1 **Changes in microbial community during hydrogen and methane production in two-**

2 **stage thermophilic anaerobic codigestion process from biowaste**

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NOMENCLATURE

AD anaerobic digestion

COD chemical oxygen demand

FISH fluorescence *in situ* hybridization

GP gas production

HP hydrogen production

HRT hydraulic retention time

MP methane production

OFMSW organic fraction of municipal solid waste

OLR organic loading rate

rRNA ribosomal RNA

SHP specific hydrogen production

SMP specific methane production

TKN total Kjeldahl nitrogen

TP total phosphorus

TS total solids

VS volatile solids

WAS waste activated sludge

Abstract

The aim of the paper was to investigate the microbial community in a two-phase thermophilic anaerobic co-digestion process for hydrogen and methane production, treating waste activated sludge and the organic fraction of municipal solid waste. In the acidogenic phase, in which hydrogen is produced, *Clostridium* sp. clusters represented 76% of total *Firmicutes*. When feeding the acidogenic effluent to the methanogenic reactors, these acid conditions negatively influenced the methanogens microorganisms: *Methanosaeta sp.,* (most *Methanobacteriales, Methanomicrobiales, Methanococcales)* decrease by 75%, 50%, 38% and 52%, respectively. At the same time methanogenic digestion produced a decrease in *Clostridium* sp. clusters due to both pH increasing and substrate reduction, and an increasing of *Firmicutes* genera (non *Clostridium*) and methanogens, especially in *Methanosaeta sp.* (208%); this was in accordance with the decrease in the acetic (98%) and butyric (100%) acid contents. To ensure the activity of the acetate-utilizing methanogens (AUM) and the acetogens, high ratios H_2-utilizing methanogens (HUM)/AUM (3.6) have been required.

Keywords: biohydrogen, thermophilic, anaerobic digestion, co-digestion, two phases, biogas, organic waste..

1. Introduction

In recent years, the phase-separated anaerobic digestion (AD) process including two-stage process has been widespread throughout Europe for the treatment of the biowaste (Pavan et al., 2000; Schievano et al., 2012). This system is normally composed by an hydrolysis-acidogenesis step carried out in the first-phase (dark fermentation) and an acetogenenesis-methanogenesis step carried out in the second phase (Zahedi et al., 2013a). The different growth rates and pH optima for hydrogen producing microorganisms (between 5.5 and 6.5) and methanogenic microorganisms (around pH 7), and thus different requirements regarding reactor conditions, have led to the development of two-stage AD (De la Rubia et al., 2009). The two-stage approach has been finalized to the hydrogen production (HP) in the first phase reactor and methane production (MP) in the second phase reactor, with the final purpose of mixing the two gasses to achieve bio-Hythane (50-55% CH_4, 5-10% H_2 and 35-40% CO_2) that allows a better combustion with a reduced greenhouse gasses emission compared with fossil fuels (Cavinato et al., 2011; Liu et al., 2012).

Due to the advantages of the AD, lots of research have be done on the optimization of the AD of organic fraction of municipal solid waste (OFMSW), including the interesting option of co-digestion process. Benefits of codigestion include: dilution of potential toxic compounds, improved balance of nutrients, synergistic effect of microorganisms, increased load of biodegradable organic matter and higher biogas yield (Callaghan et al., 1999). Hamzawi et al. (1998) and Sosnowski et al. (2003) found an average enhanced value of biogas production from the codigestion of wastewater treatment sludge and OFMSW.

Conventional bioconversion of waste activated sludge (WAS) and OFMSW in AD

55 systems is usually characterized by hydrolysis, acidogenesis, acetogenesis and
56 methanogenesis. The first three steps are carried out by *Eubacteria* domain, while the
57 fourth step (methanogenesis) is undertaken by *Archaea* domain. *Eubacteria* constitute a
58 large domain of prokaryotic microorganisms. The most *Eubacteria* identified in
59 anaerobic digesters are covered by the following phyla: *Firmicutes* (Ariesyady et al.,
60 2007), *Actinobacteria* (Ariesyady et al., 2007)*, Spirochaetes* (Lee et al., 2013)*,*
61 *Bacteriodetes* and *Proteobacteria* (Chouari et al., 2005). *Clostridia* are a highly
62 polyphyletic class of *Firmicutes*, including *Clostridium* and *Thermoanaerobacter*
63 genera. They are obligate anaerobes capable of producing endospores. *Clostridium* sp.
64 clusters are the predominant strains involved in the HP (Lee et al., 2009a). On the other
65 hand, *Archaea* constitute a domain of single-celled microorganisms. Methanogenic
66 *Archaea* is a phylogenetically diverse group of strictly anaerobic *Euryarchaeota* with an
67 energy metabolism that is restricted to the formation of CH_4 from CO_2 and H_2, formate,
68 methanol, methylamines and/or acetate (Raskin et al., 1994). *Methanococcales, Most*
69 *Methanobacteriales, Methanomicrobiales* and *Methanosaeta sp.* are considered to cover
70 most methanogens in anaerobic digesters (Yu et al., 2005; Lee et al., 2009b). Only
71 *Methanosaeta* sp. is a specialist to use acetate as sole energy source; the others three are
72 H_2-utilizing methanogens (HUM).
73 About two-stage AD process, most of researchers have been focused on the
74 optimization of gas production (GP), removal organic matter and process optimization
75 but very few reports discussed in detail the microbial population dynamics involved
76 during different stages of the thermophilic anaerobic co-digestion of WAS and OFMSW
77 for hydrogen production. Molecular tools like the fluorescence *in situ* hybridization
78 (FISH), based on sequence comparison of small-subunit ribosomal RNA (rRNA)

79 molecules, can detect specific whole cells/organisms in biological samples (Crocetti et
80 al., 2006; Ariesyady et al., 2007; Montero et al., 2009; Zahedi et al., 2013a, 2013b,
81 2014a, 2014b).
82 The aim of this study is to identify the functional *Eubacteria* and *Archaea* community
83 structures in the substrates and in the acidogenic and methanogenic effluents of a two-
84 stage thermophilic anaerobic co-digestion process treating WAS and OFMSW. In this
85 paper, microbial community structure was quantitatively investigated using different
86 specific probes employing FISH and it was related to process performance.

87 2. Materials and Methods

88 2.1 Experimental equipment and operating conditions

89 Two laboratory-scale continuously stirred tank reactors were employed. The first
90 reactor, dedicated to the HP (first phase, dark fermentation), had a 3.5 L working
91 volume, while the second reactor (second phase) dedicated to the MP had a 18.5 L
92 working volume, both heated by hot water recirculation system and maintained in
93 thermophilic condition (55°C). The system was fed semi-continuously, once per day,
94 and the organic loading rates (OLR) in the first and second phase were 16 kg TVS/m^3d
95 and 3 kg TVS/m^3d, respectively, while the corresponding hydraulic retention times
96 (HRT) were 3 and 16 d for the first and second phase reactors, respectively. The whole
97 experiment length was 70 d. After the start-up period (0-40 d), the stationary phase (41-
98 70 d) was reached.

99 2.2 Substrate

100 The wastes used to feed the acidogenic reactor were collected in the wastewater
101 treatment plant (WWTP) located in Treviso (northern Italy). This substrate was then
102 mixed with WAS with a volume ratio OFMSW:WAS of 1:5, calculated in order to have

103 an OLR of 16 kg TVS/m^3d in the first phase reactor. The OFMSW and WAS were
104 stored at -4ºC in order to avoid degradation by microorganisms in their own wastes. The
105 OFMSW was reduced in size using a grinder and mixed with WAS. The feedstock was
106 daily prepared and no pre-treatment was considered (i.e. chemical reagent or thermal
107 treatment). The characteristics of the substrate in terms of total solids (TS), volatile
108 solids (VS), chemical oxygen demand (COD), total Kjeldahl nitrogen (TKN) and total
109 phosphorus (TP) are shown in Table 1.
110
111 Table 1:
112
113 **2.3 Analytical methods**
114 The analytical determinations applied in this study can be grouped in two categories:
115 physical-chemical analysis and microbiological analysis.
116 2.3.1 Physical-chemical analysis
117 The effluent of both the reactors was monitored 2 to 3 times per week in terms of TS,
118 TVS, COD, TKN and TP. The process stability parameters, namely pH, volatile fatty
119 acid (VFA) content and speciation, total and partial alkalinity and ammonia ($NH_4^+ – N$),
120 were checked daily. All the analyses, except for VFA, were performed according to
121 APHA (1995). VFA content was monitored using a gas chromatograph (Carlo Erba
122 instruments) with H_2 as gas carrier, equipped with a Fused Silica Capillary Column
123 (Supelco NUKOL™, 15 x 0.53 x 0.5 µm film thickness) and with a flame ionization
124 detector (200 °C). The temperature during the analysis started from 80 °C and reaches
125 200 °C trough two other steps at 140 and 160 °C, with a rate of 10 °C/min. The
126 analyzed samples were centrifuged and filtrated on a 0.45 µm membrane. GP was

127 monitored continuously by a gas flow meter (Ritter Company, drum-type wet-test
128 volumetric gas meters), while the biogas composition was measured by a gas-
129 chromatograph (GC Agilent Technology 6890 N) equipped with the column HP-PLOT
130 MOLESIEVE, 30 x 0.53 mm ID x 25 um film, using a thermal conductivity detector
131 and argon as gas carrier.

132 2.3.2 Microbiological analysis and biochemical activity

133 The cellular concentration and percentages of *Eubacteria*, *Archaea*, *Firmicutes*,
134 *Clostridium* and different groups of methanogens, described below, were obtained by
135 epifluorescence method (FISH). These analyses were performed according to Montero
136 et al. (2009) and Zahedi et al. (2013a, 2013b, 2014a, 2014b). The 16S rRNA-targeted
137 oligonucleotides employed in this study are showed in the Table 2. The samples were
138 examined visually and cells counted using a DM6000 B microscope (Leica) with a
139 Leica EL6000 lamp and an x 100 oil objective.
140
141 Table 2:
142
143 The total population was calculated as the sum of the relative amounts of *Eubacteria*
144 and *Archaea* that was estimated as 100% (Montero et al., 2009; Zahedi et al., 2013a,
145 2013b, 2014a,). The others phyla *Eubacteria* (non *Firmicutes*) were calculated as
146 difference of the relative amounts of *Eubacteria* and *Firmicutes*. The others genera of
147 *Firmicutes* (*non Clostridium*) were calculated as difference of the relative amounts of
148 *Firmicutes* and *Clostridium* sp. HUM were calculated as sum of the relative's amounts
149 of microorganisms obtained by probes MB1174, MG1200 and MC1109; and acetate-

150 utilizing methanogens (AUM) were estimated as the quantity of the microorganisms
151 obtained by MX825.
152 Methanogenic activity was considered to evaluate the biochemical activity according to
153 Montero et al. (2009) and Zahedi et al. (2013a, 2013b), it was calculated as the ratio of
154 CH_4 volume generated and the number of *Archaea* inside the reactor by FISH staining.
155

156 **<u>3. Results and discussion</u>**

157 The process performances, the functional *Eubacteria* and *Archaea* community
158 structures of the two-phase anaerobic reactors for HP and MP were both investigated, as
159 well as the link between these two aspects.

160 **3.1 Process performances and yields**

161 The overall performances of the two-phase thermophilic anaerobic co-digestion process
162 are summarized in Table 3.
163

164 Table 3:

165

166 During the experiment the substrate mixture was fed to the first reactor. The low HRT
167 applied (3 d) and the high OLR used (16 kg TVS/m^3d) lead to a pH value of 4.75±0.08,
168 caused by the high VFA production (14.2 g COD/L); this pH value addressed the best
169 pH conditions for HP that is around 5.5 (Cavinato et al., 2012). VFA composition was
170 mainly due to butyric acid (10.3 g COD/L); the dominant fermentation products were
171 butyric acid and acetic acid, ranging from 62–82% and 16–21%, respectively, while
172 small average amount of propionic was detected (< 1g COD/L). The ratio of butyrate to
173 acetate was ranging from 3 to 5; it was clearly higher than those reported by Zahedi et

174 al. (2013c) in hydrolytic acidogenic AD of the OFMSW at 3 d HRT that was between 2
175 and 3 or in Cavinato et al. (2011) that it was between 1 and 2. These differences were
176 due to different factors such as type of inoculum (WAS and OFMSW), substrate
177 composition, pH, OLR and TS percentage in the feed. These factors can influence the
178 bacterial growth, fermentative pathways and bacterial community and finally determine
179 the process performance.
180 In the first phase, the biogas produced was composed by H_2 and CO_2, without CH_4
181 detection. In terms of yields, biohydrogen in the first reactor was 36±8 %, a value in
182 line with those reported in literature, typically in the range 35–40% (Cavinato et al.,
183 2011). The Hydrogen volumetric production rate (0.432±0.036 L H_2/Ld) tested were
184 significantly higher than those obtained for Romero et al. (2013) (0.210±0.015 L H_2/Ld)
185 with a similar OLR (18.5 kg TVS/m^3d) and at 4.4 d HRT. The second phase operated
186 with an HRT of 16 d and an OLR of 3 kg TVS/m^3d. pH reached a constant value of
187 7.97±0.26, this was the optimal pH for enhanced acetogenic and methanogenic activity
188 (Montero et al., 2009; De la Rubia et al., 2009). The VFA concentration was lower than
189 1 g COD/l: the removal percentages of butyric, acetic and total volatile fatty acids
190 (tVFA) were 100%, 98% and 96% respectively. The amount of propionic generated in
191 the second-stage (0.1-0.6 g COD/L) produced no inhibitory effect. As for the overall
192 performance, the COD and TVS removal observed were 73±3 % and 76±2 %
193 respectively. A similar value of removal TVS (65%), was found by Gallert and Winter
194 (1997) in AD of OFMSW in thermophilic conditions at HRT of 19 d (OLR of 9.6 g
195 TVS/Ld). The biogas produced in the second phase was H_2-free. As for yields, CH_4 in
196 biogas ranged between 50 and 68 %, a value in line with those reported in literature for
197 AD of the OFMSW (Cavinato et al., 2011). The methane production rate found

198 (0.590±0.090 l CH_4/Ld) was similar to the one obtained by Bolzonella et al. (2006) for
199 the co-digestion anaerobic process of WAS and biowaste.

200 **3.2 Analysis of the microbial communities**

201 The microorganism amounts in the substrate and in the effluents of the two-phase
202 anaerobic system were monitored. The concentration and the relative percentages of the
203 main microbial groups are shown in Table 4, Table 5.

204

205 Table 4:

206 Table 5:

207

208 The amount of *Archaea* obtained using ARC915 probe was lower than the sum of the
209 different groups of methanogens investigated using five specific methanogenic probes
210 (Table 4 and Table 5). This fact was also observed by Montero et al. (2009), and it is
211 due to one of the main problem associated with FISH technique named *Lack of*
212 *specificity:* considering that only between 0.1 and 10% of the microbial species have
213 been described, a specific probe targeting a certain group of microorganisms may target
214 other microrganisms not yet described (Amann, 1995). In complex samples containing
215 many different microbial species, this can cause overestimation of the target species
216 (Amann et al., 1995).

217 <u>3.2.1 Microbial communities in the WAS, OFMSW and mixture</u>

218 Microbiological characterization of WAS and OFMSW was determined by FISH. Table
219 4 shows the characterization of the WAS, OFMSW and mixture (5:1 based on volume)
220 fed to the AD system.

221 It is possible to observe that, the total microbial concentration in the OFMSW was
222 higher than the WAS. *Eubacteria* was the major phylogenetic domain in all waste
223 (>71.4±12.5 %). The *Firmicutes* phylum represented 82.7±10.0 % and 20.5±1.6 % of
224 the *Eubacteria* percentage in the OFMSW and WAS, respectively. In the OFMSW less
225 than 25% of *Firmicutes* phylum identified was represented by *Clostridium* sp. clusters,
226 while in WAS more than 80% of *Firmicutes* was *Clostridium*. On the other hand
227 *Archaea* domain accounted for less than 30% in all samples. The main methanogens of
228 WAS and OFMSW (*most Methanobacteriales* and *Methanosaeta sp.*) were included in
229 the mixture.

230 3.2.2 Microbial community of the two-phase thermophilic anaerobic codigestion
231 process

232 FISH analysis revealed that the *Eubacteria* was the major phylogenetic domain in both,
233 acidogenic phase and methanogenic phase (Table 5). It was according to Montero et al.
234 (2009) and Zahedi et al. (2013a, 2013b, 2014a, 2014b). However no significant
235 variation was found in *Eubacteria:Archaea* ratio (89:11) in both, first and second
236 phase, it is noteworthy that high differences in the amounts of microorganisms were
237 observed. In the acidogenic reactor, the amounts of microorganisms (non-Firmicutes
238 and non Clostridia and all the different groups of *Archaea*) were lower than in the
239 methanogenic reactor (Table 4). The lower content of microorganisms in the acidogenic
240 reactor was due to the several conditions existent and explained below (low pH and
241 HRT).

242 *3.2.2.1 Eubacteria community*

243 The AD treatment produced an increase in *Eubacteria* amount, especially others phyla
244 (non *Firmicutes Eubacteria*). This could be due to increase in phyla predominant or

245 frequently detected in anaerobic sludge digesters such as *Actinobacteria* (Ariesyady et
246 al., 2007)*, Spirochaetes* (Chouari et al., 2005; Lee et al., 2013)*, Bacteriodetes* (Chouari
247 et al., 2005) and *Proteobacteria* (Chouari et al., 2005). *Clostridium* sp. clusters
248 represented 76% of total *Firmicutes* in the acidogenic phase. The high amount of
249 *Clostridium* sp. clusters in the first phase is due to the fact that these microorganisms
250 are the predominant strains involved in hydrogen production (Lee et al., 2009a). In the
251 second phase reactor, *Clostridium* clusters populations drastically decreased due to a
252 lack of the substrate and others *genera* of *Firmicutes* (non *Clostridium*) were increased
253 due to an increase of pH and a decrease in H_2 partial pressure (it was consumed by
254 HUM). Therefore, increase on genera of *Firmicutes* (non *Clostridium*) in the second
255 phase was due to syntrophic association with HUM. Many of other genera of *Firmicutes*
256 (non *Clostridium*) in anaerobic reactors are proton-reducing-acetogenic bacteria as
257 *Syntrophomonas* sp. and *Syntrophobacter* sp. which are inhibited in acid conditions,
258 since requires very low H_2 partial pressure to favour the thermodynamics of the
259 reactions (Boone et al., 1980; Zahedi et al., 2014a).
260 *3.2.2.2 Archaea community*
261 The decline in pH in the first phase produced a decrease in all groups of methanogens
262 that were detected in the substrates; after anaerobic acid digestion, the populations of
263 *Methanosaeta sp., Most Methanobacteriales, Methanomicrobiales, Methanococcales*
264 decrease by 75%, 50%, 38% and 52% respectively. So the least and the most resistant
265 of methanogens were *Methanosaeta* sp. and *Methanomicrobiales*, respectively. These
266 results were according to Shimada et al. (2011). Shimada et al. (2011) studied the
267 microbial processes involved in two-phase AD by operating a laboratory-scale, and their
268 results indicated that the order *Most Methanobacteriales* was better able to tolerate the

269 acidified conditions than *Methanosaeta sp.* The pH increasing occurred in the second
270 phase, caused an increase of all methanogens detected: the populations of
271 *Methanosaeta, Most Methanobacteriales, Methanomicrobiales, Methanococcales*
272 increased by 208%, 133%, 50% and 144% respectively. The highest increase in the
273 second phase comparing with the first phase was observed for *Methanosaeta* sp. (208%)
274 and it was due to low concentrations of acetic acid (98% reduction) in the second phase
275 reactor. Regarding the relative amount of methanogens, the results show that HUM,
276 especially *Most Methanobacteriales*, constituted the major group of methanogens.
277 *3.2.2.3 Ratio HUM/AUM and biochemical activities in the second phase*
278 It is necessary to emphasize that not only the determination of the number of
279 microorganisms, but also the biochemical activities of microorganism and ratio
280 HUM/AUM in the reactor are key-factors that allow to understand the process
281 performances of anaerobic reactors (Zahedi et al., 2013a, 2013b).
282 Referring to the ratio of microorganism, previous studies have suggested that during the
283 MP in the anaerobic digestion process of biowaste, low ratios of HUM/AUM could
284 indicate that the H_2 generated during the acidogenic or acetogenic phase would be
285 accumulated in the system, preventing the activity of the AUM and acetogens (Zahedi et
286 al., 2013a, 2013b). For this reason it's important to establish the optimal ratio
287 HUM/AUM in the methanogenic process. Nevertheless, literature data related to the
288 optimal ratio HUM/AUM in the methanogenic process, were poor and quite varied.
289 Montero et al. (2009) and Zahedi et al. (2013b) showed that in a stable single-stage
290 anaerobic system when no H_2 was detected, the average values of the ratio *Most*
291 *Methanobacteriales*/*Methanosaeta* sp. were around 0.2 and 0.9, respectively, while in
292 the present study the average value was 1.8, slightly higher than those obtained for

293 Zahedi et al. (2013a) during the two-phase dry-thermophilic anaerobic digestion of
294 municipal solid waste. Shimada et al. (2011) and Xiao et al. (2013), in the
295 methanogenic phase of the two-phase system, obtained values of ratio
296 (*Methanomicrobiales*+*Most Methanobacteriales*)/(*Methanosaeta sp.*) of 0.9 and 56.6,
297 while in the present study the average value was 2.7.
298 Thus, the relative amount of AUM and HUM in the methanogenic phase of the two-
299 phase system may be different from that found in the conventional single stage AD
300 system and even variable in other two-phase system using other substrates.
301 In the present research work the average values of the ratio *Most*
302 *Methanobacteriales*/*Methanosaeta* sp. and HUM/AUM were 1.8 and 3.6, respectively
303 and this reflected the prevalence of the HUM. These results are consistent with previous
304 studies in two-phase AD system (Shimada et al., 2011; Xiao et al., 2013). Shimada et al.
305 (2011) and Xiao et al. (2013) attributed a high ratio HUM/AUM to the inhibition of
306 AUM. Shimada et al. (2011) reported that elevated ammonia concentrations (1.0-1.4
307 g/l) and the constant input of acetate oxidizing bacteria from the first phase in the
308 second phase may partially inhibit AUM and drives the digestion process through HP
309 by acetate oxidizing bacteria and MP by HUM. Xiao et al. (2013) established that, as
310 long as acetic acid concentration was lower than the inhibition threshold, increase in
311 acetic acid concentration promotes the AUM activity, but when acetic acid
312 concentration was higher than the inhibition threshold, it became inhibitor of the
313 activities of AUM, especially at low pH environment. In the present study, inhibition of
314 AUM in the second phase was not observed because of the high removal of acetic acid
315 (98%). Therefore, the high ratio HUM/AUM was due to other two reasons. The first

316 reason is that related to the high and constant input of HUM, especially *Most*
317 *Methanobacteriales* and *Methanomicrobiales*, from the first phase to the second phase.
318 A large amount of HUM was present in the feedstock substrates of OFMSW and WAS
319 (Table 4). They do not grow in the first-phase, hence HUM come from an external
320 source. Previous studies have demonstrated that the microbial content inside the reactor
321 was strongly influenced by the content of microorganisms in the substrate (Zahedi et al.,
322 2013a; 2014b). Therefore the input of HUM from first-phase into second-phase, come
323 from WAS and OFMSW. The average value of the ratio HUM/AUM in the substrate
324 was 2.4; during the acidic treatment, this value was increased to 5.2. This was a
325 consequence of the fact that AUM were the most affected methanogens by acidic pH
326 (decrease 75 %), while HUM, especially *Most Methanobacteriales* were better able to
327 tolerate the acidified conditions. The second reason for high values of the ratio
328 HUM/AUM was the high amount of butyric (10.3±0.4 g COD/l) generated in the
329 acidogenic phase. This fact caused an increasing of the HUM populations to archive an
330 high butyric acid removal (100%). During acetogenic process was produced large
331 amounts of H_2, which should be consumed quickly by HUM because acetogens and
332 AUM have a scarce growth in presence of H_2 in the system (Boone et al., 1980;
333 Montero et al., 2009; Zahedi et al., 2013a, 2013b). The methanogenic activity in the
334 second phase at an OLR of 3 kg TVS/m^3/d was of $109±19*10^{-13}$ l CH_4/cell/d higher than
335 those obtained by Zahedi et al. (2013b) (in the range $15–48*10^{-13}$ l CH_4/cell/d) at OLR_s
336 between 5.7 kg and 30.7 kg TVS/m^3/d (HRT of between 15 d and 3 d). To date, the
337 optimal ratio HUM/AUM in the methanogenic phase with a high ratio of butyrate to
338 acetate and high content of HUM in the feed (acidogenic effluent from WAS and
339 OFMSW) have not been studied yet. Data from this study suggest that the optimal ratio

340 *Most Methanobacteriales*/*Methanosaeta* sp. and HUM/AUM on methanogenic phase
341 for anaerobic co-digestion process from WAS and OFMSW, with high butyric loading
342 rate and high content of HUM in the feed was 1.8 and 3.6.

343 **4. Conclusion**

344 *Clostridium* sp. represented 76% of total *Firmicutes* in the acidogenic phase, while in
345 the methanogenic phase, the pH increase and the high content of the butyric an acetic in
346 the substrate produced an increase in others *genera* of *Firmicutes* (non *Clostridium*) and
347 in all methanogens studied, especially in *Methanosaeta sp.* (208%), This fact, together
348 with the increasing of others phyla (non *Firmicutes Eubacteria*) shown no variations in
349 the *Eubacteria:Archaea* ratio in both phases.
350 High ratios HUM/AUM (3.6) are required in the second phase to ensure the syntrophic
351 activity and therefore the smooth functioning of the system. *Most Methanobacteriales*
352 constituted the major group of *Archaea.*

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359 **References**

360 Amann, R. I., Binder, B. J., Olson, R. J., Chisholm, S. W., Devereux, R., &
361 Stahl, D. A. (1990). Combination of 16S rRNA-targeted oligonucleotide

362 probes with flow cytometry for analyzing mixed microbial populations.
363 *Applied and environmental microbiology*, *56*(6), 1919-1925.
364 Amann, R.I., Ludwig, W., Schleifer, K.H., 1995. Phylogenetic identification and in situ
365 detection of individual microbial cells without cultivation. Microbiol. Reviews. 59, 143-
366 169.
367 Amann, R.I., 1995. Fluorescently labelled, rRNA-targeted oligonucleotide probes in the
368 study of microbial ecology. Mol. Ecol. 4, 543-554.
369 APHA, AWWA, WEF, 1995. Standard Methods for the Examination of Water and
370 Wastewater, 19th ed. Washington, DC, New York, USA.
371 Ariesyady, H.D., Ito, T., Okabe, S., 2007. Funtional bacterial and archaeal community
372 structures of major trophic groups in a full-scale anaerobic sludge digester. Water Res.
373 41, 1554-1568.
374 Bolzonella, D., Battistoni, P., Susini, C., Cecchi, F., 2006, Anaerobic codigestion of
375 waste activated sludge and OFMSW: the experiences of Viareggio and Treviso plants
376 (Italy). Wat. Sci. Technol., 53(8), 203–211
377 Boone, R.D., Bryant, L,. 1980. Propionate-Degrading Bacterium, Syntrophobacter
378 wolinii sp. nov. gen. nov., from Methanogenic Ecosystems. Appl. Environ. Microbiol.
379 40, 626-632.

380 Callaghan, F.J., Wase, D.A.J., Thayanithy, K., Foster, C.F., 1999. Co-digestion of
381 waste organic solids: batch studies, Bioresour. Technol. 67, 117–122.
382 Cavinato, C., Bolzonella, D., Fatone, F., Cecchi, F., Pavan, D., 2011. Optimization of
383 two-phase thermophilic anaerobic digestion of biowaste for hydrogen and methane
384 production through reject water recirculation. Bioresour. Technol. 102, 8605–8611.

385 Cavinato, C., Giuliano, A., Bolzonella, D., Pavan, P., Cecchi, F., 2012. Bio-hythane
386 production from food waste by dark fermentation coupled with anaerobic digestion
387 process: A long-term pilot scale experience, International Journal of Hydrogen Energy,
388 37, 11549-11555.
389 Chouari, R., Le Paslier, D., Daegelen, P., Ginestet, P., Weissenbach, J., Sghir, A., 2005.
390 Novel predominant archaeal and bacterial groups revealed by molecular analysis of an
391 anaerobic sludge digester. Environ. Microbiol. 7, 1104–1115.
392 Crocetti, G., Murto, M., Björnsson., L., 2006. An update and optimisation of
393 oligonucleotide probes targeting methanogenic Archaea for use in fluorescence in situ
394 hybridisation (FISH). J. of Microbiol. Methods. 65, 194-201.
395
396 Daims, H., Brühl, A., Amann, R., Schleifer, K. H., & Wagner, M. (1999).
397 The domain-specific probe EUB338 is insufficient for the detection of all
398 Bacteria: development and evaluation of a more comprehensive probe
399 set. *Systematic and applied microbiology*, *22*(3), 434-444.
400 De la Rubia, M.A., Raposo, F., Rincón, B., Borja, R., 2009. Evaluation of the
401 hydrolytic–acidogenic step of a two-stage mesophilic anaerobic digestion process of
402 sunflower oil cake. Bioresour. Technol. 100, 4133–4138.
403 Franks A. H., Harmsen H. J. M., Raangs G. C., Jansen G. J., Schut F., Welling G. W.,
404 1998. Variations of bacterial populations in human feces measured by fluorescent in situ
405 hybridization with group-specific 16S rRNA-targeted oligonucleotide probes. Appl.
406 Environ. Microbiol. 64, 3336-3345.

407 Gallert, C., Winter, J., 1997. Mesophilic and thermophilic anaerobic digestion of
408 source-sorted organic wastes: effect of ammonia on glucose degradation and methane
409 production. Appl. Microbiol. Biotechnol. 48, 405–410.
410 Hamzawi, N., Kennedy, K. J., Mc Lean, D.D., 1998. Technical feasibility of anaerobic
411 co-digestion of sewage sludge and municipal solid waste. Environ. Technol. 19, 993–
412 1003.
413 Lee, M.J., Song, J.H., Hwang, S.J., 2009a. Effects of acid pre-treatment on bio-
414 hydrogen production and microbial communities during dark fermentation. Bioresour.
415 Technol. 100, *1491-1493.*
416 Lee, C., Kim, J., Hwang, K., O'Flaherty, V., Hwang, S., 2009b. Quantitative analysis of
417 methanogenic community dynamics in three anaerobic batch digesters treating different
418 wastewaters. Water Res. 43, 157-165.
419 Lee, S.H., Park, J.H., Kang, H.J., Lee, Y.H., Lee, T.J., Park, D.P., 2013. Distribution
420 and abundance of *Spirochaetes* in full-scale anaerobic digesters. Bioresour. Technol. *In*
421 *Press, Corrected Proof*.
422 Liu, X., Wang, W., Shi, Y., Zheng, L., Gao, X., Qiao, W., Zhou, Y., 2012. Pilot-scale
423 anaerobic co-digestion of municipal biomass waste and waste activated sludge in China:
424 Effect of organic loading rate. Waste Manag, 32, 2056-2060.
425

426 Meier, H., Amann, R., Ludwig, W., & Schleifer, K. H. (1999). Specific
427 oligonucleotide probes for in situ detection of a major group of gram-
428 positive bacteria with low DNA G+ C content. *Systematic and Applied*
429 *Microbiology*, *22*(2), 186-196.

430 Montero, B., García-Morales, J.L., Sales, D., Solera, R., 2009. Analysis of
431 methanogenic activity in a thermophilic-dry anaerobic reactor: Use of fluorescent in situ
432 hybridization. Waste Manage. 29, 1144–115.
433 Pavan, P., Battistoni, P., Cecchi, F., Mata-Alvarez, J., 2000. Two-phase anaerobic
434 digestion of source sorted OFMSW (organic fraction of municipal solid waste):
435 performance and kinetic study. Wat. Sci. Technol., 41(3), 111-118

436 Raskin, L., Stromley, J. M., Rittmann, B. E., Stahl, D. A., 1994. Group-specific 16S
437 rRNA hybridization probes to describe natural communities of methanogens. Appl.
438 Environ. Microbiol. 60, 1232–1240.
439 Romero, M.A., Fdez-Güelfo, L.A., Álvarez-Gallego, C.J.., Romero, L.I., 2013. Effect of
440 HRT on hydrogen production and organic matter solubilization in acidogenic anaerobic
441 digestion of OFMSW. Chem. Eng. J.219, 443-449.
442
443 Sekiguchi, Y., Kamagata, Y., Nakamura, K., Ohashi, A., & Harada, H.
444 (1999). Fluorescence in situ hybridization using 16S rRNA-targeted
445 oligonucleotides reveals localization of methanogens and selected
446 uncultured bacteria in mesophilic and thermophilic sludge granules.
447 *Applied and Environmental Microbiology*, *65*(3), 1280-1288.
448 Schievano, A., Tenca, A., Scaglia, B., Merlino, G., Rizzi, A., Daffonchio, D., Oberti,
449 R., Adani, F. (2012). Two-Stage vs Single-Stage Thermophilic Anaerobic Digestion:
450 Comparison of Energy Production and Biodegradation Efficiencies. Environ. Sci.
451 Technol., 46 (15), 8502–8510

452 Shimada, T., Morgenroth, E., Tandukar, M., Pavlostathis, S.G., Smith, A., Raskin, L.,
453 Kilian, R.E., 2011. Syntrophic acetate oxidation in two-phase (acid-methane) anaerobic
454 digesters, Water. Sci. Technol. 64, 1812–1820.
455 Sosnowski, P., Wieczorek, A., Ledakowicz, S., 2003. Anaerobic co-digestion of sewage
456 sludge and organic fraction of municipal solid wastes. Adv. Environ. Res. 7, 609–616.
457

458 Stahl, D. A. (1991). Development and application of nucleic acid probes.
459 *Nucleic acid techniques in bacterial systematics*.
460 Xiao, K., Guo, C.H., Zhou, Y., Maspolim, Y., Wang, J.Y., Ng, W.J., 2013. Acetic acid
461 inhibition on methanogens in a two-phase anaerobic process. Biochem. Eng. J. 75, 1-7.
462 Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to
463 detect methanogenic communities using quantitative real-time polymerase chain
464 reaction. Biotechnol. Bioeng. 89, 670-679.

465 Zahedi, S., Sales, D., Romero, L.I., Solera, R., 2013a. Optimisation of the two-phase
466 dry-thermophilic anaerobic digestion process of sulphate-containing municipal solid
467 waste: Population dynamics. Bioresour. Technol. 148, 443–452.
468 Zahedi, S., Sales, D., Romero, L.I., Solera, R., 2013b. Optimisation of single-phase dry
469 thermophilic anaerobic digestion under high organic loading rates of industrial
470 municipal solid waste: population dynamics. Bioresour. Technol. 146, 109–117.
471 Zahedi, S., Sales, D., Romero, L.I., Solera, R., 2013c. Hydrogen production from the
472 organic fraction of municipal solid waste in anaerobic thermophilic acidogenesis:
473 Influence of organic loading rate and microbial content of the solid waste. Bioresour.
474 Technol. 129, 85-91.

475 Zahedi, S., Sales, D., Romero, L.I., Solera, R., 2014a. Biomethanization from sulfate-
476 containing municipal solid waste: effect of molybdate on microbial consortium. J.
477 Chem. Technol. Biotechnol. 89, 1379–1387.
478 Zahedi, S., Sales, D., Romero, L.I., Solera, R., 2014b. Dark fermentation from real solid
479 waste. Evolution of microbial community. Bioresour. Technol. 152, 221–226.

480