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MICROBIAL COMMUNITY REDUNDANCE IN BIOMETHANATION SYSTEMS LEAD TO FASTER RECOVERY OF METHANE PRODUCTION RATES AFTER STARVATION

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ABSTRACT

The Power-to-Gas concept corresponds to the use of the electric energy surplus to produce H_2 by water electrolysis, that can be further converted to methane by biomethanation. However, the fluctuant production of renewable energy sources can lead to discontinuous H_2 injections into the reactors, that may interfere with the adaptation of the microbial community to high H_2 partial pressures. In this study, the response of the microbial community to H_2 and organic feed starvation was evaluated in *in-situ* and *ex-situ* biomethanation. The fed-batch reactors were fed with acetate or glucose and H_2, and one or four weeks of starvation periods were investigated. Methane productivity was mostly affected by the four-week starvation period. However, both *in-situ* and *ex-situ* biomethanation reactors recovered their methane production rate after starvation within approximately one-week of normal operation, while the anaerobic digestion (AD) reactors did not recover their performances even after 3 weeks of normal operation. The recovery failure of the AD reactors was probably related to a slow growth of the syntrophic and methanogen microorganisms, that led to a VFA accumulation. On the contrary, the faster recovery of both biomethanation reactors were related to the replacement of *Methanoculleus* sp. by *Methanobacterium* sp., restoring the methane production in the *in-situ* and *ex-situ* biomethanation reactors. This study has shown

that biomethanation processes can respond favourably to the intermittent H_2 addition without compromising their CH_4 production performance.

KEYWORDS

Biological Methanation, Biogas Upgrading, Hydrogen, Hydrogenotrophic methanogens, *Methanobacterium* sp.

HIGHLIGHTS

- Biomethanation reactors recovered their CH_4 production after 4-weeks starvation
- 4-week starvation period led to process failure in conventional AD reactors
- Lower VFA accumulation was found in biomethanation reactors compared to AD ones
- Methanogen's redundancy led to the CH_4 productivity recovery of biomethanation

1. INTRODUCTION

Over the past decades, the number of renewable energy plants from wind and solar sources have widely increased and their input is nowadays significant onto the global energy mix. However, the weather-dependent nature of the renewable energy sources leads to a fluctuant production and a lack of synchronicity between the energy production periods and the peak demands, causing energy lost due to the impossibility to directly store the electrical energy (Bailera et al., 2017). Therefore, technological solutions for long-term storage are nowadays required to achieve the successful transition towards an economy fully based on renewable energy (Thema et al., 2019). The Power-to-Gas concept (PtG) corresponds to the use of the electrical energy surplus to produce H_2 through water electrolysis. H_2 can be further converted into methane by catalytic or biological methanation processes (Angelidaki et al., 2018). Methane can then be directly injected into the gas grid, used as vehicle fuel, or stored into the gas form (Götz et al., 2016). The biological methanation, so-called biomethanation, is based in the anaerobic digestion process and uses microorganisms as

52 catalysers for producing CH_4 from CO_2 and H_2. Using microorganisms allows to proceed at
53 milder conditions (temperature between 35°C and 65°C, atmospheric pressures) than
54 catalytic processes, with a smaller carbon footprint besides the potential of transforming CO_2
55 into CH_4 or other interesting biomolecules (Fu et al., 2021). Biomethanation can be applied in
56 two configurations: (i) the *in-situ* biomethanation configuration, which consists in the direct
57 injection of H_2 into an anaerobic digester treating organic substrate resources, to convert the
58 CO_2 from the biogas into CH_4, resulting in an upgrade of the CH_4 content and (ii) the *ex-situ*
59 biomethanation configuration, where biogas and H_2 are concomitantly injected into a
60 dedicated reactor, separated from the anaerobic digester, in which the biogas upgrade takes
61 place (Lecker et al., 2017). Besides, biogas, external CO_2 provided by other sources can be
62 injected into the *in-situ* or *ex-situ* reactors, such as syngas or from other industrial gas
63 streams (Lecker et al., 2017; Rittmann et al., 2015; Tao et al., 2020). In both process, the
64 production of CH_4 is carried out by hydrogenotrophic methanogens, which have capacity to
65 directly reduce CO_2 into CH_4 using H_2 as electron donor (Zabranska and Pokorna, 2018).
66 These microorganisms are ubiquitous in anaerobic digesters.
67 Anaerobic digestion (AD) is a microbial process, which consists of successive microbial
68 steps to degrade organic substrates into a biogas mainly composed of CH_4 and CO_2 (Aryal
69 et al., 2018). First, hydrolytic microorganisms depolymerise the complex organic matter, as
70 polysaccharides or proteins, into their smaller units which are further fermented into volatile
71 fatty acids (VFA), alcohols, H_2 and CO_2 by acidogenic bacteria. These molecules are then
72 used as substrates by acetogenic bacteria and methanogenic archaea which syntrophically
73 degrade them into CH_4 and CO_2 (Merlin Christy et al., 2014). A stable methane production is
74 the result of a well-balanced process between the different microbial populations and the
75 products (or substrates) generated in each step of the AD pathway. The direct H_2 injection
76 into the AD reactor during *in-situ* biomethanation can cause a disturbance of the microbial
77 community as acetogens are extremely sensitive to dissolved H_2 (Schink, 1997). Excess H_2
78 may lead to VFA accumulation and pH drop, which can negatively affect the methanogenic
79 activity and eventually cause a process failure (Cuff et al., 2020). Nonetheless, because of

80 their high diversity, the microbial AD ecosystems have the ability to handle disturbances
81 (Carballa et al., 2015). Anaerobic digesters are often exposed to different types of
82 disturbances such as starvation or overloads (Hwang et al., 2010; Regueiro et al., 2015),
83 which deeply affect the microbial community. During the recovery period, the reactors are in
84 general less performant or present unstable CH_4 production rates (de Jonge et al., 2017;
85 Hwang et al., 2010). For instance, Wang et al. (2019) have observed a recovery period of 10
86 days after 59-days of starvation period, while de Jonge et al. (2017) have reported an
87 instability of the AD process during a 45-day recovery period after a starvation of 55 days.
88 Hwang et al. (2010) have observed that the total methane production after a 4-month
89 starvation period was 1.7-fold lower than before starvation. De Jonge et al. (2017), Hwang et
90 al. (2010) and Wang et al. (2019) have reported alterations in the microbial community
91 composition and abundance, mostly affecting the syntrophic bacteria and methanogens
92 interactions. Therefore, the observed recovery periods during conventional AD could be
93 related to the fact that methane production in AD depends on the coordination of several
94 microbial groups (de Jonge et al., 2017; Hwang et al., 2010). Meanwhile, during *ex-situ* and
95 *in-situ* biomethanation, the methane production mostly depends on one microbial group, the
96 hydrogenotrophic methanogens, whose direct substrate is added at no limiting conditions
97 (Savvas et al., 2018).
98 Hydrogenotrophic methanogens have been reported to dominate the archaeal microbial
99 community during *in-situ* and *ex-situ* biomethanation processes when achieving high-CH_4-
100 content biogas. For instance, Wang et al. (2013) have reported the dominance of
101 *Methanoculleus* sp. during *in-situ* biomethanation, when producing a biogas with a methane
102 content of 98.8%, while Logroño et al. (2021) have found *Methanobacterium* sp. to be
103 dominant during *ex-situ* biomethanation, when achieving 97.56 % CH_4 content in their
104 biogas. Besides, these authors have observed that the persistence of *Methanobacterium* sp.
105 was crucial for recovering the efficiency of the process after a 14-day starvation period
106 (Logroño et al., 2021). On the other hand, H_2 can be also consumed by homoacetogens to
107 produce acetate, which will be detrimental for CH_4 production. Although, the association

108 between homoacetogens and acetoclastic methanogens have been reported to alleviate
109 acetate accumulation and contribute to CH_4 production (Agneessens et al., 2018; Wang et
110 al., 2013). Therefore, the selection and maintenance of a microbial community adapted to a
111 high H_2 partial pressures is crucial for an efficient methane production during biomethanation.
112 In a PtG context, *in-situ* or *ex-situ* biomethanation reactors may face discontinuous H_2
113 injection, inducing variable periods of starvation that can likely affect the recovery of the
114 process (Strübing et al., 2018). Savvas et al. (2018) have reported a fast recovery period (24
115 h) for four *ex-situ* biomethanation reactors (three were operated at 37°C and one at 55°C)
116 after a gas feeding starvation of 13 and 45 days (mixing and temperature control were
117 stopped). Strubing et al. (2018) have observed that *ex-situ* biomethanation reactors have a
118 faster recovery in mesophilic conditions (25°C) than in thermophilic conditions (55°C) (4.5 h
119 and 12.4 h, respectively). Besides, these authors have reported no significant difference
120 between the CH_4 production or H_2 consumption rates after 1, 4 or 8 days of starvation.
121 Conversely to these findings, Logroño et al., (2021) have reported a significant lower H_2
122 consumption and CH_4 production rate after 7 days of starvation during *ex-situ*
123 biomethanation at mesophilic temperature (37°C). Meanwhile, Agneessens et al. (2017)
124 have observed a lower H_2 uptake after 10-day H_2 starvation during *in-situ* biomethanation,
125 although the recovery period of the reactors was not reported. On the contrary, Wahid et al.
126 (2019) reported an immediate recovery of the CH_4 production rate in *in-situ* biomethanation
127 reactors, although only after three days without H_2 addition. However, the electric supply
128 from mostly wind power plants, can be stopped for periods as long as one month during the
129 summer season (wind power plants) or for maintenance stoppages (Savvas et al., 2018).
130 Hence, further investigation on the effect of discontinuous H_2 addition on the microbial
131 community of biomethanation systems is needed.
132 Therefore, the aim of this work was to evaluate and characterize the performance recovery
133 and response of the microbial community of *in-situ* biomethanation reactors and *ex-situ*
134 biomethanation reactors after being exposed to a short (1 week) and a long (4 weeks)
135 starvation period.

136

137

138

139 ## 2. MATERIALS and METHODS

140 ### 2.1. Operational conditions

141 Schott flasks of 1000 mL were inoculated with an anaerobic leachate sampled from a
142 discontinuous mesophilic dry-AD process treating cattle manure. The initial pH of the
143 inoculum was 7.92 ± 0.02, the TS concentration was 12.3 ± 0.1 gTS/L, while the VS
144 concentration was 9.2 ± 0.1 gVS/L. No pre-treatment was applied to the inoculum. The
145 reactors' working volume was 200 mL and the initial inoculum concentration was 5 gVS/L.
146 The reactors were sealed with a rubber stopper and incubated at 35°C at a stirring speed of
147 370 rpm. They were supplemented with a mineral medium composed of: NH_4Cl 859 mg/L,
148 KH_2PO_4 323mg/L, hexa-hydrated $MgCl_2$ 194 g/L, di-hydrated $CaCl_2$ 97mg/L, and an oligo-
149 element solution as described in Cazier et al. (2015). Buffer phosphate was also added at a
150 0.5 M concentration, at pH 7.5. The reactors were exposed to 5 different experimental
151 conditions: (i) AD, in which the reactors were fed with glucose, (ii) AD, in which the reactors
152 were fed with acetate, (iii) *In-situ* biomethanation: reactors were fed with glucose and H_2/CO_2
153 (molar ratio of 4:1), (iv) *In-situ* biomethanation: the reactors were fed with acetate and H_2/CO_2
154 (molar ratio of 4:1, and (v) *Ex-situ* biomethanation, in which the reactors received only a gas
155 mixture composed by H_2 and CO_2 (molar ratio 4:1). The AD reactors have principally function
156 as control reactors. The addition of a mixture of H_2 and CO_2 in a 4:1 proportion was made in
157 order to avoid a sudden pH increase as observed in other biomethanation studies
158 (Agneessens et al., 2018, 2017; Braga Nan et al., 2020). The experiments were carried out
159 in duplicates for 99 days.

160 ### 2.2. Operational strategy

161 Figure 1 shows the operational diagram. The operation consisted first in a one-week
162 acclimation period (S0), and in three stages of normal operation (Stage 1 = 2-weeks

163 operation, Stage 2 = 3-weeks operation and Stage 3 = 3-weeks operation) separated by two
164 starvation periods (P1 = 1-week starvation period and P2 = 4-week starvation period). During
165 the acclimation week, the reactors were fed only with an organic substrate (glucose or
166 acetate) at an organic loading rate of 0.01 ± 0.004 gCOD/L_R/day. During stages 1, 2 and 3,
167 the AD reactors were fed with 0.02 ± 0.005 gCOD/L_R/day of glucose or acetate. The *in-situ*
168 biomethanation reactors started receiving 1,0 ± 0.2 gCOD/L_R/d of H_2 (H_2/CO_2 gas mixture),
169 besides the glucose or acetate they were already receiving. The *ex-situ* biomethanation
170 reactors were fed with 1,1 ± 0.2 gCOD/L_R/d of H_2 (H_2/CO_2 gas mixture). The reactors were
171 fed in a semi-continuous mode (every day). The H_2/CO_2 (1:4) gas mixture was manually
172 injected into the reactors. At t0, the reactors were flushed with N_2 for 10 minutes to achieve
173 an anaerobic environment, afterwards, the gas in the reactors was released until reaching
174 the atmospheric pressure. The H_2/CO_2 gas mixture was then injected into the reactors until
175 reaching a maximum pressure of 1.5 bar, for security reasons. For the subsequent gas
176 injections, the procedure was the same: the biogas was first released until reaching
177 atmospheric pressure, then the substrate gas was added to the reactors until reaching a 1.5
178 bar pressure in the reactors. During starvation period, the reactors were not fed with organic
179 substrates nor with H_2, in order to evaluate the effect of starvation in all the anaerobic
180 digestion chain.

181 ***Figure 1 – Operational diagram***

182 ### 2.3. Analytical methods

183 Gas pressure was manually measured with a manometer Keller LEO 2 (KELLER AG,
184 Winterthur, Switzerland). The gas composition was determined by gas chromatography using
185 GC Perkin Elmer model Clarus 580, with thermal conductivity detector as described by
186 Moscoviz et al. (2016). Gas pressure and composition were measured twice a day, before
187 and after H_2 feeding. Liquid samples were taken every day and centrifuged (13500 rpm, 15
188 min). The supernatant was used to analyse the Volatile Fatty Acid (VFA) and glucose
189 concentration while the pellet was kept at – 20°C for further molecular biology analysis.

190 Glucose concentration of the sample was analysed by YSI 2900D biochemistry analyser,
191 with the corresponding membrane and buffer, according to manufacturer instructions (YSI
192 Inc. Yellow Springs, USA) while VFA were analysed by gas chromatography (Perkin Elmer,
193 Clarus 580) coupled with a flame ionization detector as described in Cazier et al. (2015).

194

195 **2.4. Calculations**

196 The metabolite yield defined as the total accumulated amount of each individual metabolite
197 divided by the total amount of substrate added, was assessed over a period of one week.

198 The yields for the acetate-fed or glucose-fed AD reactors were calculated as follows:

199 $$Y_{CH_4} = \frac{CH_4\,prod}{HAc_{add}\ or\ glc_{add}} \quad \text{(eq. 1)}$$

200 $$Y_{HAc} = \frac{HAc\,prod}{glc_{add}} \quad \text{(eq. 2)}$$

201 $$Y_{HPr} = \frac{HPr\,prod}{HAc_{add} or glc_{add}} \quad \text{(eq. 3)}$$

202 Yields for the acetate-fed or the glucose-fed *in-situ* biomethanation reactors were calculated
203 as follows:

204 $$Y_{CH_4} = \frac{CH_4\,prod}{(HAc_{add}\ or\ glc_{add} + H_{2\,add})} \quad \text{(eq. 4)}$$

205 $$Y_{HAc} = \frac{HAc\,prod}{(HAc_{add}\ or\ glc_{add} + H_{2\,add})} \quad \text{(eq. 5)}$$

206 $$Y_{HPr} = \frac{HPr\,prod}{(HAc_{add}\ or\ glc_{add} + H_{2\,add})} \quad \text{(eq. 6)}$$

207 Yields for the *ex-situ* biomethanation reactors were calculated as follows:

208 $$Y_{CH_4} = \frac{CH_4\,prod}{H_{2\,add}} \quad \text{(eq. 7)}$$

209 $$Y_{HAc} = \frac{HAc\,prod}{H_{2\,add}} \quad \text{(eq. 8)}$$

210 Where Y_{CH_4} is the methane yield, Y_{HAc} is the acetate yield and Y_{HPr} is the propionate yield, the
211 metabolites yields were expressed as gCOD of produced metabolite/gCOD of added
212 substrate. $CH_4\,prod$ represents the produced methane expressed in gCOD, while $HAc\,prod$
213 represents the produced acetate and $HPr\,prod$ represents the produced propionate, both
214 also expressed in gCOD. HAc_{add} represents the added acetate, glc_{add} represents the added
215 glucose and $H_{2\,add}$ represents the added H_2, all in gCOD.

216 **2.5. Microbial community analysis**

217 The microbial community composition was analysed by Illumina Miseq sequencing. For the
218 sequencing, the inocula, one sample before and after starvation and one sample from each
219 operation week were analysed. The DNA extraction was made with a FastDNA™ SPIN kit in
220 accordance with the manufacturer's instructions (MP biomedicals, LCC, California, USA).

221 **2.5.1. Sequencing of Bacterial and Archaeal Communities**

222 The *Bacteria* members were identified by amplification of the V3-V4 region of the 16S rRNA
223 gene as reported by Carmona-Martínez et al. (2015). The following degenerated primers
224 were designed by our laboratory in order to amplify the V4-V5 region of the 16S rRNA gene
225 to target *Archaea* members: 5'-CAGCMGCCGCGGKAA-3' (F504 – 519) and 5'-
226 CCCGCCWATTCCTTTAAGT-3' (R910 – 928). Adapters and bar codes for Miseq
227 sequencing were already included in the primer sets. The PCR mix contained MTP™ Taq
228 DNA Polymerase (Sigma-Aldrich, Inc., Merck, Germany) (0.05 u/µL) with its enzyme buffer,
229 forward and reverse primers (0.5 mM), dNTP (0.2 mM), sample DNA (0.04 to 0.2 ng/µL) and
230 water with a 60µL final volume. The PCR amplification program was the following: 35 cycles
231 of denaturation (95°C, 1 min), annealing (set at 59°C, 1 min) and elongation (72°C, 1min). At
232 the end of 35 amplification cycles, a final extension step was carried out for 10 min at 72°C.
233 PCR reactions were carried on in a Mastercycler® thermal cycler (Eppendorf, Hamburg,
234 Germany). The sequencing reaction was carried out in Illumina Miseq sequencer at the
235 GenoToul platform, Toulouse, France (www.genotoul.fr). Reads cleaning, assembly and

236 quality checking was performed in Mothur version 1.39.5. SILVA release 128 was used for
237 alignment and taxonomic outline (Venkiteshwaran et al., 2016).

238 The generated sequencing datasets are registered in the Sequence Read Archive
239 (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA735449,
240 with SRA accessions numbers SRR14743890 to SRR14743946 for the Bacteria-targeted-
241 sequencing dataset and SRR14743947 to SRR14744003 for the Archaea-targeted-
242 sequencing dataset.

243 2.6. Statistical analysis

244 All statistical analyses were performed with R software version 4.0.2 using Rstudio version
245 1.2.5001. The Kruskal–Wallis tests, the Wilcoxon test, and the Bonferroni correction method
246 to adjust the p-values for pairwise comparisons, were performed with the "rstarix" version
247 0.6.0. The microbial community data analyses were made using PhyloSeq package v 1.32.0
248 (McMurdie and Holmes, 2013). While the PCA analysis was performed with the package
249 "FactoMineR" v 2.4.

250 3. RESULTS and DISCUSSION

251 3.1. Reactor performances before and after starvation

252 3.1.1. Methane production

253 Figure 2A shows the cumulated volumes of methane throughout the total duration of the
254 experiments. At the end of the operation, the *in-situ* biomethanation reactors fed with glucose
255 produced 307 ± 4.2 mL CH_4/gCOD$_{add}$, and the methane content in the reactors was 83.5 ±
256 0.1 %, while the *in-situ* biomethanation reactors fed with acetate produced 327 ± 25.4 mL
257 CH_4/gCOD$_{add}$ achieving a CH_4 content of 89.7± 0.4% (Figure 2B). Meanwhile, the e*x-situ*
258 biomethanation reactors produced 345 ± 10 mL CH_4/gCOD$_{add}$ and reached a CH_4 content of
259 92.3 ± 0.2%. Whilst the acetate-fed AD reactors and the glucose-fed AD reactors produced
260 318 ± 1 mL CH_4/gCOD$_{add}$ (CH_4 content = 72.6 ± 4.1%) and 258 ± 6.3 mL CH_4/gCOD$_{add}$ (CH_4
261 content = 39.8 ± 1.2%), respectively.

262 ***Figure 2 – The cumulated methane volume at 35°C along the operation (A) and the total volume***
263 ***of CH_4 produced per $gCOD_{add}$ (B) is shown for the in-situ biomethanation reactors fed with***
264 ***acetate (Ac-in-situ), the glucose-fed in-situ biomethanation reactors (glc-in-situ), the ex-situ***
265 ***biomethanation reactors (ex-situ) and the acetate-fed and glucose-fed AD reactors (Ac-AD and***
266 ***glc-AD, respectively). In A, S0 represents the acclimation stage, S1, S2 and S3 represent stages***
267 ***1, 2 and 3, respectively and each stage duration is delimited by the discontinous lines. The***
268 ***periods in between the operation stages represent the duration of each starvation period (P1=***
269 ***1 week; P2= 4 weeks).***

270 To observe the effect of the starvation periods on the reactor performances, the daily
271 methane production rates (MPRs) were calculated over a period of one week and plotted in
272 Figure 3A for AD reactors fed with glucose and acetate and in Figure 3B for both *in-situ* and
273 *ex-situ* biomethanation reactors. A statistical analysis of the methane production rates was
274 also performed to assess the performance before and after the starvation periods. A Kruskal-
275 Wallis test followed by a Wilcoxon test, using the Bonferroni's p-value correction method was
276 used to compare the MPRs measured the week before starvation periods to the MPRs of the
277 three weeks after the starvation periods.

278 It was observed that most of the reactors (i.e., AD, *in-situ* and *ex-situ* biomethantion reactors)
279 were not affected by a 1-week of starvation period (P1), since no statistically significant
280 differences were detected between the MPR of the last week of stage 1 and the MPRs of the
281 three weeks of stage 2 (Figure 3A and 3B). Only the AD reactors fed with acetate showed a
282 significantly lower MPR during the first week of stage 2 compared to the MPR of the last
283 week of operation of stage 1. Nonetheless, the methane productivity was recovered at the
284 second operation week of stage 2 (Figures 3A).

285 Different behaviours of performance recovery were observed after a 4-week starvation period
286 (P2). Indeed, a lower methane productivity was observed in most of the conditions (i.e., AD,
287 *in-situ* and *ex-situ* biomethanation) during the first week of stage 3 regarding the last-week of
288 operation of stage 2 (Figures 3A and 3B). In particular, performances between stages 2 and

289 3 were statistically different ($p<0.05$) for the AD reactors fed with acetate or glucose, the
290 acetate-fed *in-situ* biomethanation reactors and the *ex-situ* biomethanation reactors.
291 However, the MPR of *ex-situ* biomethanation reactors were recovered by the second week of
292 operation of stage 3, while the MPR of acetate-fed AD reactors and acetate-fed *in-situ*
293 biomethanation reactors were recovered by the third week of operation in stage 3. The
294 glucose-fed AD reactors did not recover their MPR after three weeks of normal operation
295 along stage 3. Meanwhile, regarding the performances of the *in-situ* biomethanation reactors
296 fed with glucose, a decrease in the MPR by the first week of operation of stage 3 was
297 detected (figure 3B), however, it was not statistically different ($p>0.05$) from the MPR of the
298 last-week-of-operation of stage 2, suggesting that the process was not affected by the four
299 weeks of starvation.

300 In the literature, syntrophic microorganisms and methanogens are described as more
301 affected by starvation periods than fermentative bacteria. Hwang et al. (2010) observed a
302 better activity of the acidogenic microorganisms one month before the methanogens
303 recovered their activity during the anaerobic digestion of swine wastewater. Besides,
304 methanogens depend also on the activity of slow-growing syntrophic microorganisms, which
305 can delay the recovery of the methanogenic activity (Amani et al., 2010; de Jonge et al.,
306 2017). Regarding, the slow recovery of the MPR in acetate-fed *in-situ* biomethanation
307 reactors was likely due to a specific inhibition of the hydrogenotrophic methanogens caused
308 by the high acetate concentrations (Zhang et al., 2018).

309 Comparatively, in a biomethanation context in which H_2 and CO_2 are added in a
310 stoichiometric relation, hydrogenotrophic methanogens do not depend on other
311 microorganisms to produce methane (Savvas et al., 2018). That can explain the faster
312 performance recovery observed in the *ex-situ* biomethanation and the glucose-fed *in-situ*
313 biomethanation reactors. Consistently, the VFA concentrations in these last reactors were
314 low regarding the other reactors (data non shown).

315 ***Figure 3 – Methane production rate (MPR) of the AD (A), in-situ and ex-situ biomethanation***
316 ***reactors (B) calculated by operation week. The p-value of the statistically significant different***
317 ***MPRs are shown. The grey rectangles between stages indicate the starvation periods P1 (1***
318 ***week) and P2 (4 weeks).***

319 ### 3.1.2. Substrate consumption and metabolite production

320 The COD mass balance was estimated for each condition (Supplementary material 1) and
321 showed that no major metabolite was omitted in this study, considering that at least 10 to
322 12% of the COD contributed to the production of microbial biomass and a reasonable
323 variability error of 10% (Angelidaki and Sanders, 2004; Cohen et al., 1979; Paillet et al.,
324 2019). The conversion yields of the substrates into the main metabolites were assessed
325 (CH_4, acetate and propionate) for each week of operation during stages 1, 2 and 3. The
326 consumption of the organic substrates and H_2 was also estimated (Figure 4A, 4B, 4C, 4D
327 and 4E).

328 After the first period of 1-week starvation, the AD reactors fed with glucose showed an
329 increase of the acetate and propionate yields at the expense of the methane yield. Similar
330 behaviour was observed in the *in-situ* biomethanation reactors fed with glucose (Figure 4A
331 and 4B, respectively). Glucose was completely consumed in both conditions, indicating a
332 good activity of acidogens. However, the acetate and propionate accumulation detected in
333 these reactors indicates that syntrophs and acetotrophic methanogens were at some extent
334 affected by a 1-week starvation period.

335 After the 4-week starvation period, a decrease in glucose consumption during the first week
336 of operation of the AD reactors in stage 3 was observed, as well as an accumulation of
337 acetate, and mainly propionate. The substrate consumption rapidly recovered at the second
338 week of operation in stage 3, while the propionate yield increased (from 0.14 ± 0.01
339 $gCOD_{propionate}/gCOD_{glucose}$ in the first week of stage 3 to 0.37 ± 0.06 $gCOD_{propionate}/gCOD_{glucose}$
340 on the third week) at the expense of the methane yield (from 0.25 ± 0.06
341 $gCOD_{CH4}/gCOD_{glucose}$ to 0.21 ± 0.01 respectively). Propionate is thermodynamically more

342 difficult to degrade than the others VFA such as acetate, as the reactions are more
343 endergonic (Müller et al., 2010). Propionate accumulation could have been caused by an
344 increase in the H_2 partial pressure in these glucose-fed AD reactors, if acetogenesis
345 reactions occurred faster than the acetogenesis ones, hence producing H_2 at a faster rate
346 than its consumption rate. However, the H_2 partial pressure in these reactors was during
347 stage 3 was in average $9.2 \times 10^{-5} \pm 1.8 \times 10^{-5}$ atm, which was lower than the required to
348 cause inhibition (> 5×10^{-3} atm) (Kaspar and Wuhrmann, 1977). Accumulation of propionate
349 during AD clearly indicated an imbalance between the production and consumption of
350 propionate, likely due to a slower recovery of the syntrophic microorganisms capable of
351 consuming propionate (Wang et al., 2019). The increasing accumulation of acetate during
352 stage 3 (from 0.08 ± 0.03 $gCOD_{acetate}/gCOD_{glucose}$ to 0.15 ± 0.05 $gCOD_{acetate}/gCOD_{glucose}$) was
353 likely resulting from the inhibition of acetotrophic methanogens due to propionate
354 accumulation (Amani et al., 2011). Although such VFA accumulation caused a pH decrease
355 (final pH of 6.61 ± 0.01), the pH was always within a range favourable to the growth of
356 methanogens, i.e. between 6.0 and 8.5 (Zabranska and Pokorna, 2018). In contrast, for the
357 *in-situ* biomethanation reactors fed with glucose, no decrease of the substrate consumption
358 was observed during the first week of operation in stage 3. A minor decrease in glucose
359 consumption was observed for the second and third week of operation in stage 3 (93 ± 0.3 %
360 and 97 ± 0.2 % of glucose consumption, respectively), probably due to a slight inhibition of
361 acidogens. Even though a propionate accumulation from the first week to the third week of
362 operation of stage 3 was observed (0.04 ± 0.01 $gCOD_{propionate}/gCOD_{glucose+H2}$ to 0.08 ± 0.03
363 $gCOD_{propionate}/gCOD_{glucose+H2}$), it was lower than in the AD reactors fed with glucose. The low
364 propionate accumulation during *in-situ* biomethanation was attributed to the rapid
365 consumption of H_2 by the enhanced activity of the hydrogenotrophic methanogens after H_2
366 addition, which helped decreasing the partial pressure of H_2 in the media, thus avoiding the
367 inhibition of syntrophic microorganisms (Luo and Angelidaki, 2013).

368 ***Figure 4 – Metabolite yields and substrate consumptions for A) glucose-fed AD reactors, B)***
369 ***glucose-fed in-situ biomethanation reactors, C) acetate-fed AD reactors, D) acetate-fed in-situ***
370 ***biomethanation reactors and E) ex-situ biomethanation reactors.***

371 Figures 4C and 4D show the methane yield and acetate consumption in the acetate-fed AD
372 and *in-situ* biomethanation reactors. Acetate consumption and methane yield of the acetate-
373 fed reactors were not affected after the first starvation period. Even though the methane yield
374 of the acetate-fed AD reactors during the first two weeks of operation in stage 2 seemed
375 slightly lower than in stage 1, this difference was not statistically significant (t-test, $p>0.05$).
376 After the second period of starvation, a significant decrease of the acetate consumption was
377 observed. Acetate consumption recovered in the acetate-fed AD reactors during the second
378 week of stage 3, probably due to an enzymatic reactivation of the acetotrophic methanogens.
379 Hao et al. (2012) reported that the inactivation of acetotrophic methanogens was more
380 probable than cell lysis during starvation periods. Inactivated cells can be rapidly reactivated
381 when exposed to favourable environmental conditions (Hao et al., 2012). Meanwhile, the
382 acetate consumption did not recover in the acetate-fed *in situ* biomethanation reactors. This
383 was probably caused by an inhibition of acetotrophic methanogens and syntrophic acetate
384 oxidizing bacteria, due to the high H_2 partial pressure in these conditions (Schink et al., 1997,
385 Mulat et al., 2017, Agneessens et al., 2017).

386 Finally, for the *ex-situ* biomethanation reactors, a slightly increase in the acetate yield was
387 observed mostly during the first week of each operation stage (Figure 4E). Such transitory
388 acetate accumulation was already reported in the literature (Strubing et al., 2018; Rachbauer
389 et al., 2016) and is probably a consequence of a low syntrophic acetate oxidizing activity due
390 to the high H_2 partial pressure and/or a high homoacetogenic activity. Nevertheless, during
391 the first week of stage 3, acetate accumulation in *ex-situ* biomethanation remained at low
392 level (0.06 ± 0.02 gCOD/gCOD$_{H2}$) and acetate was then rapidly consumed.

393 ## 3.2. Microbial community analysis

394 ### 3.2.1. Bacterial community composition

395 From all samples, 2113 distinct OTUs were detected and affiliated to 19 bacterial phyla,
396 from which 7 had an abundance higher that 1% in all reactors and all along the operation
397 (*Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Tenericutes, Spirochaetae* and
398 *Cloacimonetes*). The inoculum was dominated by *Firmicutes* (63%), *Bacteroidetes* (16%),
399 followed by the *Proteobacteria* (7,4%), *Actinobacteria* (6,0%) and *Tenericutes* (2,3%). The
400 dominance of *Firmicutes*, *Bacteroidetes* and *Protobacteria* have been reported in previous
401 studies operating AD, *ex-situ* and *in-situ* biomethanation reactors inoculated with manure-
402 based sludge (Bassani et al., 2015; Calusinska et al., 2018; Treu et al., 2018). In Figure 5,
403 the relative abundance at the bacterial order level is presented. In the inoculum, *Clostridiales*
404 (37%) and a *Clostridia*-like bacteria group MBA03 (13%), both affiliated to the *Firmicutes*
405 phylum and the *Clostridia* class, were the most abundant groups, followed by the order of the
406 *Bacteroidales* (15%). From stage 1, the bacterial community of the reactors evolved
407 differently according to the experimental conditions, in particularly regarding the supplied
408 organic feeding (Supplementary material 2). The reactors fed with glucose (AD and *in-situ*
409 biomethanation) showed a drastic change, as *Bacteroidales* became the most dominant
410 group of the microbial community. The relative abundance of *Bacteroidales* increased up to
411 59% outcompeting the *Clostridiales* (17% in the AD reactors to 13% in the *in-situ*
412 biomethanation reactors at the end of the operation) and the *Clostridia*-like bacteria group
413 MBA03 (2% and 4%, respectively). *Bacteroidales* and *Clostridiales* have similar functions in
414 anaerobic digestion, as they correspond to hydrolytic and fermentative bacteria using
415 carbohydrates and proteins (Hahnke et al., 2016). Besides, they are widespread in anaerobic
416 environments and are able to survive at a wide range of pH (De Vos et al., 2011; Krieg et al.,
417 2010). Nonetheless, *Bacteroidales* could efficiently outcompete other bacteria for organic
418 molecules within the same niche (Ju et al., 2017), explaining therefore their predominance in
419 the glucose-fed reactors. In the *in-situ* biomethanation reactors fed with glucose, one OTU
420 affiliated to *Bacteroidales* have increased its relative abundance stepwise along the
421 operation, from 3% to 44% at the end of the operation, becoming the most dominant OTU in
422 the bacterial community. By performing a search against the NCBI database using the 16S

423 rRNA sequence database, this OTU was affiliated to the species *Proteiniphilum*
424 *saccharofermentas*. Although the sequence identity score (89.81%) was low to confirm the
425 microorganisms belong to the same species or genus (Yarza et al., 2014). Nevertheless,
426 these microorganisms likely belong to the same bacterial family (sequence identity threshold
427 > 86.5%): the *Dysgonomonadaceae* family (Maus et al., 2020; Yarza et al., 2014). Similarly,
428 in the glucose-fed AD reactors, this OTU was also found dominant in their bacterial
429 community (25% relative abundance at the end of the operation). While a second most
430 dominant OTU of *Bacteroidales* (22% relative abundance at the end of the operation) was
431 affiliated to *Fermentimonas caenicola* (sequence identity score of 97.38%.) was also found in
432 these reactors. Both microorganisms have been identified as members of the
433 *Dysgonomonadaceae* family which have been reported to play a role in the acidogenesis
434 stage of the AD being able to encode all enzymes of the methylmalonyl-CoA pathway which
435 allow them to produce propionate from pyruvate, which may explain the increasing
436 propionate yield in these reactors (Hahnke et al., 2016; Maus et al., 2017). Although, a pH
437 decrease was detected in the glucose-fed AD and in-situ biomethanation reactors, from 7.13
438 ± 0.04 to 6.61 ± 0.00 and from 7.09 ± 0.01 to 6.96 ± 0.02, it was within the pH range of
439 growth of these microorganisms (6.3-9.1) (Hahnke et al., 2016).

440 In contrast, in the acetate fed-reactors, the *Clostridiales* members remained dominant,
441 reaching a relative abundance of 39% and 51% at the end of the operation for the *in-situ*
442 biomethanation and AD reactors, respectively. The second more abundant order in these
443 reactors was affiliated to a *Clostridia*-like bacteria group MBA03 (23% and 24%,
444 respectively), representing more than 74% and 63% of members from the *Clostridia* class in
445 these reactors, respectively. Interestingly, the dominant bacterial orders in the *ex-situ*
446 biomethanation were also *Clostridiales* and the *Clostridia*-like bacteria group MBA03,
447 reaching a relative abundance of 42% and 11%, respectively at the end of the operation.
448 This *Clostridia*-like bacteria group MBA03 was previously found in AD-reactors and *in-situ*
449 biomethanation reactors treating farm waste or inoculated with manure-based inocula, as

450 well as in *ex-situ* biomethanation lab-scale reactors (Braga Nan et al., 2020; Calusinska et
451 al., 2018; Logroño et al., 2020).

452 *Clostridiales* have wider metabolic capabilities, being able to perform homoacetogenesis or
453 acetogenesis (Schink, 1997), as well as the oxidation of acetate with a syntrophic partner
454 (Müller et al., 2016). This likely explains their high dominance in the AD and *in-situ*
455 biomethanation reactors fed with acetate and in the *ex-situ* biomethanation reactors. The
456 dominant OTU related to *Clostridiales* in the acetate-fed reactors (AD and *in-situ*) was related
457 to *Alkaliphilus* sp., reaching 24% and 12% of relative abundance at the end of the operation
458 in the AD and *in-situ* biomethanation reactors respectively. *Alkaliphilus* members have been
459 identified in biogas reactors inoculated with pig manure and feed with maize silage (Wirth et
460 al., 2012). They are able to growth in a wide range the pH depending on the species (from
461 5.5 to 12.5) (De Vos et al., 2011; Fisher et al., 2008). Some species have been reported to
462 growth with lactate and acetate as electron donors (Fisher et al., 2008; Ye et al., 2004). The
463 most dominant OTU belonging to *Clostridiales* in the *ex-situ* biomethanation reactors was not
464 possible to be assigned any further than the order phylogenetic level.

465 From stages 2 to 3, a sharp increase of the relative abundance of *Lactobacillales* was
466 observed in the reactors fed with glucose (both AD and *in-situ* biomethanation), although
467 only transient low lactate accumulation was detected during stage 3 (data non shown).
468 Several species affiliated to the order *Clostridiales* have the ability to consume lactate to form
469 mainly acetate, propionate or butyrate and release H_2 (Muñoz-Tamayo et al., 2011; Seeliger
470 et al., 2002; Stolz et al., 2007). In the present work, one of the more abundant OTU affiliated
471 to *Clostridiales* in glucose-fed reactors was identified as *Alkaliphilus ormelandii* (94.99%
472 BLASTn sequence identity), which is able to use lactate as electron donors (Fisher et al.,
473 2008).

474 ***Figure 5 – Relative abundance of the bacterial community at the order level along the***
475 ***operation. One sample of each operation week was analysed. The starvation periods are***
476 ***represented with grey bars. References: Ac-, glc-AD: AD reactors fed with acetate or glucose,***

respectively; Ac-, glc-in-situ: in-situ biomethanation reactors fed with acetate or glucose, respectively; ex-situ: ex-situ biomethanation reactors.

3.2.2. Archaeal community composition

From the 135 OTU belonging to the Archaea domain found in all reactors, 17 have an abundance higher than 1% in all samples. They were clustered into 7 genera within the *Euryarchaeota* phylum (*Methanosarcina* sp., *Methanoculleus* sp., *Methanobacterium* sp., *Methanosaeta* sp., *Methanobrevibacter* sp., *Methanomasiilicoccus* sp., *Methanothermobacter* sp.) and 4 other taxons belonged to the order *Thermoplasmata*, the *Bathyarchaeota* phylum and the class WSA2/Arc1, also known as "*Candidatus Methanofastidiosa*" class, which are thought to perform methylotrophic methanogenesis (Nobu et al., 2016). The relative abundance of the archaeal community at the genus level is shown in Figure 6.

The inoculum was dominated by *Methanosarcina* sp. (34%), followed by *Methanoculleus* sp. (24%), while *Methanobaterium* sp. (11%) and *Methanobrevibacter* sp. (9%) were found in lower proportions. In AD reactors *Methanosarcina* sp. and *Methanobacterium* sp. were outcompeted principally by *Methanoculleus* sp. and *Bathyarchaeota*. In the acetate-fed AD reactors, the relative abundance of *Methanoculleus* sp. increased stepwise along the operation, reaching 41% by the end of the operations, while the relative abundance of *Bathyarchaeota* reached 18% at the end of the operation. By the end of the operation, in the glucose-fed AD reactors, the relative abundance of *Methanoculleus* sp. and *Bathyarchaeota* was 33% and 36%, respectively. Co-occurrence of *Methanoculleus* sp. and *Bathyarchaeota* was previously reported in batch reactors treating cow manure, in which the inferred function of *Bathyarchaeota* was the consumption of lignocellulose (Li et al., 2020). The *Bathyarchaeota* phylum has been described as a widely distributed phylum in anaerobic environments, with a highly diverse metabolism. Although, no microorganisms belonging to this phylum has been cultivated, the "omics" approaches enabled to identify several genes belonging to *Bathyarchaeota* members and inferred their putative functions within the

504 ecosystems (Zhou et al., 2018). These microorganisms seem to be capable of performing
505 carbohydrate-based heterotrophic metabolism and consuming proteins. They may also be
506 involved in homoacetogenesis and in syntrophic interactions with methanogens (Evans et al.,
507 2015; He et al., 2016; Maus et al., 2018). Likely, the taxon affiliated to the *Bathyarchaeota*
508 phylum was involved in heterotrophic consumption of glucose, although their implication of
509 the acetate metabolism cannot be disregarded as they were the second dominant
510 microorganisms in acetate-fed reactors.

511 ***Figure 6** – **Relative abundance of the archaeal community at the genus level along the***
512 ***operation. One sample of each operation week was analysed. The starvation periods are***
513 ***represented with grey bars. References: Ac-, glc-AD: AD reactors fed with acetate or glucose,***
514 ***respectively; Ac-, glc-in-situ: in-situ biomethanation reactors fed with acetate or glucose,***
515 ***respectively; ex-situ: ex-situ biomethanation reactors.***

516 The archaeal community of the *in-situ* and *ex-situ* biomethanation reactors clearly shifted to
517 hydrogenotrophic methanogenesis, as *Methanoculleus* sp. became dominant after H_2
518 injection. In addition, an increase of the relative abundance of *Methanobacterium* sp. was
519 also observed. *Methanobrevibacter* sp. and *Methanothermobacter* sp., which are also
520 hydrogenotrophic methanogens, were outcompeted by *Methanoculleus* sp. and
521 *Methanobacterium* sp. The dominance of *Methanoculleus* sp. and *Methanobacterium* sp.
522 was already reported in *in-situ* and *ex-situ* biomethanation, as well as in AD systems
523 (Bassani et al., 2015; Kern et al., 2016; Li et al., 2020; Rachbauer et al., 2017; Treu et al.,
524 2018a). After 1-week of starvation, *Methanosarcina* sp. was outcompeted by *Methanoculleus*
525 sp. and *Methanobacterium* sp., confirming the shift of the archaeal community towards
526 hydrogenotrophic methanogenesis. A slight increase in the *Bathyarchaeaota* relative
527 abundance was also observed in all reactors. After the 4-weeks starvation period,
528 *Methanoculleus* sp. was still dominant, until the second week of the stage 3 where an
529 increase of the relative abundance of *Methanobacterium* sp. was observed, outcompeting
530 *Methanoculleus* sp. and *Bathyarchaeota* in the *in-situ* biomethanation reactors. By the end of
531 the operation, the relative abundance of *Methanobacterium* sp. was 60% and 87% for the

532 glucose-fed and acetate-fed *in-situ* biomethanation reactors, respectively. Such increase in
533 the relative abundance of *Methanobacterium* sp. have likely led to the recovery of the
534 methane production rate after the long starvation period. The substitution of *Methanoculleus*
535 sp. by *Methanobacterium* sp. was already observed in long-term operation of AD reactors
536 with H_2-addition (Zhu et al., 2020). The *Methanobacteriaceae* family and the
537 *Methanomicrobiaceae* family share the core genes for methane evolution, although their
538 hydrogenases complexes for H_2 uptake are different (Porat et al., 2006). These differences
539 may confer to the *Methanobactericeae* family an advantage for long-term exposure to high
540 H_2 partial pressure (Zhu et al., 2020). Besides, during stage 3 an increase in the VFA
541 accumulation was detected in the *in-situ* biomethanation reactors, which have most likely led
542 to the replacement of *Methanoculleus* sp. by *Methanobacterium* sp., as it was suggested that
543 members of the *Methanobacteriaceae* family were more resistant to high VFA
544 concentrations, while the *Methanoculleus* sp. were mostly found to be dominant at low VFA
545 concentrations (Hori et al., 2006). Indeed, *Methanoculleus* sp. remained dominant in the *ex-*
546 *situ* biomethanation reactors, where such VFA accumulation was not detected. The
547 dominance of *Methanobacterium* sp. in the *in-situ* biomethanation reactors was reflected in a
548 drop in the archaeal community diversity (Supplementary material 3).

549 **3.3. Reactors performances vs. microbial community composition**

550 Overall, the reactors performances as well as the microbial community were clearly impacted
551 by the starvation periods to which they were exposed, and more particularly after the 4-
552 weeks starvation period. Therefore, a principal component analysis (PCA) was performed
553 considering the process performance parameters and microbial community of all the reactors
554 in stage 3. These process indicators were the methane production rate, the methane yield
555 and acetate and propionate concentrations in the reactors, while the more abundant
556 microorganisms of the archaeal and bacterial community were used as microbial indicators.

557 ***Figure 7 – Principal components analysis of the performances and the microbial community of***
558 ***stage 3. [ace] and [prop] stands for acetate and propionate concentration, while CH4_PR***
559 ***stands for methane production rate.***

560 This PCA allows to visualize 68% of the variation. Regarding the bacterial population,
561 *Clostridiales* and *Bacteroidales* were correlated to acetate and propionate concentrations, as
562 the relative abundance of these microorganisms increased in the acetate-fed and glucose-
563 fed reactors, where acetate and propionate accumulated, respectively.

564 Regarding the archaeal populations, *Methanosarcina* sp. did not correlate with methane
565 production, probably due to the change of the microbial community to hydrogenotrophic
566 methanogenesis in the *in-situ* and *ex-situ* biomethanation reactors. While in the AD reactors
567 it was replaced by *Bathyarchaeota*. The later microorganism was correlated to the propionate
568 yield and *Bacteroidales* because its abundance highly increased in the glucose-fed reactors
569 while *Bacteroidales* was also dominant, and propionate accumulated.

570 *Methanobacterium* sp. was the only methanogen correlated to the CH_4 production rate and
571 yield, while *Methanoculleus* sp. was anti-correlated to these parameters, likely due to its
572 substitution by *Methanobacterium* sp.. The substitution of *Methanoculleus* sp. by
573 *Methanobacterium* sp. was already reported during long time operation of AD reactors (Ács
574 et al., 2019; Zhu et al., 2020). Such phenomenon could be attributed to the high functional
575 redundancy among the methanogens community, where one microorganism could be
576 replaced by another better adapted to the environmental conditions but conserving the same
577 function to the system (Treu et al., 2018a).

578 **4. CONCLUSION**

579 Overall, a one-week starvation period did not affect the methane productivity and yield for
580 any of the conditions tested. Meanwhile, a 4-weeks starvation period affected all reactors
581 regarding their metabolite production, substrate consumption, methane yield and methane
582 production rate. The AD reactors did not recover their MPR or yield due to the accumulation

583 of VFA in their bulk phase. Interestingly, both the *in-situ* and *ex-situ* biomethanation reactors
584 recovered their MPR after approximately 1-week of operation due to the replacement of
585 *Methanoculleus* sp. by *Methanobacterium* sp., recovering the function of the system. This
586 work showed that *ex-situ* and *in-situ* biomethanation processes are able to recover from
587 short and long starvation periods without compromising their methane producing
588 performance and therefore, they are suitable to receive H_2 in an intermittent mode, being
589 able to be coupled to the power-to-gas concept.

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