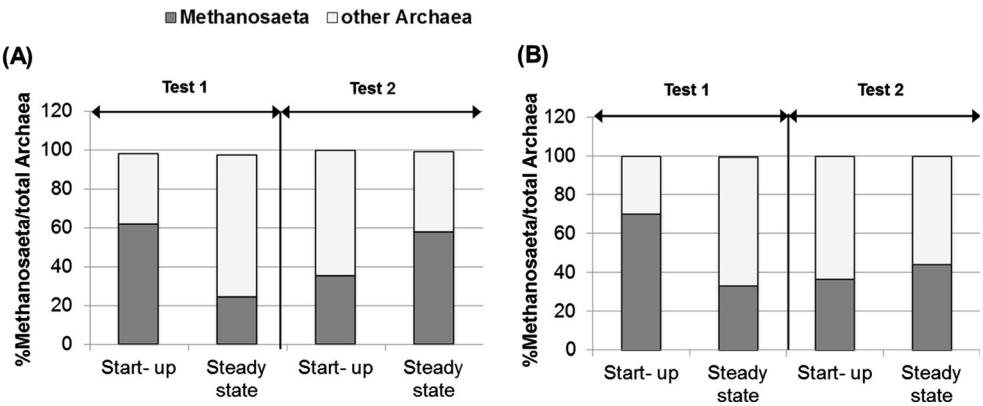

Fig. 5 Relative abundance of archaea (ARC915 probe) and *Methanosaeta* (MX825a,b,c probes) out of total cells in mesophilic reactors fed with untreated (**a**) and sonicated (**b**) sludge for the two digestion tests, at the beginning and at the end of each digestion test

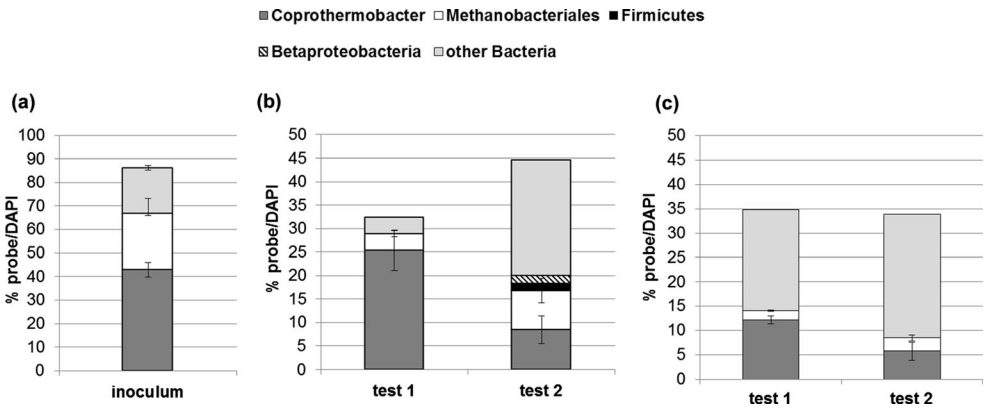

Fig. 6 Relative abundance of bacteria (EUB338mix probes), *Coprothermobacter* (CTH485 probe), *Betaproteobacteria* (BETA42A probe), *Firmicutes* (LGC354a,b,c), and *Methanobacteriales* (MB311 probe) out of total cells in thermophilic reactors fed with untreated (**b**) and sonicated (**c**) sludge for the two digestion tests, at the end of each digestion period. In (**a**), the microbial composition of thermophilic inoculum is reported

variations in relative abundances of *Methanothermobacter* and *Coprothermobacter* revealed that microbial community was strongly affected by the overall operative conditions. The decrease of *Coprothermobacter,* together with the higher presence of other bacteria, was in line with the likely occurrence of SAO as methane production pathway. The identity of other bacteria needs to be further investigated to fully describe the methane production process occurring in the thermophilic stage of this dual stage system.

Biodiversity of mesophilic and thermophilic microbial communities

The relative abundance of each microbial component was utilized to estimate two different parameters commonly employed to describe the biodiversity of mixed microbial communities. ShannonWeaver diversity index (H') and Pielou's evenness index (E) estimates with FISH data showed a decrease of biodiversity and evenness under thermophylic conditions (Table 3). H' index was lower than 0.7 under thermophilic conditions (compared with 1.3–1.4 estimated in mesophilic samples), and it was flanked by similar trend of the evenness. This finding indicated that thermophilic anaerobic biomass could be therefore more susceptible to sudden changes and less prompt to adapting to operative variations. The strong impact of microbial biodiversity on the process evolvement was highlighted in the previous sections: during mesophilic stage, microbial population quickly changed in response to variations in HRT and OLR, switching from methane to VFA production (Figs. 3 and 4), with negligible variations of microbial composition and relative abundance. Conversely, during thermophilic stage, the startup microbial population slowly evolved encountering operative variations, changing the way of fermentative hydrogen production.

Differences between mesophilic and thermophilic microbial communities in anaerobic digesters are largely reported (Ike et al. 2010; Shi et al. 2013; Pervin et al. 2013a; Zamanzadeh et al. 2013). A systematical analysis of 21 mesophilic and thermophilic full-scale anaerobic digesters highlighted that bacterial and archaeal community composition was mainly related to the temperature of the process (Sundberg et al. 2013). Looking at the distribution of microbial population within archaea and bacteria domains, in the study of Nelson et al. (2011) a meta analysis of microbial diversity in several AD systems revealed that species richness of bacteria is higher than archaea. Generally, in studies based on in situ identification, archaeal relative abundance is lower than bacterial one (Ariesyady et al. 2007; Montero et al. 2009; Krakat et al. 2010). Indeed, hydrolytic and fermentative steps require the cooperation of different bacterial groups that degrade the wide range of

Table 2 Soluble COD and proteins during steady state of dual-digestion tests 1 and 2

	Untreated			Sonicated		
	Feed	1° stage	2° stage	Feed	1° stage	2° stage
Test 1						
CODsol (mg/L)	56±9	210±25	930±135	520±79	252±36	871±99
Proteins (mg/L)	65±19	258±52	650±164	279±59	264±46	664±117
Test 2						
CODsol (mg/L)	134±26	1,520±198	2,180±247	2,082±609	2,892±308	2,482±267
Proteins (mg/L)	89±21	661±53	1,388±198	1,103±75	818±130	1,471±312

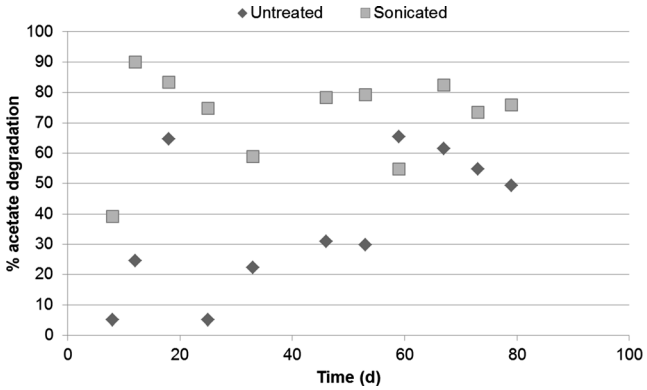

Fig. 7 Acetate degradation in thermophilic stage during test 2 in reactor fed with untreated and sonicated sludge

soluble organics and convert them into end-products of fermentation. On the other hand, methanogens have a very complex metabolic system that comprises all of enzymes required for methane production, and they modulate this machinery in relation to substrate availability. Moreover, they have different growth kinetics with respect to bacteria. For example, the hydrogenotrophic methanogen *Methanothermobacter thermoautotrophicus,* did not grow when a very low H_2 was supplied, although methanogenesis continued and transcription of genes for methanogenesis was stimulated (Morgan et al. 1997); in this manner, it can optimize H_2 utilization obtaining high yield of methane in H_2-limiting environments, without exponential growth. This means that the abundance or the number of components of *Methanothermobacter* population is not directly related to methane yield.

Under mesophilic conditions, acetotrophic methanogenesis is the main way of acetate production. Only the genera *Methanosarcina* and *Methanosaeta* are able to transform acetate into methane (Smith and Ingram-Smith 2007). Their growth kinetics are mainly related to acetate and proportional to its concentration. In low acetate environments, *Methanosaeta* prevails on *Methanosarcina* because of its higher affinity for the substrate (Berger et al. 2012), and vice versa. Nevertheless, their simultaneous presence can benefit the process due to the higher metabolic versatility of *Methanosarcina*, as it can use almost all substrates for methanogenesis, with the exception of formate (Braguglia et al. 2012). *Methanosarcina* and *Methanosaeta* were found also in thermophilic processes, but their activity is often limited because of acetate utilization is mostly exploited by fermentative bacteria during SAO. Indeed, the syntrophs of acetate oxidizing bacteria and their partner hydrogenotrophic methanogens are able to successfully outcompete the aceticlastic methanogens (Hao et al. 2011). In thermophilic conditions, in the absence of acetotrophic methanogens, microbial communities could still maintain a highly efficient and stable performance without acetate accumulation (Krakat et al. 2010). For this reason, acetate was converted via SAO, and the high hydrogenotrophic activity retrieved in several biogas reactors using acetate as carbon source supported this opinion (Hao et al. 2011). In this study, FISH analysis highlighted the presence of aceticlastic methanogens during the mesophilic stage, while *Methanothermobacter* population was the only species identified during thermophilic stage. As acetate was the main substrate during thermophilic stage in test 2, the original syntrophic association between *Methanothermobacter* and *Coprothermobacter* was replaced by a different hydrogenotrophic pathway, likely related to SAO.

Table 3 Shannon–Weaver (H') and evenness (E) values calculated for the two digestion tests in dual-stage system at the end of each digestion period

	Mesophilic stage				Thermophilic stage			
	Untreated		Sonicated		Untreated		Sonicated	
	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
H'	1.4	1.3	1.4	1.4	0.5	0.7	0.6	0.5
E	0.7	0.7	0.70	0.7	0.7	0.5	0.8	0.7

Conclusions

Study of microbial populations during the innovative dual-stage mesophilic/thermophilic AD indicated HRT of the mesophilic stage as crucial parameter to improve the performance of the following thermophilic stage. Shortening the HRT, a shift from methane to VFA production was observed, in particular by pretreating the sludge with ultrasounds. In thermophilic stage, substrate composition and availability strongly influenced the composition of the microbial population. In particular, the proteolytic *Coprothemobacter* deriving from thermophilic inoculum drastically decreased, and other bacteria, likely involved in syntrophic acetate oxidation, took place. Species richness was lower under thermophilic conditions compared with the values estimated in mesophilic AD and it was flanked by similar trend of the evenness, indicating that thermophilic microbial communities may require a longer acclimation period before obtaining a stable microbial population being more susceptible to sudden changes and less prompt to adapting to operative variations.

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