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Title:

Performance and stability of sewage sludge digestion under CO₂ enrichment: a pilot study.

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1 Abstract

2 Carbon dioxide (CO₂) injection in anaerobic digestion has recently been proposed as an
3 interesting possibility to boost methane (CH₄) recovery from sludge and organic waste
4 by converting a greenhouse gas into a renewable resource. This research assessed the
5 effects of exogenous CO₂ injection on performance and process stability of single-phase
6 continuous anaerobic digesters. Two pilot scale reactors treating sewage sludge were
7 operated for 130 days. One reactor was periodically injected with CO₂ while the other
8 acted as control. Two injection frequencies and injection devices were tested. The
9 results indicated that CO₂ enrichment allowed an increase in CH₄ production of *ca.*
10 12%, with a CH₄ production rate of $371 \pm 100 \text{ L}/(\text{kg VS}_{\text{fed}} \cdot \text{d})$ and a CH₄ concentration of
11 *ca.* 60% when dissolved CO₂ levels inside the test reactor were increased up to 1.9-fold.
12 Results also indicated an improvement in process resilience to temporary overloads and
13 no impacts on stability parameters.

14
15 **Keywords:** anaerobic digestion; carbon dioxide utilisation; sewage sludge; pilot scale;
16 process stability.

18 1. Introduction

19 Anaerobic digestion (AD) has recently been proposed as a promising system to
20 biochemically convert exogenous carbon dioxide (CO₂) into methane (CH₄) (Bajón
21 Fernández *et al.*, 2014; Salomoni *et al.*, 2011) and this option is finding growing interest
22 thanks to the possibility of developing carbon negative renewable energy production
23 (Cheah *et al.*, 2016; Budzianowski, 2012). CO₂ reduction to CH₄ in the AD process is
24 traditionally associated with the activity of hydrogenotrophic methanogens (Demirel

25 and Scherer, 2008). Homoacetogens can also play a role in reducing CO₂ and H₂ into
26 acetic acid that is then transformed into CH₄ by acetoclastic methanogens (Liu *et al.*,
27 2016) or through syntrophic acetate oxidation followed by hydrogenotrophic
28 methanogenesis (Schnürer and Nordberg, 2008). Whilst the biochemical mechanisms
29 for exogenous CO₂ bioconversion in AD have not been fully elucidated, various authors
30 have assessed the possibility to enhance CH₄ production from AD by CO₂ enrichment.
31 Alimahmoodi and Mulligan (2008) studied, at lab scale, the possibility of converting
32 CO₂ into CH₄ by using an up-flow anaerobic sludge blanket (UASB) reactor fed with a
33 solution composed of dissolved CO₂ and volatile fatty acids (VFAs). The same authors
34 observed a 69–86% CO₂ uptake, reporting that VFAs were used as source of H₂ for
35 hydrogenotrophic methanogens to perform the CO₂ conversion to CH₄. Salomoni *et al.*
36 (2011) studied at pilot scale the injection of CO₂ into the fermentation phase of a two-
37 phase anaerobic digestion (TPAD) plant. Off gases from the fermentation phase were
38 recirculated into the methanogenic phase to sustain CO₂ reduction to CH₄ and a 25%
39 increase in CH₄ yield was observed. Similarly, Yan *et al.* (2016) studied the
40 recirculation of off-gases from a TPAD reactor for food waste digestion. These authors
41 utilised an acidogenic leach bed reactor, as first phase, and diverted off-gases (rich in
42 CO₂ and H₂) and leachate from this reactor into a methanogenic UASB, used as second
43 digestion phase. Results indicated an improvement of CH₄ production thanks to CO₂
44 and H₂ conversion to CH₄ that was assumed to be carried out by hydrogenotrophic
45 methanogens.

46 These results highlight the biological feasibility of CO₂ bioconversion into CH₄ even
47 though most of the studies utilised exogenous H₂ to support this bioprocess. The current
48 lack of an inexpensive H₂ supply system and the low water solubility of H₂ are

49 challenges that hinder the full exploitation of CO₂ bioconversion into CH₄ at AD sites
50 by the use of exogenous H₂ (Bassani *et al.*, 2016). Similarly, the use of TPAD
51 configuration could limit a large implementation of CO₂ bioconversion, considering that
52 the majority of AD assets are single phase plants (De Baere and Mattheeuws, 2010).
53 To overcome these limitations, an alternative approach could be based on the injection
54 of CO₂ directly into digesters without any additional fermentation phase and without
55 addition of exogenous H₂. Recent studies have assessed this procedure and indicated
56 encouraging results. Bajón Fernández *et al.* (2014) studied the possibility to improve
57 AD performance by direct CO₂ injection in single phase digestion, without the
58 availability of exogenous H₂. Results from batch tests indicated an increase of CH₄
59 yields between 5 to 13% for food waste digestion and a speed up of CH₄ production for
60 sewage sludge leading to an increase of *ca.* 100% on CH₄ production within the first 24
61 h of digestion, if compared to control experiments. A positive influence of exogenous
62 CO₂ on AD performance during biochemical methane potential (BMP) tests was also
63 reported by Koch *et al.* (2015; 2016), that observed an increase of CH₄ yields
64 proportional to the CO₂ concentration of gases used to flush reactors head space. The
65 benefit of direct injection of CO₂ on AD was also observed at pilot scale for food waste
66 digestion (Bajón Fernández *et al.*, 2015). Results from this investigation indicated a 2.5-
67 fold increase in H₂ concentration in the digester enriched with CO₂, that could support
68 the conversion of exogenous CO₂ into CH₄, and resulted in a *ca.* 20% higher CH₄
69 production when comparing performance of test reactor before and after CO₂ injection.
70 These results therefore support that biochemical conversion of exogenous CO₂ to CH₄
71 can be obtained in AD also without external supplementation of H₂. This option opens
72 the possibility to exploit such biological process in various industrial sectors where AD

73 is already an implemented technology. This could be further facilitated by the growing
74 application of biogas upgrading to biomethane (Sun *et al.*, 2015) that is leading to the
75 large availability of CO₂, directly on the digestion sites, that can be converted into CH₄,
76 as promising approach to convert a waste stream into a commodity (Koch *et al.*, 2016).
77 Enhancement of CH₄ production from sewage sludge AD supplemented with exogenous
78 CO₂ has only been proved at batch scale (Bajón Fernández *et al.*, 2014) and further
79 confirmations at larger scale are needed to proof the concept and clarify the long-term
80 impacts of CO₂ injection on AD performance and stability. This research was therefore
81 aimed at assessing, at pilot scale, the effects of exogenous CO₂ injection on single phase
82 continuous AD of sewage sludge, without exogenous H₂ addition. The research focused
83 on understanding the impacts of moderate and intense exogenous CO₂ injections on
84 CH₄ production, biogas quality and AD process stability parameters.

85

86 **2. Material and methods**

87 **2.1. Reactors configuration and operation**

88 Two identical pilot scale AD reactors were used for the research study. The reactor used
89 for CO₂ enrichment is hereafter referred to as Test reactor while the other is referred to
90 as Control reactor. A scheme of the experimental rig is presented in Figure 1. Each unit
91 was composed of a cylindrical reactor with a cone base having a total volume of 165 L.
92 Working liquid volume was set to 90 L. Mixing of digestion material was performed by
93 an external peristaltic pump (series 600, Watson Marlow, Cornwall, UK). Pump rate
94 was set to have a full recirculation of the working liquid volume in 30 minutes. The AD
95 process was carried out at mesophilic conditions. Temperature of digestion liquid was

96 maintained at 38.5 ± 1 °C by using heating jackets (LMK Thermosafe, Haverhill, UK)
97 placed over the cylindrical section of each reactor.

98 The reactors were operated semi-continuously with feeds carried out once a day. The
99 feeding regime was repeated weekly as follows: 6 L of sewage sludge from the 1st to the
100 4th day of the week, 12 L of sewage sludge on the 5th day and no feed on the 6th and 7th
101 day of the week. Micronutrients were added during any feed at a dosing rate of 0.05 mL
102 of TEA 310 solution (Omex Environmental Ltd., King's Lynn, UK) per kg of volatile
103 solids (VS) fed. The pH of feeding sewage sludge was not adjusted. The weekly
104 average Hydraulic Retention Time (HRT) was 17.5 d and the average Organic Loading
105 Rate (OLR) was 2.1 ± 0.4 kgVS/m³·d. The two reactors were fed in parallel at the same
106 time of the day and were maintained at the same feeding conditions for the entire
107 experimental period.

108 The Test reactor was equipped with an external column retrofitted as a side process to
109 perform the CO₂ enrichment of the digestion liquid. The column was connected to the
110 Test reactor in the mixing loop only during each CO₂ enrichment (Figure 1). Test and
111 Control reactors operated similarly during the rest of the time. No CO₂ injections were
112 carried out on Test reactor until day 42.

113 Biogas production, biogas composition, pH and temperature of the digestion liquid were
114 monitored five times per week. Samples of digestate from both reactors were collected
115 up to 5 times a week to measure: Total Solid (TS), VS, Ammonium Nitrogen (NH₄⁺),
116 Partial Alkalinity (PA), Intermediate Alkalinity (IA), Total Alkalinity (TA), H₂CO₃
117 Alkalinity and total Volatile Fatty Acids (VFAs) concentration. The following single
118 VFAs were also monitored: acetic acid, propionic acid, butyric acid and valeric acid.
119

120 **2.2. Feeding material and inoculum of reactors**

121 Sewage sludge was used as feedstock for the reactors. The sewage sludge used in this
122 study was a mixture of primary sludge and waste activated sludge produced in a
123 municipal wastewater treatment works (WwTW) located in the Midlands area of UK.
124 Sludge was collected from the inlet flow of a full-scale AD plant located in this
125 WwTW. After collection, samples were stored at 4 °C until use. Four batch samples of
126 sludge were collected at different times during the experiment and are named Sample 1,
127 Sample 2, Sample 3 and Sample 4. During the entire experiment, both reactors were fed
128 with the same sludge sample. Phases of the experiment during which the four samples
129 of sludge were used are reported in Figures 2, 3, 5 and 6.

130 The composition of each sample of sludge was monitored for the following parameters:
131 TS, VS, NH_4^+ , TA, H_2CO_3 alkalinity, total and single (acetic acid, propionic acid,
132 butyric acid and valeric acid) VFAs concentration. Average characteristics of each
133 sample are reported in Table 1.

134 Reactors were inoculated with digestate collected from a full-scale mesophilic
135 anaerobic digester located in the same WwTW. TS and VS concentrations of the
136 inoculum were 30 ± 2 gTS/L and 18 ± 1 gVS/L, respectively.

137

138 **2.3. Carbon dioxide injection procedure**

139 CO_2 enrichment of digestion liquid was performed by using a 1 m tall and 10 cm
140 diameter column located in the recirculation loop of the Test AD reactor (Figure 1). The
141 column was operated with a liquid working volume of 7 L. CO_2 was injected at the
142 bottom of the column through a perforated plate. A metallic mesh with 0.5 mm hole size
143 was placed on top of the perforated plate to generate small gas bubbles enhancing CO_2

144 dissolution into the digestion liquid. The contact between digestion liquid and CO₂ was
145 performed in co-current mode.

146 In order to assess the impact of dissolved CO₂ levels in AD operation, two different
147 column configurations were used. The first was a bubble column configuration with
148 internal space of the column empty. The second was a packed column configuration in
149 which the internal space was filled with small perforated plastic media of cylindrical
150 shape and various dimensions (length = 5 cm, diameters = 1, 2 and 4 cm) having
151 rectangular openings of *ca.* 2 x 10 mm evenly distributed on the surface.

152 The moderate CO₂ enrichment was carried out between day 42 and day 76, with three
153 CO₂ injections per week using the bubble column configuration. The intense CO₂
154 enrichment was performed between day 91 and day 127 with five CO₂ injections per
155 week using the packed column configuration. Between these two phases, Test reactor
156 was operated without CO₂ injection for 14 days.

157 During both phases, the CO₂ injection was carried out for 1 hour at a time maintaining a
158 fixed CO₂ flow rate into the column of 1.5 L/min by means of a mass flow controller
159 (MFC) (Premier Control Technologies, Norfolk, UK). CO₂ was supplied from gas
160 cylinders (BOC, Manchester, UK). The mixing pump speed was reduced during
161 injection in order to increase the gas to liquid contact time in the column and to
162 circulate the entire digestion liquid through the column during the 1-hour operation. The
163 same speed reduction was applied to the mixing pump of the Control reactor for the
164 length of the CO₂ injection procedure. CO₂ enrichment was performed at the same time
165 of the day and always before feeding both the reactors. The experimental set up used
166 was similar to the one reported by Bajón Fernández *et al.* (2015).

167 Dissolved CO₂ concentration and pH were measured in the digestion liquid of the Test
168 reactor at the beginning and at the end of any CO₂ enrichment, while dissolved CO₂
169 concentration and pH of the liquid entering and exiting the CO₂ injection column were
170 measured every 10 minutes. Concentrations of CO₂ and CH₄ in the column gas exhaust
171 (Figure 1) were measured every 5 minutes. At the end of any CO₂ enrichment, biogas
172 composition in the Test reactor head space was also measured.

173

174 **2.4. Analytical methods and statistical analysis**

175 Biogas production was measured by drum-type gas meters (Ritter TG 05/5, Germany).
176 Biogas composition was measured by means of a portable gas analyser (LMSXi
177 multifunction gas analyser, Gas Data, Coventry, England) and data on biogas mixing
178 ratio are reported as concentrations expressed in %. Dissolved CO₂ concentrations were
179 measured by means of CO₂ sensors (InPro®5000(i), Mettler-Toledo AG, Switzerland)
180 connected to a multiparameter transmitter (M400, Mettler-Toledo AG, Switzerland).
181 Concentrations of CO₂ and CH₄ in the column gas exhaust (Figure 1) were measured by
182 means of gas sensors (BCP sensors, Bluesens, Herten, Germany) and recorded in a
183 computer using BacVis software (Bluesens, Herten, Germany).
184 TS and VS were measured on raw samples according to Standard Methods (APHA,
185 2005). NH₄⁺, IA, PA, TA, H₂CO₃ alkalinity and total and single VFAs, were measured
186 on the supernatant of samples centrifuged for 20 minutes at 8000 g and 20 °C. NH₄⁺
187 was quantified by using Spectroquant test kits (Merck, Germany). Alkalinities and total
188 VFAs were measured by titration with 0.06 N HCl acid on supernatants diluted 1:10 in
189 deionised water. IA and PA were measured by titration to pH values of 5.75 and 4.30,
190 respectively, and IA/PA ratio was calculated as ratio between titration volumes (Ripley

191 *at al.*, 1986). TA, H₂CO₃ alkalinity and total VFAs were measured by titration at 8 pH
192 points as reported by Lahav *et al.* (2002). The ratio between total VFAs and H₂CO₃
193 alkalinity measured by this titration procedure is referred as VFA/Alk ratio in the
194 present study.

195 To measure single VFAs, supernatants were filtered through 0.45 µm pore size syringe-
196 drive filters (Millipore™, Billerica, United States). High performance liquid
197 chromatography (HPLC) (Shimadzu VP Series unit, Milton Keynes, UK) was utilised
198 to quantify concentration of acetic acid, propionic acid, butyric acid and valeric acid.
199 The methodology is reported in Soares *et al.* (2010) with the only exception that a
200 HPLC run time of 60 minutes was used in this research.

201 Results from both Control and Test reactors were statistically evaluated by means of
202 sign test. Sign test is a non-parametric test with dependent samples ordered in pairs. A
203 confidence level of 95% was selected for all statistical comparisons.

204

205 **3. Results and Discussion**

206 **3.1. Sewage sludge digestion performance and effects of CO₂ injection**

207 A comparison of Control and Test reactors performance during the different phases of
208 the experimental work is presented in Table 2. Control and Test reactors are compared
209 for results before the CO₂ injection started and during the two phases of CO₂ injection
210 performed at different frequencies and column configurations. Trends of CH₄ and H₂
211 concentrations for the entire experimental period are reported in Figure 2. Trends of
212 NH₄⁺ concentration in digestate and H₂CO₃ alkalinity are presented in Figure 3a while
213 pH trends are presented in Figure 3b. Figure 4 presents the average change in pH on
214 digestate exiting the injection column during CO₂ enrichment and the average increase

215 in dissolved CO₂ concentration compared to the starting point (C/C_0). The final C/C_0
216 achieved in the Test AD after completing the CO₂ injection is also reported.

217 The Control reactor showed unstable performance during the first two weeks (data not
218 shown), therefore it was reseeded and feeding started again, at the same feed rate of
219 Test reactor, on day 19. From day 19 onwards, both reactors showed stable operational
220 conditions with similar process performance ($p>0.05$). During the period without CO₂
221 enrichment (first 42 days) average CH₄ concentration was $65 \pm 3\%$ for both reactors
222 (Table 2 and Figure 2) and specific CH₄ production was 373 ± 169 and 384 ± 175
223 L/(kgVS_{fed}·d) for Control and Test reactors, respectively (Table 2), H₂ concentrations
224 followed similar patterns with a slight increase in concentration after day 30 for both
225 reactors (Figure 2).

226 The decreasing trend of H₂CO₃ alkalinity (Figure 3a) was probably due to a change in
227 organic nitrogen content of feed sludge as also indicated by the decreasing trend of
228 NH₄⁺ concentration in the reactors. Degradation of organic nitrogen to NH₄⁺ is in fact
229 the main way in which alkalinity is generated during biodegradation of organic matter
230 (Rittmann and McCarty, 2001). IA/PA and VFA/Alk ratio remained below 0.4 and 0.2,
231 respectively (Figure 5). Acetic and propionic acids showed similar trends for both Test
232 and Control reactors with no peaks in concentration (Figure 6a) during the initial phase
233 of the research without CO₂ enrichment, indicating a stable operational condition.

234 Overall, the differences between monitoring parameters (Table 2) did not result
235 statistically different ($p>0.05$).

236 The first phase of CO₂ injection started on Test reactor on day 42, with 3 injections per
237 week by means of a bubble column.

238 The dissolution of a weak acid during CO₂ enrichment produced a temporary reduction
239 in pH and this effect can be observed on the decreasing trend of pH in the effluent from
240 the injection column (Figure 4a and 4b). On average, the use of a bubble column
241 (Figure 4a) produced a pH reduction of about 0.10 points while injections with a packed
242 column (Figure 4b) reduced the pH by 0.15 points. The use of a packed column in fact
243 allowed a higher CO₂ dissolution, as confirmed by the higher C/C₀ ratio reached during
244 the second phase of CO₂ injection (Figure 4b).

245 Both reactors showed a decreasing trend of pH (Figure 3b) that can be associated to the
246 reduction in organic nitrogen content on feed sludge as confirmed by the lowering
247 pattern of NH₄⁺ concentrations (Figure 3a), as already discussed. The Test reactor did
248 not show any additional decreasing trend of pH during CO₂ enrichment, indicating that
249 the system was able to recover after the temporary pH reduction in digestion liquid
250 exiting the column. CO₂ injection did not impact therefore H₂CO₃ alkalinity of the Test
251 reactor (Figure 3a). These results confirm observations reported by Bajón Fernández *et al.*
252 *al.* (2014) where CO₂ enrichment of batch tests treating sewage sludge and food waste
253 indicated that the initial acidification associated with CO₂ injection was overcome
254 within one day. Bajón Fernández *et al.* (2015) during pilot scale digestion of food waste
255 did not observe a reduction on digestion pH with a CO₂ enrichment frequency of 3
256 injections per week, similarly to the moderate frequency on the present study. Al-
257 mashhadani *et al.* (2016) also indicated a short-term effect of pH reduction during CO₂
258 injection, followed by a recovery phase when injection was not performed, in a gaslift
259 digester sparged with pure CO₂ for 5 minutes a day. An overall increasing pH trend was
260 also observed for this reactor, but a comparison with a control unit was not reported.

261 These results therefore suggest that the CO₂ enrichment procedure has no long term
262 impacts on pH under continuous operating conditions.

263 During the first phase of CO₂ injection, a variable H₂ concentration for Test reactor was
264 observed, with peaks up to 220 ppm (Figure 2 and Table 2). On the contrary H₂
265 concentration for Control reactor remained stable at values close to 110 ppm from day
266 42 onwards. In the first phase of CO₂ injection, CH₄ concentration in Test reactor
267 resulted rather variable (Figure 2). Average concentration for Test reactor was $59 \pm 3\%$
268 while for Control reactor was $62 \pm 2\%$ ($p < 0.05$) (Table 2). During the second phase of
269 CO₂ injection, started on day 92 with 5 injections per week and a packed column
270 configuration, H₂ concentration of Test reactor showed a higher average concentration
271 ($p < 0.05$) than the Control, 138 ± 26 ppm and 107 ± 10 ppm, respectively, and average
272 CH₄ concentration was slightly lower ($p < 0.05$), with an average of $61 \pm 2\%$ and $63 \pm$
273 2% in Test and Control reactors, respectively (Table 2).

274 An increasing concentration of H₂ in biogas together with growing concentrations of
275 organic acids in digestate is typically reported as an indicator of overloading or
276 inhibitory conditions for anaerobic bioreactors (Voolapalli and Stuckey, 2001;
277 Ketheesan and Stuckey, 2015). Accumulation of intermediates indicates in fact an
278 unbalanced condition between the activity of acetogens and methanogens due to a fast
279 change of process conditions. The peaks in H₂ concentration observed after the start of
280 CO₂ injection, could be associated to a release of protons when carbonic acid
281 dissociates into carbonate and bicarbonate (Bajón Fernández *et al.*, 2015) but could also
282 suggest that this procedure introduced a disturbance in the biological process affecting
283 the activity of H₂ consuming microorganisms or be related to a boost of H₂ producing
284 metabolisms. Increase of H₂ concentration due to a reduction of hydrogenotrophic

285 activity is usually simultaneous to increases of propionate or butyrate acids due to
286 syntrophic degradation of these intermediates (Voolapalli and Stuckey, 2001). As no
287 reduction of biogas or CH₄ production (Table 2) or indications of process instability
288 were recorded, it is likely that the increase of H₂ production and of these acids was
289 associated to an increased acidogenic activity stimulated by the CO₂ injection rather
290 than an inhibition of hydrogenotrophic activity. No clear trends of VFA concentration
291 were anyway observed, suggesting that further work is needed to elucidate the
292 mechanisms of utilization of the injected CO₂. The CO₂ injection, both at moderate and
293 intense frequency, did not lead to increasing levels of H₂, but to a new H₂ baseline
294 which, for Test reactor, stabilised at *ca.* 138 ppm (Table 2). The fact that the H₂
295 concentration reached a new baseline rather than maintaining an increasing trend,
296 suggests that hydrogenotrophic activity was stimulated because of a higher substrate
297 availability. Bajón Fernández *et al.* (2015) also measured an increasing trend of H₂
298 concentration with a new baseline being reached at 320 ± 153 ppm in biogas during
299 CO₂ enrichment of a pilot scale food waste AD. In that study, the higher H₂ production
300 was attributed to either a chemical process of proton formation due to CO₂ dissolution
301 into carbonate/bicarbonate, or to a biologically enhanced acetogenesis. The increased
302 H₂ consumption (new H₂ baseline rather than a rising trend) was in this case attributed
303 to a potential increase in homoacetogenesis via the Wood-Ljungdahl pathway (Bajón
304 Fernández *et al.*, 2015). Al-mashhadani *et al.* (2016) suggested that the addition of CO₂
305 in an anaerobic gaslift bioreactors of kitchen waste, deploying microbubbles generated
306 by fluidic oscillation, could boost H₂ production (and consequently CH₄ production)
307 due to an improved hydrolysis of organics given by the collapse of microbubbles
308 generating radicals able to facilitate the disruption of slowly biodegradable organics.

309 This hypothesis could explain both the higher H₂ concentration observed during the
310 experimental period and the increased CH₄ production (Table 2). The injection of CO₂
311 could therefore increase H₂ levels as a result of improved hydrolysis but this assumption
312 needs further confirmation as the equipment utilised in this research study was not
313 designed to generate microbubbles.

314 CH₄ production resulted differently affected during the two injection phases (moderate
315 and intense) (Table 2). In the first phase, characterised by 3 injections per week with a
316 bubble column, average specific CH₄ productions resulted similar. During the intense
317 phase of CO₂ injection, 5 injections per week with a packed column, average specific
318 CH₄ production in the Test reactor ($371 \pm 100 \text{ L}/(\text{kgVS}_{\text{fed}} \cdot \text{d})$) was *ca.* 12% higher than
319 for the Control Reactor ($332 \pm 94 \text{ L}/(\text{kgVS}_{\text{fed}} \cdot \text{d})$) and in this case productions over time
320 were statistically different (paired sign test, $p < 0.05$). The increase in CH₄ production
321 could be explained by an increased hydrogenotrophic methanogenesis, by an increased
322 acetoclastic methanogenesis or by an increased methylotrophic methanogenesis. An
323 increased hydrogenotrophic methanogenesis could be a result of a stimulation of H₂
324 production pathways as a response to the increased inorganic carbon availability, as
325 previously described, while a boost in acetate availability because of utilisation of CO₂
326 in the Wood-Ljungdahl mechanism can explain an increase in activity of acetoclastic
327 methanogens leading to higher CH₄ productions (Bajón Fernández *et al.*, 2015). The
328 reduction of exogenous CO₂ and H₂ to methanol is also another possible route for
329 higher CH₄ production that is linked to conversion of methanol to CH₄ by
330 methylotrophic methanogens (Guo *et al.*, 2015).

331 A higher CH₄ production was also observed by Salomoni *et al.* (2011) during CO₂
332 injection on TPAD of sewage sludge at pilot scale. These authors achieved a 25%

333 increase in CH₄ production, if compared to a full-scale single phase digestion plant, by
334 injecting CO₂ into the acidogenic stage of the TPAD process. In the present study, the
335 improvement of CH₄ production associated with CO₂ enrichment was *ca.* 12%. Even
336 though the two systems have similar HRTs (~17 d), differences as OLR (1.05 ± 0.04 vs.
337 2.1 ± 0.4 kgVS/m³·d in the present study), plant configuration (double phase vs. single
338 phase in the present study), injection procedure (continuous vs. intermittently in the
339 present study), and the specific conditions of the digestion liquid during injection
340 (acidic vs. neutral-alkaline in the present study) limit the comparability of results.
341 Enhancement of CH₄ production was also reported by Al-mashhadani *et al.* (2016)
342 during pure or diluted biogas recirculation, or CO₂ injection in anaerobic gaslift
343 bioreactors of kitchen waste, using microbubbles generated by fluidic oscillation. These
344 authors described that the injection of recirculated biogas (with CO₂ concentration of 40
345 or 80%) increased CH₄ production between 10 and 14% while the injection by
346 microbubbles of pure CO₂ increased CH₄ production by more than 100%. It is
347 suggested that this procedure stimulates CH₄ production due to two processes. The first
348 is a faster removal of CH₄ from the liquid phase that reduces its partial pressure and
349 thermodynamically enhances reactions having CH₄ as final product. The second process
350 links the higher CH₄ production to an increased hydrolysis. In the present study, no net
351 difference was recorded on VS concentrations between the two reactors ($p > 0.05$) (Table
352 2) therefore it is not possible to confirm an improved solids degradation even though
353 CH₄ production was higher with injection of CO₂, if compared to Control reactor.
354 Further studies are therefore necessary to gain a better understanding of this aspect.

355

356 **3.2 Anaerobic digestion process stability under CO₂ injection**

357 Variations over time of process stability parameters (IA/PA and VFA/Alk ratios) are
358 reported in Figure 5. Concentrations of acetic and propionic acids are reported in Figure
359 6a, concentrations of butyric and valeric acids are reported in Figure 6b.

360 During the first 42 days in which both reactors were maintained at the same loading
361 conditions and CO₂ injection was not performed on Test reactor, IA/PA and VFA/Alk
362 parameters remained within ranges indicating good stability of the biological process,
363 (IA/PA < 0.4 and VFA/Alk < 0.2, Li *et al.*, 2014; Vannecke *et al.*, 2014) and VFAs
364 concentration showed comparable trends between the two reactors (Figures 5 and 6).

365 From day 42, both reactors showed some peaks of both IA/PA and VFA/Alk ratios.
366 Control reactor showed peaks of these parameters on day 50, 65 and 108. On day 50,
367 IA/PA and VFA/Alk ratios for Control reactor reached values of 0.55 and 0.6,
368 respectively, while during the other two events IA/PA ratio resulted close to or higher
369 than 0.5 and VFA/Alk ratio higher than 0.3. Test reactor also showed peaks of these
370 parameters on the same days, but the increase resulted less intense (Figure 5). During
371 the moderate phase of CO₂ injection characterised by 3 injections per week, IA/PA ratio
372 of the Test reactor reached peaks of about 0.45 on days 50 and 65, while VFA/Alk ratio
373 increased to values of about 0.25 on the same days. During the intense phase of the
374 injection procedure, characterised by 5 injections per week, results from the Test reactor
375 indicated that IA/PA never exceeded 0.4 and VFA/Alk remained stable around 0.1
376 (Figure 4).

377 Observing the trends of concentration of VFAs (Figure 6a and 6b), an increase in acetic,
378 propionic and butyric acids was recorded during the days in which peaks in stability
379 parameters (IA/PA, VFA/Alk) were measured. Similarly, a reduction of H₂CO₃
380 alkalinity was also observed during these events (Figure 3a).

381 As these variations in process parameters were observed for both reactors, it is
382 presumable that they were a response to a temporary unbalanced process condition
383 caused by a change of feeding load or composition. Even though reactors were fed with
384 the same volume of sewage sludge (see paragraph 2.1), variations in solids
385 concentrations and sludge composition over time could have imposed changes on
386 loading rates on reactors. Both reactors recovered quickly from these temporary
387 unbalanced conditions without requiring any reduction in feeding regime. However, it is
388 of note that the Test reactor showed lower peaks of stability parameters than the Control
389 reactor during all these events, while it was subjected to CO₂ enrichment. In fact, IA/PA
390 and VFA/Alk ratios for the Test reactor never exceed 0.45 and 0.25, respectively, in all
391 these occasions, while the Control reactor reached values of IA/PA and VFA/Alk ratios
392 up to 0.55 and 0.6, respectively. Similarly, acetic acid concentrations in the Test reactor
393 resulted always lower than those for Control reactor (Figure 6a).

394 This increased resilience of the Test reactor is particularly evident during the third event
395 around day 105. IA/PA and VFA/Alk ratios remained at high values for about 10 days
396 for the Control reactor, while only small variations were recorded for the same
397 parameters for the Test reactor (Figure 5). Acetic acid concentrations also remained
398 above 500 mg/L for about ten days in the Control reactor, while a moderate peak, below
399 500 mg/L, and a fast recovery, less than 5 days, was observed for the Test reactor
400 (Figure 6a).

401 These observations suggest that the injection of CO₂ on Test reactor induced a higher
402 resilience to temporary overloads caused by sudden variations of feed composition at
403 constant volumetric loads. Improved resilience as an effect of CO₂ injection was also
404 observed by Bajón Fernández *et al.* (2015) during anaerobic digestion of food waste at

405 pilot scale. The AD reactor enriched with CO₂ faced a sudden temperature drop of 12.5
406 °C that caused a decrease of both biogas production and pH. No VFA accumulation was
407 observed and the reactor recovered from the stress condition much faster than the
408 Control reactor, subject to a similar temperature drop, which required a partial re-seed
409 to recover. No other studies have investigated the effect of CO₂ injection on AD process
410 resilience, but the similar results obtained in this research study and by Bajón Fernández
411 *et al.* (2015) observed from different stress conditions, suggest that the CO₂ enrichment
412 procedure not only can be applied to boost CH₄ production but also can enhance process
413 stability and resilience.

414 The higher resilience observed for the Test reactor could be associated with a higher
415 heterogeneity or functional redundancy of microbial populations within the process
416 stimulated by CO₂ enrichment. A more diversified microbial community expressing a
417 high degree of redundancy for trophic pathways, is suggested to maintain a high rate of
418 degradation activity and process stability even under variability of feed composition or
419 organic load (Briones and Raskin, 2003). This could explain why stability parameters
420 showed lower peaks and faster recovery for the Test reactor in this study. Strategies to
421 control or recover digesters from hydraulic or loading shock currently focus on
422 stimulating either methanogenic activity or propionate and butyrate consumption by
423 microbial bioaugmentation in an attempt to maximise intermediate consumptions and
424 speed up process recovery (Ketheesan and Stuckey, 2015). Lerm *et al.* (2012) indicated
425 that the coexistence of hydrogenotrophic and acetoclastic methanogens is necessary to
426 respond to process perturbations and leads to stable process performance during shock
427 load conditions. Shifts from acetoclastic to hydrogenotrophic methanogens were in fact

428 reported during organic overloads as a response to high H₂ availability (Lerm *et al.*,
429 2012).

430 From an overall point of view, CO₂ injection did not produce negative impacts on
431 biological stability of the Test reactor. Excluding the three events during which an
432 overload of both reactors was observed, IA/PA and VFA/Alk remained within values
433 normally reported for stable performance (Ketheesan and Stuckey, 2015; Ripley *et al.*,
434 1986). No accumulation of VFAs was observed during both moderate and intense CO₂
435 enrichment phases (Figure 6). On the contrary, average acetic acid concentration in the
436 Test reactor (200 ± 120 mg/L) resulted lower than in the Control reactor (320 ± 180
437 mg/L) and butyric acid concentration in the Test reactor remained below concentrations
438 measured in the Control reactor, in particular during the second (intense) phase of CO₂
439 injection (Figure 6b). These observations further support the hypothesis that a higher
440 CH₄ production could be a result of an increased acidogenic activity. These results also
441 suggest that the implementation of CO₂ enrichment in full scale AD operations can
442 improve process resilience and potentially accommodate extra-loading capacity.

443 Moreover, CO₂ enrichment could potentially represent a controlling strategy for
444 digestion plants in which feed composition variability can easily create overloading
445 conditions and inhibit the biological process. Further studies are required to understand
446 whether CO₂ enrichment can enable an increased process capacity by supporting stable
447 operation at higher OLR and lower HRT in single-phase continuous digestion
448 processes.

449

450 4. Conclusions

451 This study confirmed at pilot scale the possibility to enhance AD of sewage sludge by
452 CO₂ enrichment without exogenous H₂ addition. The injection of exogenous CO₂ into
453 AD represents a promising option to improve CH₄ production in a single-phase digester.
454 Specific CH₄ production was increased by *ca.* 12% and no impacts were observed on
455 the AD stability parameters that remained within typical ranges. CO₂ enrichment also
456 allowed an increased process resilience to temporary overloads. CO₂ enrichment of
457 sludge ADs has potential to enable a carbon-negative sewage sludge management with
458 limited changes in process operation and control.

459

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463

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547 recovery from two-phase anaerobic digestion of food waste through reutilization of
548 acidogenic off-gas in methanogenic reactor. *Bioresource Technol.* 217, 3–9.

549 **Table captions**

550

551 Table 1. Average physical and chemical composition of the samples of sewage sludge
552 used as feedstock. Temporal reference on when samples were used during the
553 experiments are reported in Figure 2, 3, 5 and 6.

554

555 Table 2. Average data (\pm Standard Deviation) obtained from the Control and Test
556 reactors during the different phases of the experimental period. Star (*) indicates
557 statistically different data ($p < 0.05$) between the same experimental condition.

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570 **Table 1.** Average physical and chemical composition of the samples of sewage sludge
 571 used as feedstock. Temporal reference on when samples were used during the
 572 experiments are reported in Figure 2, 3, 5 and 6.

Parameter	Sample 1	Sample 2	Sample 3	Sample 4
Total Solids (%)	5.4	4.1	6	4.5
Volatile Solids (% of TS)	77	79	80	81
pH	6.06	5.61	5.51	5.98
NH ₄ ⁺ (mgN/L)	435	370	210	90
Total alkalinity (mgCaCO ₃ /L)	3500	5500	2900	4200
H ₂ CO ₃ alkalinity (mgCaCO ₃ /L)	820	1180	600	1050
Total VFAs (mgCH ₃ COOH/L)	3100	4800	2600	3350
Acetic acid (mg/L)	500	1250	1500	1800
Propionic acid (mg/L)	800	2200	2800	1200
Butyric acid (mg/L)	620	1400	1600	1150
Valeric acid (mg/L)	900	1420	1900	3600

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574

575 **Table 2.** Average data (\pm Standard Deviation) obtained from the Control and Test reactors during the different phases of the experimental
 576 period. Star (*) indicates statistically different data ($p < 0.05$) between the same experimental condition.

Parameter	No CO ₂ injection		3 CO ₂ injections/week		5 CO ₂ injections/week	
	Control	Test	Control	Test	Control	Test
pH	7.68 \pm 0.08	7.69 \pm 0.08	7.46 \pm 0.08	7.38 \pm 0.10	7.35 \pm 0.06	7.28 \pm 0.05
TS (g/L)	26.6 \pm 2.3	27.8 \pm 0.8	24.8 \pm 0.7	24.6 \pm 1.9	21.1 \pm 2.2	22.0 \pm 2.9
VS (g/L)	16.4 \pm 1.5	17.1 \pm 0.4	15.5 \pm 0.5	15.6 \pm 1.0	13.7 \pm 1.1	13.8 \pm 1.3
NH ₄ ⁺ (mgN/L)	1608 \pm 124	1575 \pm 141	1219 \pm 220	1239 \pm 131	944 \pm 33	989 \pm 61
Biogas production (L/d)	132 \pm 35	141 \pm 33	*119 \pm 41	*140 \pm 33	*126 \pm 25	*147 \pm 31
CH ₄ production (L/d)	86 \pm 24	91 \pm 21	74 \pm 26	83 \pm 20	*80 \pm 17	*90 \pm 21
Specific CH ₄ production (L/(kgVSfed·d))	373 \pm 169	384 \pm 175	290 \pm 107	333 \pm 112	*332 \pm 94	*371 \pm 107
CH ₄ concentration (%)	65 \pm 3	65 \pm 3	*62 \pm 2	*59 \pm 3	*63 \pm 2	*61 \pm 2
H ₂ concentration (ppm)	80 \pm 23	72 \pm 23	*113 \pm 11	*126 \pm 36	*107 \pm 10	*138 \pm 26

577

578

Figure captions

Figure 1. Scheme of the experimental rig. (a) Control reactor and (b) Test reactor configuration during CO₂ injection. (1) Anaerobic reactor, (2) heating jacket, (3) peristaltic pump, (4) biogas sample point, (5) biogas meter, (6) bubble column, (7) mass flow controller, (8) gas pressure regulator, (9) CO₂ cylinder, (10) CH₄-CO₂ analyser, (11) digestate sampling point.

Figure 2. Methane (CH₄) production, CH₄ and hydrogen (H₂) concentration in Test and Control reactors during the experimental period. Black vertical lines divide the phases of the experimental period between: no CO₂ injections phase (No CO₂ inj.), phase of moderate CO₂ enrichment at 3 injections per week with a bubble column (3 CO₂ inj./week) and phase of intense CO₂ enrichment at 5 injections per week with a packed column (5 CO₂ inj./week). Top grey line identifies when different samples of sludge were used.

Figure 3. Ammonium nitrogen (NH₄⁺), H₂CO₃ Alkalinity concentrations (a) and pH (b) for Test and Control reactors during the different phases of the experimental period. Vertical lines divide the phases of the experimental period. Top grey line identifies when different samples of sludge were used.

Figure 4. Evolution of the parameter C/C_0 representing the ratio between the initial CO₂ concentration in digestate (C_0) and the concentration on the effluent of the CO₂ injection column (C). Evolution of pH in the effluent of the CO₂ injection column.

The C/C_0 achieved in the Test reactor at the end of the injection is marked as “X”.

Graph a) is for the use of a bubble column, graph b) is for the use of a packed column.

Figure 5. Intermediate to Partial Alkalinity (IA/PA) ratio and volatile fatty acids to H_2CO_3 Alkalinity (VFA/Alk) ratio for Test and Control reactors during the different phases of the experimental period. Vertical lines divide the phases of the experimental period. Top grey line identifies when different samples of sludge were used.

Figure 6. Acetic and propionic acid concentrations (a) and butyric and valeric acid concentrations (b) for Test and Control reactors during the different phases of the experimental period. Vertical lines divide the phases of the experimental period. Top grey line identifies when different samples of sludge were used.

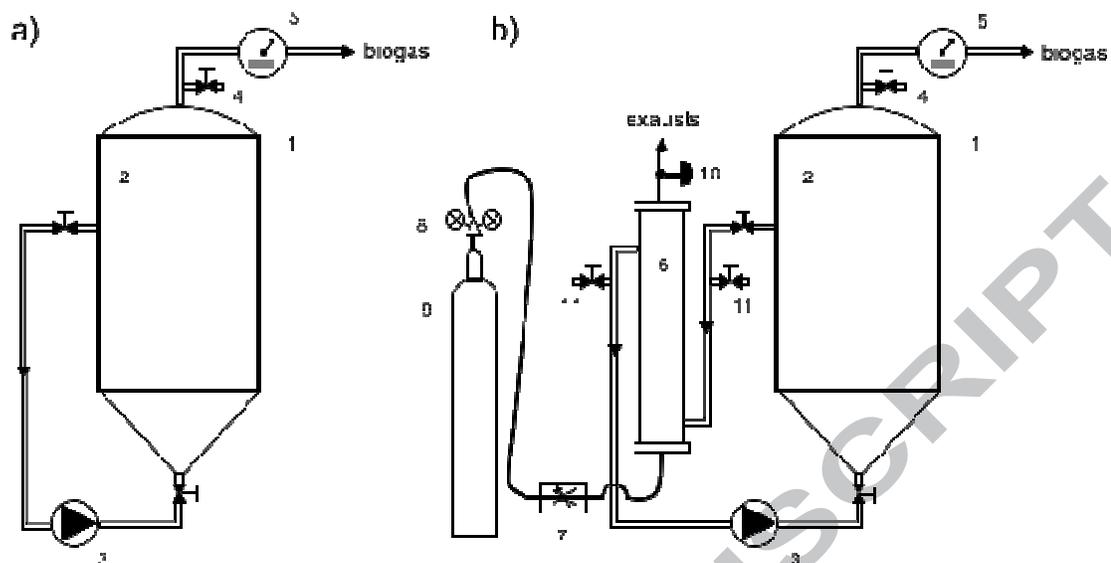


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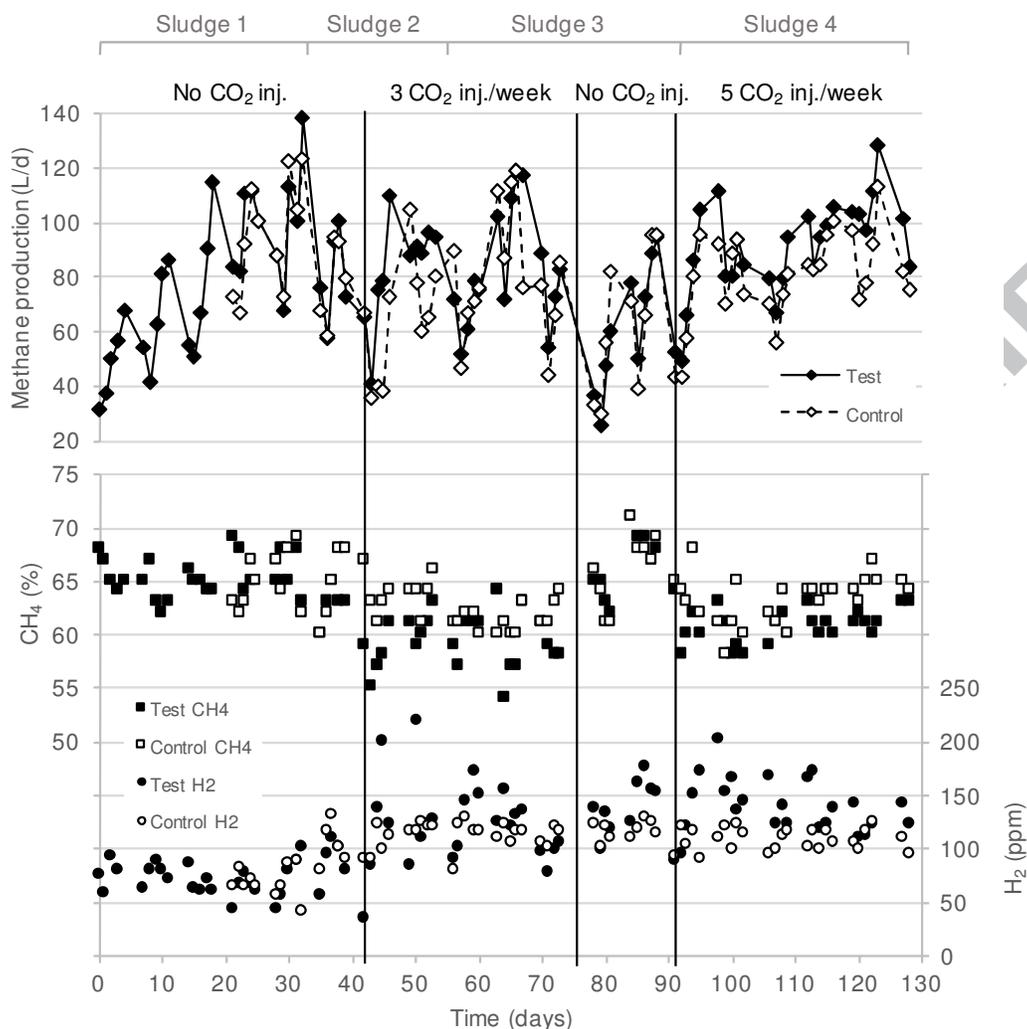


Figure 2. Methane (CH_4) production, CH_4 and hydrogen (H_2) concentration in Test and Control reactors during the experimental period. Black vertical lines divide the phases of the experimental period between: no CO_2 injections phase (No CO_2 inj.), phase of moderate CO_2 enrichment at 3 injections per week with a bubble column (3 CO_2 inj./week) and phase of intense CO_2 enrichment at 5 injections per week with a packed column (5 CO_2 inj./week). Top grey line identifies when different samples of sludge were used.

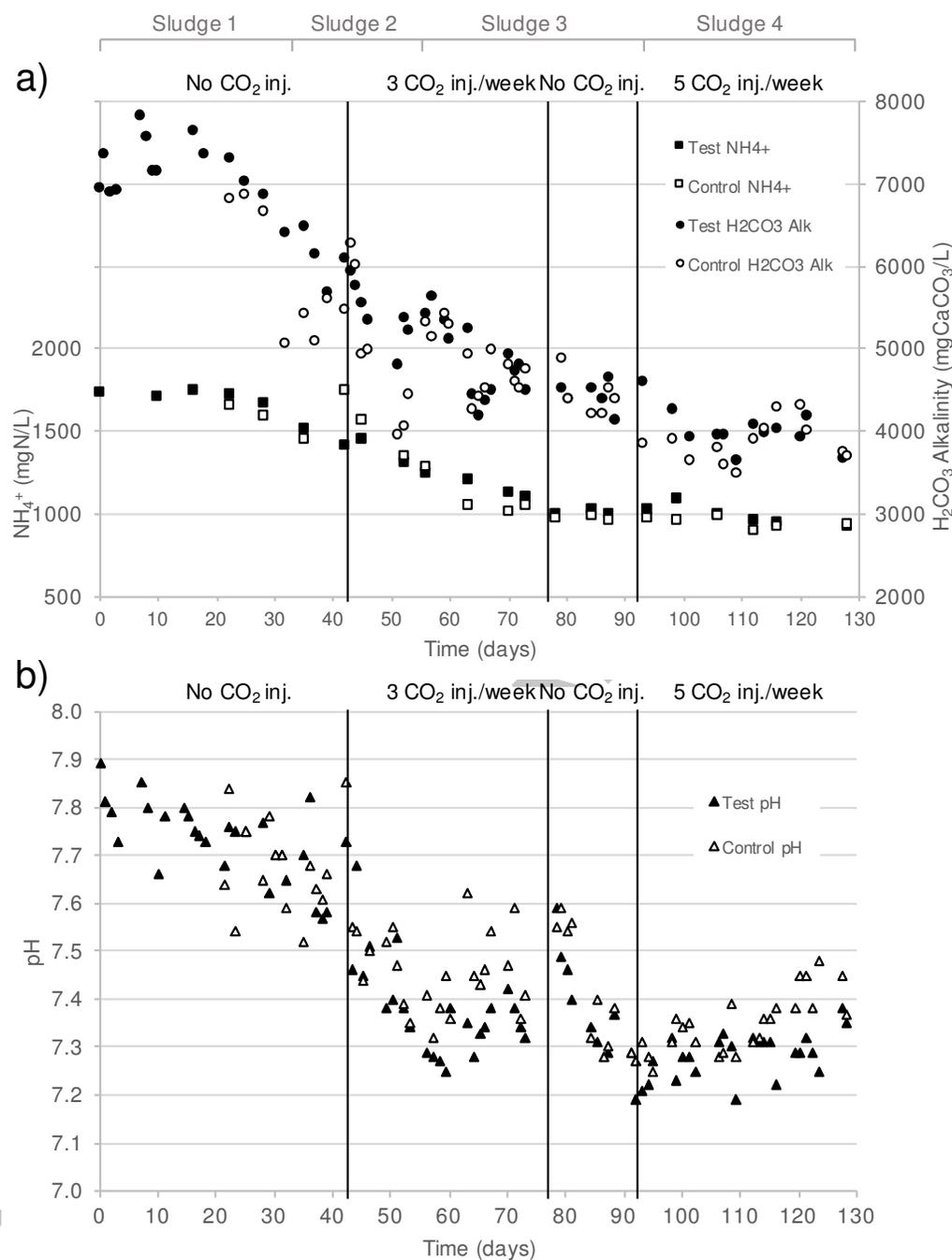


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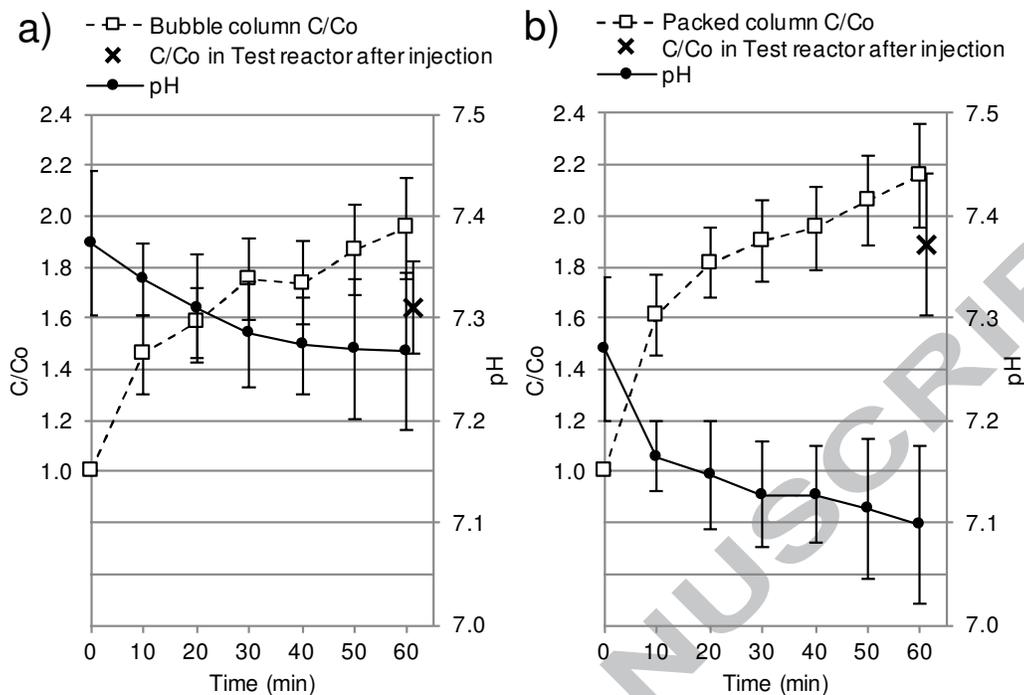


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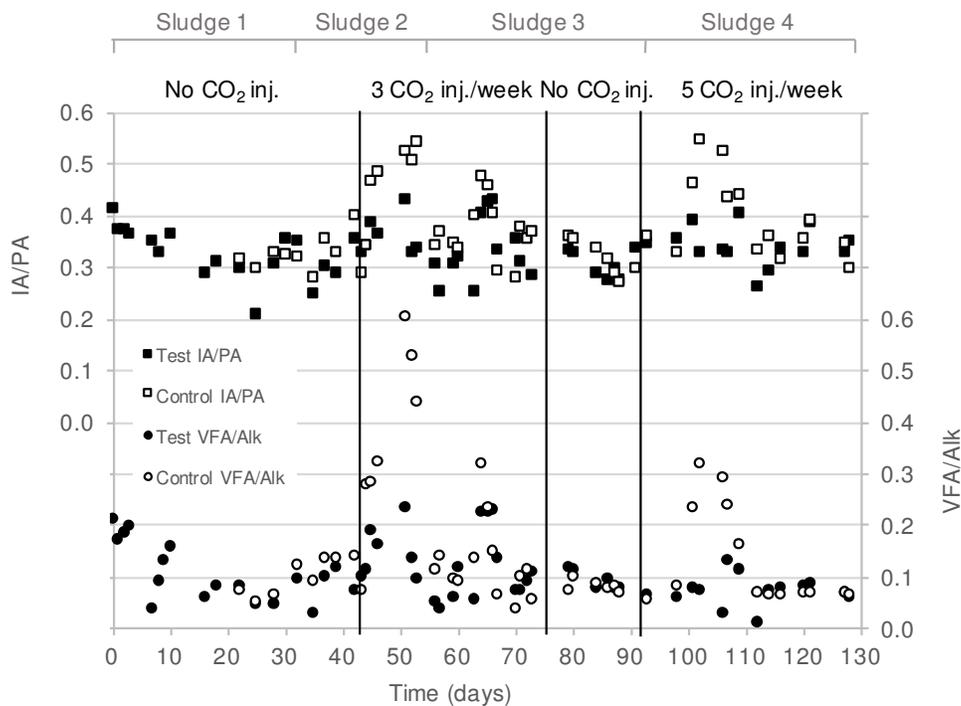


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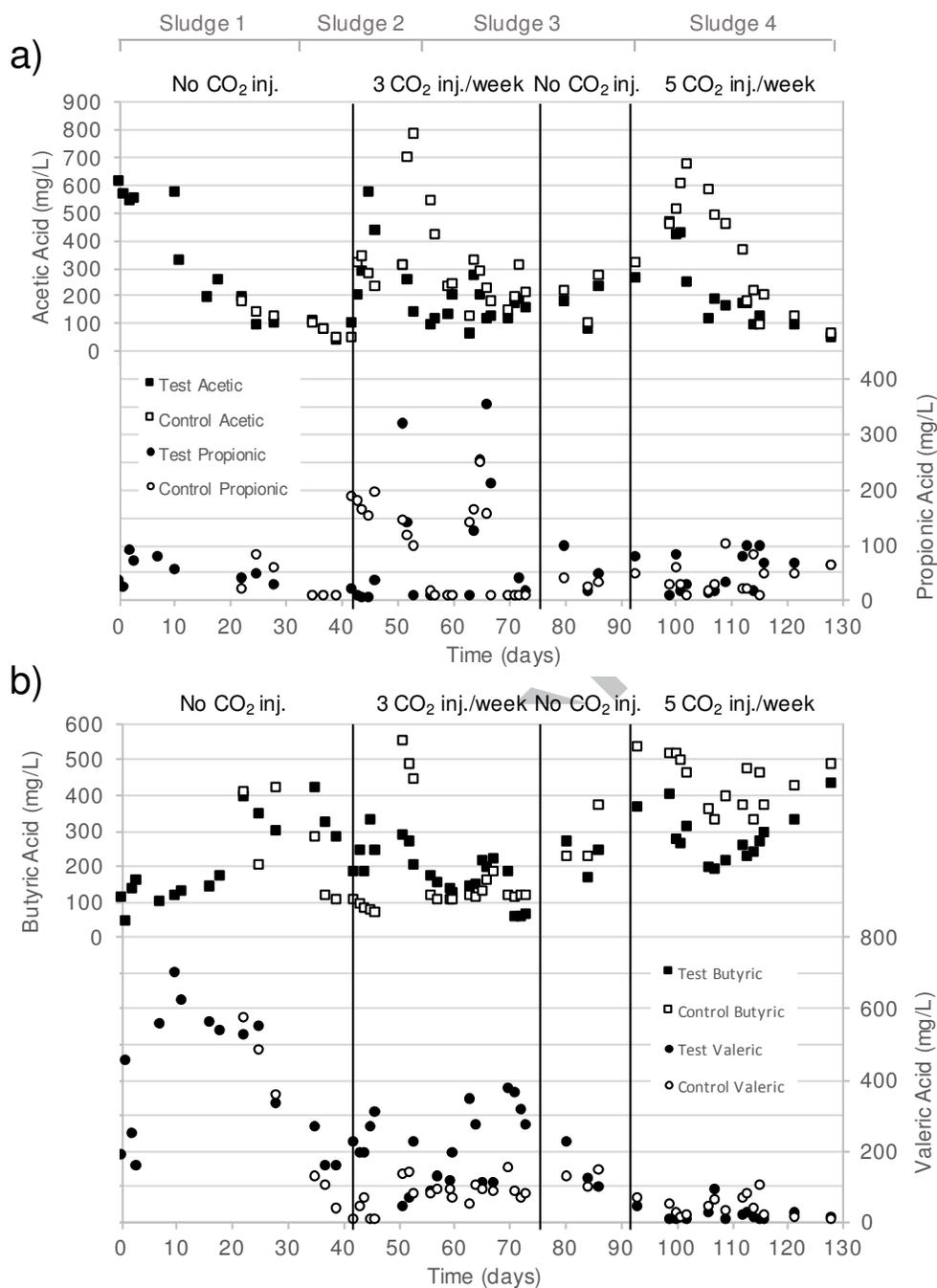


Figure 6. Acetic and propionic acid concentrations (a) and butyric and valeric acid concentrations (b) for Test and Control reactors during the different phases of the experimental period. Vertical lines divide the phases of the experimental period. Top grey line identifies when different samples of sludge were used.

HIGHLIGHTS

- CO₂ enrichment was tested on sewage sludge anaerobic digestion at pilot scale.
- CO₂ enrichment enhanced CH₄ production under moderate and intense injections.
- CO₂ injection had no negative effects on anaerobic digestion process stability.
- Benefits of CO₂ enrichment were proved without exogenous H₂ addition.

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Performance and stability of sewage sludge digestion under CO₂ enrichment: a pilot study

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