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**Environmental advances due to the integration of food industries and anaerobic digestion for biogas production: perspectives of the Italian milk and dairy product branch**

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1 **TITLE:**

2 Assessing archeal **indicators** of performance by RT-qPCR methods during anaerobic co-digestion of  
3 organic wastes.

4

5 **Authors:** Deborah Traversi<sup>1\*</sup>, Chiara Capone<sup>1</sup>, Silvia Villa<sup>1</sup>, Romanazzi Valeria<sup>1</sup>, Biancamaria Pietrangeli<sup>2</sup>,  
6 Giorgio Gilli<sup>1</sup>

7

8 **Affiliations:**

9 <sup>1</sup>Department of Public Health and Pediatric Sciences, Università di Torino

10 via Santena 5 bis 10126, Torino, Italy

11 phone +390116705810

12 fax +390112605810

13 e-mail: [deborah.traversi@unito.it](mailto:deborah.traversi@unito.it)

14 <sup>2</sup>Department Productive Plants & Environment Interaction (DIPIA), National Institute of Occupational  
15 Safety & Prevention INAIL ExISPESL, , I-00184 Rome, Italy

16 \*corresponding author

17

18 **KEYWORDS:**

19 methanogens, anaerobic digestion, biogas production, renewable energy, archaeal communities

20

21 **ABSTRACT:**

22 Biogas is a renewable energy resource produced during the anaerobic digestion of various organic  
23 substrates. A wide community of microorganisms is involved, including methanogens. These **Archaea** are  
24 the biologic key to the process because they accomplish the methane-forming reaction. Despite its  
25 crucial role, the micro-biome inside the digester is poorly understood. The aim of this work is to develop  
26 bio-indicators of efficiency for the anaerobic process through the quantification and characterisation of  
27 the methanogens and sulphate-reducing bacteria. From a full-scale digester fed with organic wastes, 31  
28 samples were collected. Temperature, pH, acidity, alkalinity and biogas quantity and quality were  
29 monitored over time. The methanogens were detected from the samples both in total and as belonging  
30 to different taxa units. These evaluations, by qPCR-RT methods, produced valuable results for  
31 *Methanosarcina*, *Methanosaeta*, Methanocorpusculaceae and sulphate-reducing bacteria.  
32 *Methanosarcina* was the most abundant family, followed by Methanocorpusculaceae and then  
33 *Methanosaeta*. The methanogen taxa are significantly and directly correlated with each other ( $p < 0.05$ ).  
34 *Methanosaeta* and Methanocorpusculaceae are present in significantly different amounts at different  
35 temperatures. While *Methanosaeta* levels also change when the organic load increases (T-test,  $p < 0.05$ ),  
36 *Methanosarcina* is more tolerant, and its levels are quite constant. *Methanosarcina* and *Methanosaeta*  
37 are proposed to be bio-indicators of the stability of the process (the first) and of susceptibility (the  
38 second) to detect early sufferance conditions in the digester. These methods will be useful in the control  
39 and optimisation of an eco-friendly waste-to-energy system.

40

41

42 **1. INTRODUCTION**

43 The anaerobic digestion of organic waste removes organic pollutants, produces biogas and partially  
44 reduces the volume of organic waste [1, 2]. For this reason, many European countries have established  
45 favourable conditions for electricity production from biogas [3]. In Italy today, there are more than 500

46 anaerobic digesters, and 58% of these plants use a co-digestion configuration, including organic  
47 materials that are currently suitable [4].

48 The progress of the technology generally depends on lifestyles, the waste generation rate, the  
49 characteristics of the waste, the awareness level, acceptability, market value and other economic  
50 factors. Regulatory directives and stringent energetic and environmental policies are the main reasons  
51 for the development of these technologies [2, 5].

52 The potential evaluated biomethane from the biodegradable fraction of municipal solid waste, including  
53 biowaste, in Europe, is equal to 10 billion  $m^3$ , and the estimated percentage of biomethane in the total  
54 amount of methane that will be utilised as gas by 2020 is 40% [6].

55 This biogas can be used to generate heat or electricity and/or can be used as a vehicular fuel. In recent  
56 years, several researchers have evaluated the possibility of upgrading the biogas for biomethane  
57 production, with particular regard to corrosive molecules, such as hydrogen sulphide, mercaptans and  
58 siloxanes [7].

59 Recently, anaerobic digestion by microorganisms was compared in terms of life cycle assessments  
60 (LCAs), which quantify end-of-life management options, with other systems for organic waste treatment,  
61 such as aerobic composting, gasification, combustion, incineration and mechanical biological treatments.  
62 Although wider evaluations are needed, anaerobic digestion is environmentally preferable to other  
63 waste-to-energy technologies, especially in terms of climate change impacts [8].

64 The four stages of anaerobic digestion are hydrolysis, acidogenesis, acetogenesis and methanogenesis  
65 [1, 9]. The major bacterial taxa involved in anaerobic digestion belong to Clostridia and Bacteroidetes,  
66 while the archaeal community is dominated by the methanogenic archaea of the orders  
67 Methanomicrobiales and Methanosarcinales [1, 10]. The community composition is mainly influenced by  
68 the feedstock type and temperature shifts (from mesophilic to thermophilic) [11] but also by other  
69 factors, generally chose when the plant was installed, such as the reactor configuration, batch or  
70 continuous, the hydraulic retention time, the organic load rate and eventually biomass pre-treatments

71 [2, 12].

72 The majority of the work reported in the literature has focused on the chemical-physical control of the  
73 process but has overlooked the microbial aspects [1]. This area has gained influence only recently [10,  
74 13, 14]. The evidence shows that methanogenesis is often the rate-limiting step, after a good level of  
75 hydrolysis in a constant feed process. This phase is the most sensitive to temperature and toxic  
76 chemicals. Low methanogen activity increases the H<sub>2</sub> partial pressure in the gas phase and the levels of  
77 short-chain fatty acids, with a consequent decrease in pH. Therefore, enhancing methanogenesis is a  
78 promising mechanism for improving the performance of anaerobic reactors. However, diverse microbe  
79 groups, such as sulphate-reducing bacteria, are responsible for the weak quality of biogas [15]. There are  
80 three primary useful substrates for methanogens: acetate, methyl-group-containing compounds and  
81 CO<sub>2</sub>. Acetate is the predominant intermediate in the anaerobic food chain, and as much as two-thirds of  
82 all biologically generated methane is produced from this molecule [16]. Although each pathway begins  
83 differently, they all end with the reaction of methyl-coenzyme M (HS-CoM) with a second thiol  
84 coenzyme (coenzyme B) to form methane and a mixed disulphide between coenzyme M and coenzyme  
85 B. This reaction is catalysed by methyl-coenzyme M reductase (Mcr); thus, Mcr is the key enzyme in  
86 methanogenesis [17]. As HS-CoM has been found in all methanogens examined, it has been proposed as  
87 a sensitive biomarker for quantitative and qualitative methanogen identification in different anaerobic  
88 environments. Mcr is exclusive to methanogens, with the exception of methane-oxidising archaea [17-  
89 19], and specific primers have been developed for the Mcr  $\alpha$ -subunit gene sequence (*mcrA*) [20-22].  
90 Anaerobic methanotrophic Archaea clades have been detected in waste-water treatment sludge, but  
91 their physiology remains unknown. Studies of the bioreactor biomass in its early phase have shown that  
92 these microorganisms are very poorly represented in comparison with methanogens [18, 23]. *mcrA*  
93 analysis can be used, either in conjunction with or independently of, the *16S rRNA* gene to detect  
94 methanogens [10, 24-26]. Biomolecular methods to detect other groups of microorganisms have been  
95 developed using functional genes. In particular, anaerobic digestion by sulphate-reducing bacteria is

96 interesting because of their correlation to hydrogen sulphide production. Various target genes can be  
97 amplified to evaluate sulphate-reducing bacteria in the samples, and among these is the adenosine-5'-  
98 phosphosulphate reductase gene (Apr) [27, 28].

99 Various recently studies were focussed on a chemical and biological integrate control of the anaerobic  
100 processes and they highlighted some microorganisms as key control point [29, 30]

101 The aim of this work was to apply a method for assessing methanogens by real-time quantitative PCR  
102 (RT-qPCR) as a useful indicator of the biogas production efficiency in a full-scale reactor during co-  
103 digestion of organic wastes. The purpose is to evaluate the methanogens in total and the sulphate-  
104 reducing bacteria, more specifically, during the digestion process. Moreover, the goal is to evaluate the  
105 impact of methanogen sub-groups on the early diagnosis of sufferance conditions.

106

## 107 **2. METHODS AND MATERIALS**

### 108 **2.1 Digestion process**

109 A full-scale reactor placed in the Alessandria's territory in northwest Italy was used for this study. The  
110 anaerobic reactor was fed with a pre-treated organic fraction of municipal solid waste (OFMSW) and  
111 sporadically with discarded material from neighbouring food industries. The feed was prepared with a  
112 preliminary pre-treatment consisting of a mechanical splitting method (turbo-mixing). Through  
113 centrifugation, the organic fraction was separated from the inert fraction containing inert materials,  
114 such as plastics. The organic fraction dissolved in a liquid phase had a total concentration of solids  
115 varying from 4% to 11%. In stable conditions, the volatile solids were 93% of the total solids. The reactor  
116 functioned in mesophilic conditions, and all of the phases of the anaerobic digestion occurred in the  
117 same tank, so it consisted of a mono-step configuration. The feeding was continuous, and the hydraulic  
118 retention time was approximately 20 days. The OFMSW included in the daily feedings was variable  
119 (**table 1**), it conditions the organic load rate (OLR) (**figure 1A**). The carbon-to-nitrogen (C/N) ratio for the  
120 feed mixtures was, on average, 19 and was evaluated once a month by the plant lab following standard

121 methods. The biogas yield was approximately 21,000 cubic meters of biogas daily; the conversion into  
122 electric energy from the middle of December to the end of January was approximately 1,761 MW. The  
123 composition of the produced biogas during the sampling period **was evaluated by gas chromatograph**  
124 **Varian Chrompack CP-4900 model and it** is shown in **table 2**.

125

## 126 **Sampling**

127 The sampling was performed once every five days from October 11<sup>th</sup>, 2011 to December 20<sup>th</sup>, 2011. For  
128 every sampling day, two samples were collected at two different positions in the digester. Polyethylene  
129 terephthalate sterile bottles with a 1-litre capacity were used for the sampling. The total samples  
130 collected were 31, plus 2 samples of pre-treated OFMSW before their introduction into the digester.  
131 **Table 1** shows the main physical and chemical characteristics of the collected samples from the reactor  
132 **(figure 1B)**. The chemical measurements were performed at the internal laboratory of the treatment  
133 plant involved in the study, following standard methods. Samples were stored at -18°C until the  
134 extraction procedure.

135

## 136 **2.2 DNA extraction and purification**

137 The digester sample aliquots were thawed at 4°C overnight and centrifuged at 3000 x g for 20 minutes;  
138 the supernatant was then removed, and the semi-dry aliquots were used in the subsequent steps. The  
139 total DNA was extracted from 0.25 g of particulate matter (residual humidity of 31±5%) with the  
140 PowerSoil DNA Isolation Kit, followed by the UltraClean Soil DNA Kit (MoBio Laboratories Inc. 2746 loker  
141 Ave West, Carlsbad) [31, 32]. **The DNA quantity extracted was 4.97± 1.97 ng/µl (mean), and its quality**  
142 **was evaluated by gel electrophoresis prior to PCR. Generally only the samples with a concentration**  
143 **greater than 1 ng/µl were used for the analysis [33]. All of the samples were extracted within one month**  
144 **from the sampling day, and all met the DNA quantity and quality standards [34, 35].**

145

### 146 2.3 RT-qPCR analysis

147 After DNA extraction and purification, we evaluated the total methanogen, the sulphate-reducing  
148 bacteria and the presence in the sample of specific methanogen families. The total methanogen content  
149 was quantified using methanogen-specific short primers (ThermoBiopolymer) for the *mcrA* sequence  
150 described by Steinberg and Regan [22]. The reactions used a standard super-mix (Bio-Rad SsoFast™  
151 EvaGreen SuperMix) and the RT-PCR Chromo4 (Bio-Rad) with Opticon Monitor 3 Software. The reaction  
152 conditions have been previously described [22]. The standard reference was a *Methanosarcina*  
153 *acetivorans mcrA* sequence of approximately 470 base pairs [36] inserted into a pCR21 vector  
154 (Invitrogen), supplied by L.M. Steinberg and J.M. Regan of Pennsylvania State University. The plasmid  
155 was amplified by transforming *Escherichia coli* Top10 cells, according to the manufacturer's instructions.  
156 The transformed cells were selected on LB agar in the presence of ampicillin, and the plasmid was  
157 extracted with a plasmid DNA purification kit (NucleoSpin Plasmid, Macherey-Nagel). The standard  
158 curve had six points and was calculated according to the threshold cycle method; for the highest yield, 2  
159 µl of plasmid was amplified ( $\sim 2.52 \times 10^7$  plasmid copies). There was a 1:10 dilution between each  
160 standard curve point. The resolution limit for the method was  $2.52 \times 10^2$  copies of *mcrA*. The number of  
161 methanogen cells in the sample was proportional to the number of *mcrA* gene copies in the DNA  
162 extract. The PCR efficiency was 97%, and we used 2 µl of a 1:10 dilution of each DNA extract sample for  
163 the amplification [32].

164 Standard references were not used for the next PCR evaluation of the work, and thus, the data could  
165 only be relative. The sulphate-reducing bacteria were quantified using specific short primers for the  
166 *aprA* gene, as previously described [17, 27, 37]. The different methanogens were quantified using the  
167 same methanogen-specific short primers, adding specific probes synthesised by ThermoBiopolymer that  
168 have been previously described [38]. Members belonging to *Methanosarcina*, *Methanosaetaceae*,  
169 *Methanobacteriaceae*, *Methanocorpusculaceae*, *Methanospirillaceae*, the Uncultured MCR-7 group, the  
170 Uncultured MCR-2 group, MCR-2b Uncultured and Fen Uncultured were determined with the following

171 probes: msar, msa, mrtA and mbac-mcrA, mcp, msp, MCR-7, MCR-2a, MCR-2b, and Fen.  
172 The reactions were conducted in singleplex with a standard super-mix (Bio-Rad iQ™ Multiplex  
173 Powermix) using the RT-PCR Chromo4 (Bio-Rad) and Opticon Monitor 3 Software. The reaction  
174 conditions have been previously described [38].  
175 The efficiency of the PCR reactions was determined with a serial 1:10 dilution of the sample and was  
176 determined for all primers and probes to be between 87 and 102%. The results for these methanogen  
177 groups and sulphate-reducing bacteria were expressed as cycle threshold (Ct) or as 1/Ct (=1/cycle  
178 threshold) so its value is proportional to the concentration of the gene target into the sample.  
179 The standards, when present, and samples were tested in triplicate. The triplicate value was accepted  
180 only if the coefficient of variation was below 20%.

181  
182 **2.4 Statistics**  
183 Statistical analyses were performed with the SPSS Package version 17.0 for Windows. A Spearman  
184 correlation coefficient was used to assess the relationship between the variables. A t-test of  
185 independent variables was used for the mean evaluations. The differences and correlations were  
186 considered to be significant at  $p < 0.05$  and highly significant at  $p < 0.01$ . No significant differences were  
187 detected when the chemical and microbiological variables of the samples coming from the two positions  
188 of samplings in the reactor were compared, demonstrating the homogeneity of the mass inside the  
189 digester, so the two samples were not separated for the subsequent analyses.

190  
191 **3. RESULTS AND DISCUSSION**

192  
193 **Total methanogens**  
194 All of the samples were extracted and quantified within the standard curve. The results are shown in  
195 **figure 2**. The concentration of the total methanogens is quite constant, especially since November, as

196 confirmed by **figure 2** and by the descriptive analysis shown in **table 3**. The higher concentration  
197 detected during October could be largely influenced by the higher temperature. A few degrees of  
198 temperature shift towards thermophilic conditions results in a selection of methanogens [39, 40]; this  
199 result will be discussed further for each detected family. The total mean of the methanogens detected  
200 in the digested sludge (approximately  $8 \cdot 10^8$  cells/g SV) is comparable to the levels evaluated in other  
201 studies using this type of reactor [41, 42]. The organic material, after pre-treatment and before its  
202 introduction into the reactor, contained methanogens, but on average, only at low concentrations  
203 ( $9.60 \cdot 10^3 \pm 2.62 \cdot 10^3$  *mcrA* copies/ $\mu$ l DNA extract that is equivalent to approximately  $5 \cdot 10^5$  *mcrA*  
204 copies/g SV).

205

#### 206 **Methanogen families**

207 Methanogens belonging to the Methanosarcinaceae, Methanosaetaceae, Methanocorpusculaceae and  
208 Methanospirillaceae families were identified with a threshold cycle (Ct) that was inversely proportional  
209 to the concentration of the samples, while for the MCR-2b uncultured methanogens, relative  
210 quantification was possible for only 45% of the samples. Finally, the Methanobacteriaceae, uncultured  
211 MCR-2, uncultured MCR-7 and Fen Uncultured groups were identified at levels below the detection  
212 limit.

213 The most abundant methanogens belong to *Methanosarcina*, followed by Methanocorpusculaceae,  
214 *Methanosaeta*, and, finally, the Mrc-2b group (as illustrated in **figure 3**). Moreover, the concentrations  
215 are directly and significantly correlated (**table 4**). *Methanosarcina* increased significantly with the  
216 increase in the total methanogens ( $p < 0.05$ ). This evidence is confirmed in the literature based on the  
217 metabolic and optimal growth conditions, which are similar for these methanogens. *Methanosarcina*,  
218 *Methanosaeta* belong to Methanosarcinaceae and Methanocorpusculaceae belong to  
219 Methanomicrobiales [19]. The substrates used by Methanosarcinaceae for methanogenesis are  $H_2 +$   
220  $CO_2$ , acetate, and methyl compounds; formate is never used. The substrates used by

221 Methanocorpusculaceae for methanogenesis are H<sub>2</sub> + CO<sub>2</sub>, formate, and sometimes alcohols. The use of  
222 acetate seems to be the main biochemical pathway in this kind of system for this reason our attention is  
223 focussed on the Methanosarcinaceae.

224

#### 225 **Total sulphate-reducing bacteria**

226 Sulphate-reducing bacteria are present in 90% of the samples; they are less abundant than  
227 methanogens and are quite constant (**figure 2, table 3**). The level of substrate-reducing bacteria in the  
228 digester is proportionally coherent - at least 3 orders of magnitude – with respect to the methanogens,  
229 as has been previously observed in other studies on these types of digesters [14, 42]. No significant  
230 correlation was observed with methanogens, both in total and as sub-groups. The correlation between  
231 the concentration of sulphate-reducing bacteria and hydrogen sulphide in the biogas is significant  
232 (Spearman rho = - 0.849 p<0.01); moreover, the evaluation of biogas quality was suitable only for 30% of  
233 the samples. The hydrogen sulphide concentration was less than 300 ppm and quite constant (**table 2**),  
234 with few exceptions.

235

#### 236 **Chemico-physical parameter influence on the methanogen community**

237 The anaerobic digestion system can be considered to be quite stable with an increase in the organic load  
238 to levels above 10% SST. A wider variation in the methanogen population was observed for changes in  
239 temperature, but we also observed a relevant change in relation to the organic load. In **figure 4**, it is  
240 shown that only *Methanosaeta* and Methanocorpusculaceae were significantly affected by the  
241 temperature, while the correlation of temperature with *Methanosarcina* and sulphate-reducing bacteria  
242 is not significant. *Methanosarcina* seems to be quite constant with the exception of two days in the first  
243 sampling period. Moreover, *Methanosaeta* appeared to be the more sensitive genus among those  
244 investigated as showed on the figure 3. In fact, the presence of this microorganism varied in relation to  
245 an increase in the input organic load from 4 to 11% of total solids fed (T-test; p<0.05) and before of the

246 acidity increase (figure 1A and figure 3). The methanogens decreased during the time before the acidity  
247 increase especially when we look at the *Methanosaeta* and Methanocorpuscolaceae (around 20<sup>th</sup>  
248 October versus the begin of November). The total methanogens hadn't this clear trend for the major  
249 contribution of *Methanosarcina* that seems to be less variable during the time. There is an inverse,  
250 highly significant correlation between *Methanosaeta* concentration and the organic waste feedings  
251 amounts (Spearman' rho = .492; p<0.01) and between Metanocorpuscolaceae and OLR (Spearman' rho  
252 = .424; p<0.05).

253 We choose *Methanosaeta*, as susceptibility indicator, for its relevance, suggested in the literature,  
254 together with *Methanosarcina* in such anaerobic digestion system (De Vrieze, 2012) on the other hand  
255 Methanocorpuscolaceae showed a quite similar trend to *Methanosaeta* but also more similar also to the  
256 total methanogens and *Methanosarcina* (table 4 and figure 3). An ideal indicator has to be simply and  
257 operative into the application so we propose only one indicator for stability (*Methanosarcina*) and one  
258 for susceptibility (*Methanosaeta*).

259 The relevance of *Methanosarcina* and *Methanosaeta* has been recently discussed in the literature [14,  
260 43], and it is here confirmed for this type of digester and feedings. The Methanocorpusculaceae family is  
261 generally more relevant in psychrophilic conditions and when waste water sludge is present as organic  
262 materials [44, 45]. Moreover the complex microbiome in anaerobic digesters has to be in-depth studied  
263 developing a knowledge able to confirm or to correct the archeal indicators here proposed.

264

#### 265 **4. CONCLUSIONS**

266

267 Bio-molecular methods can result in assistance and improvement to the routine physical-chemical  
268 control of the digestion process. For each type of system, the prevalent population can be determined,  
269 and this knowledge can be used to develop microbiological indicators. Those indicators can reveal, in  
270 advance, the status of the anaerobic population, to produce high quantity and good quality biogas.

271 Moreover, these indicators make it possible to perform alarm evaluations sooner than the chemical  
272 variations, such as pH or acidity. However, biomolecular methods are expensive, so it is necessary to  
273 choose useful bioindicators with a positive economic impact on the process. This can be translated in  
274 terms of sufferance condition removal and compensation. Our work shows that:

- 275 • Methanomicrobiales is the major taxonomic order of methanogens and, in particular,  
276 *Methanosarcina* is the most abundant and quite stable in concentration family in a system fed  
277 mainly by OFMSW, so for this type of system, this last family may be proposed as a stability  
278 bioindicator;
- 279 • *Methanosaeta* and Methanocorpusculaceae exhibited a higher sensitivity to various process  
280 stresses (such as temperature and OLR); for this reason, the genus *Methanosaeta*, more  
281 common in this type of system, may be proposed as a susceptibility bioindicator; and
- 282 • no relevant information can be observed about sulphate-reducing bacteria, likely due to the low  
283 variation of hydrogen sulphide in the biogas and the lack of information about the sulphur  
284 species in the system.

285 Therefore, in our opinion, few bioindicators can be chosen for a specific digestion process, and they can  
286 then be applied in parallel with the physical-chemical monitoring of the process. The early identification  
287 of the sufferance condition can be a real advantage in developing corrective methods.

288

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291 Steinberg and J. Regan for the supply of plasmid for standardisation. Finally, the authors thank all of the  
292 collaborators employed in the anaerobic digestion plant and, obviously, the owners of the company.

293

## 294 **FIGURE LEGENDS**

295 **Figure 1 – A Quantity of the organic waste added to the digester during the time. B Acidity and alkalinity**

296 into the digester determined during the sampling period.

297 Figure 2 - Total methanogen quantification by qPCR-RT (expressed as the mean and standard deviation  
298 of the technical triplicate); for each data point, two samples were collected.

299 **Figure 3** - Methanogen taxa and sulphate-reducing bacterial concentration in the samples during the  
300 sampling period. The samples were numbered from 1 (the first) to 31 (the last). The Y axis unit is the  
301 reciprocal of the Ct (=1/cycle threshold) so its value is proportional to the concentration of the gene  
302 target into the sample.

303 **Figure 4** - Correlation between temperature and microorganism taxa concentrations. A statistically  
304 significant correlation was observed for *Methanosaeta* ( $y = 0,0004x + 0,0173$   $R^2 = 0,6144$ ; Spearman's  
305 rho = 0.697  $p < 0.01$ ) and Methanocorpusculaceae ( $y = 0,0005x + 0,0163$   $R^2 = 0,5103$ ; Spearman's rho =  
306 0.666  $p < 0.01$ ). 1/Ct is equal to the reciprocal of the cycle threshold (1/Ct) so its value is proportional to  
307 the concentration of the gene target into the sample.

308

#### 309 TABLE LEGENDS

310

311 **Table 1:** Chemical-physical characteristics of the process during the sampling period.

312 **Table 2:** Biogas quality analysis by percentage of methane in the biogas and concentration of hydrogen  
313 sulphide and molecular hydrogen (ppm).

314 **Table 3:** Descriptive analysis of the micro-organisms detected by qRT-PCR in the samples. The results are  
315 expressed as mcrA gene copies with respect to the standard curve included in the analytical session or  
316 as the threshold cycle. The threshold cycle is inversely proportional to the concentration in the samples.

317 **Table 4:** Spearman's rho correlation between the total methanogens and the 1/Ct evaluation for each of  
318 the methanogen families. ns = not significant; \*  $p < 0.05$ ; \*\*  $p < 0.01$ .

319

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