IRIS AperTO

UNIVERSITÀ DEGLI STUDI DI TORINO

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

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

This is the author's manuscript

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/133515 since 2015-09-07T14:37:53Z

Published version:

DOI:10.1007/s12155-013-9341-4

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

25 April 2024

UNIVERSITÀ DEGLI STUDI DI TORINO

***This is an author version of the contribution published on**:*

Questa è la versione dell'autore dell'opera:

BIOENERGY RESEARCH, Bioenerg. Res. 7:720 ‑727(2014),

10.1007/s12155-013-9401-9

The definitive version is available at:

La versione definitiva è disponibile alla URL:

[http://link.springer.com/article/10.1007/s12155-013-9401-9]

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[1*], Chiara Capone[1], Silvia Villa[1], Romanazzi Valeria[1], Biancamaria Pietrangeli[2],

6 Giorgio Gilli[1]

7

8 **Affiliations:**

9 [1]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

14 [2] 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_2 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_2. 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 α-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 11th, 2011 to December 20th, 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 SsoFastTM
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 (~$2.52x10^7$ plasmid copies). There was a 1:10 dilution between each
160 standard curve point. The resolution limit for the method was $2.52x10^2$ 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 $8*10^8$ 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 ($9.60*10^3 \pm 2.62*10^3$ *mcrA* copies/µl DNA extract that is equivalent to approximately $5*10^5$ *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 $H_2 + CO_2$, 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 20th
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.

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

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

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

Acknowledgements

The authors wish to thank the INAIL (ex ISPESL) for funding. Special acknowledgments are due to L. Steinberg and J. Regan for the supply of plasmid for standardisation. Finally, the authors thank all of the collaborators employed in the anaerobic digestion plant and, obviously, the owners of the company.

FIGURE LEGENDS

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

320 **BIBLIOGRAPHY**

321 1. Amani T, Nosrati M, Sreekrishnan TR: **Anaerobic digestion from the viewpoint of**
322 **microbiological, chemical, and operational aspects - a review**. *Environmental Reviews* 2010,
323 **18**:255-278.
324 2. Mata-Alvarez J, Dosta J, Mace S, Astals S: **Codigestion of solid wastes: A review of its uses and**
325 **perspectives including modeling**. *Critical Reviews in Biotechnology* 2011, **31**(2):99-111.
326 3. EUROSTAT: **Energy, transport and environment indicators**. In: *Eurostat pockedbook.*
327 Luxembourg: Publications Office of the European Union, 2012; 2012 edition.
328 4. Dinuccio E, Balsari P, Gioelli F, Menardo S: **Evaluation of the biogas productivity potential of**
329 **some Italian agro-industrial biomasses**. *Bioresource Technology* 2010, **101**(10):3780-3783.
330 5. Balat M: **Progress in biogas production processes**. *Energy Education Science and Technology*
331 2008, **22**(1):15-36.
332 6. EUROSTAT: **Sustainable development in the European Union**. In: ***2011 monitoring report of the***
333 ***EU sustainabledevelopment strategy.*** Edited by Commission E. Luxembourg Publications Office
334 of the European Union; 2011.
335 7. Ryckebosch E, Drouillon M, Veruaeren H: **Techniques for transformation of biogas to**
336 **biomethane**. *Biomass & Bioenergy* 2011, **35**(5):1633-1645.
337 8. Morris J, Matthews HS, Morawski C: **Review and meta-analysis of 82 studies on end-of-life**
338 **management methods for source separated organics**. *Waste Management* 2013, **33**(3):545-
339 551.
340 9. Lozano CJS, Mendoza MV, de Arango MC, Monroy EFC: **Microbiological characterization and**
341 **specific methanogenic activity of anaerobe sludges used in urban solid waste treatment**.
342 *Waste Management* 2009, **29**(2):704-711.
343 10. Cardinali-Rezende J, Debarry RB, Colturato LFDB, Carneiro EV, Chartone-Souza E, Nascimento
344 AMA: **Molecular identification and dynamics of microbial communities in reactor treating**
345 **organic household waste**. *Applied Microbiology and Biotechnology* 2009, **84**(4):777-789.
346 11. Ziganshin AM, Liebetrau J, Proter J, Kleinsteuber S: **Microbial community structure and**
347 **dynamics during anaerobic digestion of various agricultural waste materials**. *Applied*
348 *Microbiology and Biotechnology* 2013, **97**(11):5161-5174.
349 12. Azbar N, Ursillo P, Speece RE: **Effect of process configuration and substrate complexity on the**
350 **performance of anaerobic processes**. *Water Res* 2001, **35**(3):817-829.
351 13. Cardinali-Rezende J, Colturato LFDB, Colturato TDB, Chartone-Souza E, Nascimento AMA, Sanz
352 JL: **Prokaryotic diversity and dynamics in a full-scale municipal solid waste anaerobic reactor**
353 **from start-up to steady-state conditions**. *Bioresource Technology* 2012, **119**:373-383.
354 14. De Vrieze J, Hennebel T, Boon N, Verstraete W: **Methanosarcina: The rediscovered**
355 **methanogen for heavy duty biomethanation**. *Bioresource Technology* 2012, **112**:1-9.
356 15. Muyzer G, Stams AJM: **The ecology and biotechnology of sulphate-reducing bacteria**. *Nature*
357 *Reviews Microbiology* 2008, **6**(6):441-454.
358 16. Liu YC, Whitman WB: **Metabolic, phylogenetic, and ecological diversity of the methanogenic**
359 **archaea**. *Incredible Anaerobes: From Physiology to Genomics to Fuels* 2008, **1125**:171-189.
360 17. Friedrich MW: **Methyl-coenzyme M reductase genes: Unique functional markers for**
361 **methanogenic and anaerobic methane-oxidizing Archaea**. *Environmental Microbiology* 2005,
362 **397**:428-442.
363 18. Knittel K, Boetius A: **Anaerobic Oxidation of Methane: Progress with an Unknown Process**.
364 *Annual Review of Microbiology* 2009, **63**:311-334.
365 19. Whitman WB, Bowen TL, Boone DR: **The Methanogenic Bacteria**. *Prokaryotes* 2006, **3**(Chapter
366 9):165-207.
367 20. Franke-Whittle IH, Goberna M, Insam H: **Design and testing of real-time PCR primers for the**

368 **quantification of Methanoculleus, Methanosarcina, Methanothermobacter, and a group of**
369 **uncultured methanogens**. *Canadian Journal of Microbiology* 2009, **55**(5):611-616.
370 21. Luton PE, Wayne JM, Sharp RJ, Riley PW: **The mcrA gene as an alternative to 16S rRNA in the**
371 **phylogenetic analysis of methanogen populations in landfill**. *Microbiology-Sgm* 2002,
372 **148**:3521-3530.
373 22. Steinberg LM, Regan JM: **Phylogenetic Comparison of the Methanogenic Communities from an**
374 **Acidic, Oligotrophic Fen and an Anaerobic Digester Treating Municipal Wastewater Sludge**.
375 *Applied and Environmental Microbiology* 2008, **74**(21):6663-6671.
376 23. Ettwig KF, Shima S, van de Pas-Schoonen KT, Kahnt J, Medema MH, op den Camp HJM, Jetten
377 MSM, Strous M: **Denitrifying bacteria anaerobically oxidize methane in the absence of**
378 **Archaea**. *Environmental Microbiology* 2008, **10**(11):3164-3173.
379 24. Bergmann I, Mundt K, Sontag M, Baumstark I, Nettmann E, Klocke M: **Influence of DNA isolation**
380 **on Q-PCR-based quantification of methanogenic Archaea in biogas fermenters**. *Systematic and*
381 *Applied Microbiology* 2010, **33**(2):78-84.
382 25. Bergmann I, Nettmann E, Mundt K, Klocke M: **Determination of methanogenic Archaea**
383 **abundance in a mesophilic biogas plant based on 16S rRNA gene sequence analysis**. *Canadian*
384 *Journal of Microbiology* 2010, **56**(5):440-444.
385 26. Nettmann E, Bergmann I, Mundt K, Linke B, Klocke M: **Archaea diversity within a commercial**
386 **biogas plant utilizing herbal biomass determined by 16S rDNA and mcrA analysis**. *Journal of*
387 *Applied Microbiology* 2008, **105**(6):1835-1850.
388 27. Meyer B, Kuever J: **Molecular analysis of the distribution and phylogeny of dissimilatory**
389 **adenosine-5'-phosphosulfate reductase-encoding genes (aprBA) among sulfuroxidizing**
390 **prokaryotes**. *Microbiology-Sgm* 2007, **153**:3478-3498.
391 28. Meyer B, Kuever J: **Phylogeny of the alpha and beta subunits of the dissimilatory adenosine-5'-**
392 **phosphosulfate (APS) reductase from sulfate-reducing prokaryotes - origin and evolution of**
393 **the dissimilatory sulfate-reduction pathway**. *Microbiology-Sgm* 2007, **153**:2026-2044.
394 29. De Vrieze J, Hennebel T, Boon N, Verstraete W: **Methanosarcina: the rediscovered methanogen**
395 **for heavy duty biomethanation**. *Bioresour Technol*, **112**:1-9.
396 30. Demirel B, Yenigun O: **Changes in microbial ecology in an anaerobic reactor**. *Bioresource*
397 *Technology* 2006, **97**(10):1201-1208.
398 31. Traversi D, Villa S, Acri M, Pietrangeli B, Degan R, Gilli G: **The role of different methanogen**
399 **groups evaluated by Real-Time qPCR as high-efficiency bioindicators of wet anaerobic co-**
400 **digestion of organic waste**. *AMB Express* 2011, **1**:28.
401 32. Traversi D, Villa S, Lorenzi E, Degan R, Gilli G: **Application of a real-time qPCR method to**
402 **measure the methanogen concentration during anaerobic digestion as an indicator of biogas**
403 **production capacity**. *Journal of Environmental Management* 2012, **111**:173-177.
404 33. Nolvak H, Truu M, Truu J: **Evaluation of quantitative real-time PCR workflow modifications on**
405 **16S rRNA and tetA gene quantification in environmental samples**. *Sci Total Environ*, **426**:351-
406 358.
407 34. Botes M, de Kwaadsteniet M, Cloete TE: **Application of quantitative PCR for the detection of**
408 **microorganisms in water**. *Anal Bioanal Chem*, **405**(1):91-108.
409 35. Bustin SA: **Why the need for qPCR publication guidelines?--The case for MIQE**. *Methods*,
410 **50**(4):217-226.
411 36. Lueders T, Chin KJ, Conrad R, Friedrich M: **Molecular analyses of methyl-coenzyme M reductase**
412 **alpha-subunit (mcrA) genes in rice field soil and enrichment cultures reveal the methanogenic**
413 **phenotype of a novel archaeal lineage**. *Environmental Microbiology* 2001, **3**(3):194-204.
414 37. Meyer B, Kuever J: **Molecular analysis of the diversity of sulfate-reducing and sulfur-oxidizing**
415 **prokaryotes in the environment, using aprA as functional marker gene**. *Applied and*
416 *Environmental Microbiology* 2007, **73**(23):7664-7679.

417 38. Steinberg LM, Regan JM: **mcrA-Targeted Real-Time Quantitative PCR Method To Examine**
418 **Methanogen Communities**. *Applied and Environmental Microbiology* 2009, **75**(13):4435-4442.
419 39. Kundu K, Sharma S, Sreekrishnan TR: **Effect of operating temperatures on the microbial**
420 **community profiles in a high cell density hybrid anaerobic bioreactor**. *Bioresource Technology*
421 2012, **118**:502-511.
422 40. Solera R, Romero LI, Sales D: **Determination of the microbial population in thermophilic**
423 **anaerobic reactor: Comparative analysis by different counting methods**. *Anaerobe* 2001,
424 **7**(2):79-86.
425 41. Merlino G, Rizzi A, Schievano A, Tenca A, Scaglia B, Oberti R, Adani F, Daffonchio D: **Microbial**
426 **community structure and dynamics in two-stage vs single-stage thermophilic anaerobic**
427 **digestion of mixed swine slurry and market bio-waste**. *Water Research* 2013, **47**(6):1983-1995.
428 42. Merlino G, Rizzi A, Villa F, Sorlini C, Brambilla M, Navarotto P, Bertazzoni B, Zagni M, Araldi F,
429 Daffonchio D: **Shifts of microbial community structure during anaerobic digestion of agro-**
430 **industrial energetic crops and food industry byproducts**. *Journal of Chemical Technology and*
431 *Biotechnology* 2012, **87**(9):1302-1311.
432 43. Conklin A, Stensel HD, Ferguson J: **Growth kinetics and competition between Methanosarcina**
433 **and Methanosaeta in mesophilic anaerobic digestion**. *Water Environment Research* 2006,
434 **78**(5):486-496.
435 44. Abram F, Enright AM, O'Reilly J, Botting CH, Collins G, O'Flaherty V: **A metaproteomic approach**
436 **gives functional insights into anaerobic digestion**. *Journal of Applied Microbiology* 2011,
437 **110**(6):1550-1560.
438 45. O'Reilly J, Lee C, Collins G, Chinalia F, Mahony T, O'Flaherty V: **Quantitative and qualitative**
439 **analysis of methanogenic communities in mesophilically and psychrophilically cultivated**
440 **anaerobic granular biofilims**. *Water Research* 2009, **43**(14):3365-3374.
441
442