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**Reversibility of hydrolysis inhibition at high hydrogen partial pressure in dry anaerobic digestion processes fed with wheat straw and inoculated with anaerobic granular sludge**

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**Abstract**

In dry anaerobic digestion (AD), methanogenic performances are lowered by high solid contents. Low performances are often caused by a decrease of the gas-liquid transfer kinetics leading to local accumulation of inhibitory by-products. Hydrogen was previously identified as an inhibitor of hydrolytic and acetogenic microbial activities in dry AD. CO<sub>2</sub> is also generated but its impact on the microbial activity remains unknown. In this study, the reversibility of dry AD inhibition by high H<sub>2</sub> partial pressure ( $P_{H_2}$  of 1 bar) was investigated by adding CO<sub>2</sub> (400 mbars) after 11 and 18 days of methanogenesis inhibition, in an AD process operated at 25% TS, using wheat straw as substrate and inoculated with anaerobic granular sludge. As soon as CO<sub>2</sub> was added, the methanogenic activity rapidly recovered within 3 days, from  $0.41 \pm 0.1$  to  $3.77 \pm 0.8$  and then  $2.25 \pm 0.3$ , likely through the hydrogenotrophic pathway followed by the acetoclastic pathway, respectively. This result was confirmed by the high abundance of *Methanomicrobiales* (83%) and the emergence of *Methanosarcinales* sp (up to 17%) within the methanogens. Furthermore, the recovery kinetics were impacted by the

22 duration of the inhibition period suggesting a different impact of the high  $P_{H_2}$  on  
23 hydrogenotrophic and acetoclastic methanogens.

24

## 25 **Keywords**

26 Acidogenesis; Carbon dioxide; Gas transfer; Hydrogen; Solid-State Anaerobic  
27 Digestion

## 28 **1 Introduction**

29 In anaerobic digestion, the organic matter is converted by microorganisms into (1) a  
30 biogas composed of  $CH_4$  and  $CO_2$ , and (2) a residual digestate that may be further used  
31 as fertilizer if sanitary and environmental requirements are met. Three types of  
32 anaerobic digestion (AD) processes are distinguished according to the operational  
33 conditions: (1) the wet AD operated at a total solid content (TS) lower than 10%, (2) the  
34 semi-dry AD at a TS content between 10 and 20 % and (3) the dry AD, also called  
35 solid-state AD, at a TS content above 20% (Abbassi-Guendouz et al., 2012). Since less  
36 water is required in dry AD, the digester size as well as the energy demand are both  
37 minimized. Dry AD has gained lot of interest for industrial purposes and is now being  
38 widely implemented for the treatment of agricultural and ligno-cellulosic residues.

39 In counterpart, dry AD technologies present several disadvantages due to their high TS  
40 content, such as a decrease of the AD performances with lower methane yields and  
41 some handling difficulties due to the high heterogeneity and viscosity of the substrate  
42 (Abbassi-Guendouz et al., 2012; Motte et al., 2013). When the TS content exceeds 30  
43 %, the anaerobic digestion process can be rapidly blocked or even strongly inhibited

44 (Abbassi-Guendouz et al., 2012). Such inhibition phenomenon is characterized by a  
45 decrease of the biogas production and an increase of the Volatile Fatty Acids (VFAs)  
46 concentration (Abbassi-Guendouz et al., 2012; Motte et al., 2013). Indeed, the decrease  
47 of the free available water at high TS content results in the reduction of mass transfer  
48 kinetics of soluble molecules such as VFAs, or dissolved gases (Bollon et al., 2013).  
49 Since dissolved gas diffusion and gas-liquid transfers become rapidly limiting, local  
50 accumulation of these by-products can occur and lead to microbial local inhibition in  
51 the bulk phase where microorganisms are active (Abbassi-Guendouz et al., 2012).

52 In particular, dissolved hydrogen accumulation rapidly makes the acetogenic reactions  
53 thermodynamically unfavourable causing higher VFAs accumulation, a subsequent  
54 decrease of the pH and finally methanogenesis inhibition (Guo et al., 2010). Under  
55 anaerobic conditions,  $H_2$  is produced by acidogenic bacteria and is immediately  
56 consumed in combination with  $CO_2$  by either homoacetogenic bacteria to produce  
57 acetate, or hydrogenotrophs to produce methane (Amani et al., 2010). This latter  
58 pathway represents about 30% of the  $CH_4$  produced in anaerobic digestion (Amani et  
59 al., 2010). All these reactions are reversible in the AD process where acetate oxidation  
60 plays also a key role between methanogenic pathways (Karakashev et al., 2006). The  
61 local  $H_2$  partial pressure can transitorily increase but must remain low enough to avoid  
62 inhibition of syntrophic acetogenic bacteria. When the hydrogen partial pressure ( $P_{H_2}$ ) is  
63 high, VFAs production increases, causing a decrease of the pH to lower value than 6  
64 (Guo et al., 2010). Such variation in pH can impact biomass hydrolysis as well as the  
65 following steps of acidogenesis and methanogenesis (Siegert and Banks, 2005; Veeken  
66 et al., 2000). Indeed, the growth of methanogenic and acidogenic bacteria are strongly  
67 affected by the pH (Luo and Angelidaki, 2013).

68 In addition, Cazier et al. (2015) reported that a high initial partial pressure of  $H_2$  in the  
69 headspace was the main inhibitory factor affecting wheat straw hydrolysis in dry AD. It  
70 was suggested that  $CO_2$  played a key role since  $H_2$  inhibition occurred only in absence  
71 of remaining  $CO_2$ . Indeed, hydrolysis inhibition did not occur when  $CO_2$  was initially  
72 present with  $H_2$  in the reactor headspace. In that case,  $H_2$  was rapidly consumed by  
73 homoacetogenic bacteria and methanogens. When anaerobic digestion is efficiently  
74 working, it can be assumed that  $CO_2$  and  $H_2$  are both biologically produced during  
75 acidogenesis and acetogenesis and are continuously consumed by homoacetogenic  
76 bacteria and methanogenic archaea. More  $CO_2$  than  $H_2$  is produced, the overall  $CO_2$   
77 content ranging from 30 to 50% of the biogas.

78 However, the exact role of  $CO_2$  on the bacterial activity in AD remains unclear. On the  
79 one hand,  $CO_2$  has been reported as inhibitor of the production and degradation of  
80 VFAs, as previously shown by Hansson and Molin (1979) and Arslan et al. (2012) who  
81 worked on acetate and propionate accumulation at pH 7 and pH 4.5 and under 1 bar of  
82  $CO_2$ , respectively. Consistently, it was elsewhere reported that an inhibitory impact of  
83  $CO_2$  on acetogenic and lactic acid bacteria at pH 5.3 (Kim et al., 2006) and on  
84 acetoclastic methanogens at pH 7 (Hansson and Molin, 1981). On the other hand,  
85 acidogenesis and more particularly  $H_2$  production was shown to be improved by  
86 sparging  $CO_2$  before fermentation (at 30 to 300  $ml_{CO_2}.min^{-1}$ ) (Bru et al., 2012; Kim et  
87 al., 2006). Nonetheless, an inhibitory effect was observed when  $CO_2$  was sparged at  
88 higher rate (500  $ml_{CO_2}.min^{-1}$ ) (Bru et al., 2012). In contrast, Park et al. (2005) reported  
89 that fermentative  $H_2$  production was improved by removing the  $CO_2$ . Since all  
90 experiments were carried out under different operating conditions and different

91 microbial communities, concluding on the exact impact of CO<sub>2</sub> on the different AD  
92 microbial activities remains unclear.

93 The aim of this study was to evaluate the impact of adding CO<sub>2</sub> in mesophilic dry AD  
94 when methanogenesis was artificially inhibited by high initial H<sub>2</sub> partial pressure in  
95 headspace. Two inhibition durations (11 and 18 days) prior to CO<sub>2</sub> injection were  
96 investigated to evaluate the persistence of the inhibitory effect on acidogenic and  
97 methanogenic populations.

## 98 **2 Materials and methods**

### 99 *2.1 Substrate*

100 Wheat straw (*Triticum aestivum*) was used as substrate. After harvest and collection,  
101 wheat straw was fractionated using a cutting miller through a 1 mm grid, and then  
102 sieved to collect particles having a size between 400 µm and 1 mm. The TS content of  
103 the wheat straw particles was 95%.

### 104 *2.2 Operating conditions of the batch tests*

105 Industrial UASB anaerobic granules were used to inoculate the batch reactors. The  
106 granules were manually broken and mixed during 24 h at 35°C, and were then  
107 centrifuged (7 841 g, 20 min, and 4°C) to obtain a homogeneous anaerobic inoculum.  
108 The TS content of the inoculum ranged between 10 and 15 %. The substrate/inoculum  
109 ratio was fixed at 3 (on basis of the volatile solid contents) (Liew et al., 2012). A buffer  
110 solution of sodium bicarbonate (0.0026 g of NaHCO<sub>3</sub>·g<sup>-1</sup> of substrate) was used to keep  
111 the pH at 8 all along the experiment (data not shown). A solution of trace elements  
112 (FeCl<sub>2</sub> 2 g·L<sup>-1</sup>, CoCl<sub>2</sub> 0.5 g·L<sup>-1</sup>, MnCl<sub>2</sub> 0.1 g·L<sup>-1</sup>, NiCl<sub>2</sub> 0.1 g·L<sup>-1</sup>, ZnCl<sub>2</sub> 0.05 g·L<sup>-1</sup>,

113  $\text{H}_3\text{BO}_3$  0.05 g·L<sup>-1</sup>,  $\text{Na}_2\text{SeO}_3$  0.05 g·L<sup>-1</sup>,  $\text{CuCl}_2$  0.04 g·L<sup>-1</sup>,  $\text{Na}_2\text{MoO}_4$  0.01 g·L<sup>-1</sup>) was  
114 added (0.2 mL by flask) at start of the experiment, using the same protocol than Cazier  
115 et al. (2015). Initial TS content of the mixture (inoculum, wheat straw, sodium  
116 bicarbonate solution and trace elements) was fixed at 25% corresponding to the  
117 maximal TS value where no inhibitory effect was observed in dry AD (Abbassi-  
118 Guendouz et al., 2012; Motte et al., 2013) and to only investigate the reversibility of the  
119 inhibition only caused by high hydrogen partial pressure (Cazier et al. (2015)).

120 First, the mixture was introduced into a reactor with a working volume of 3 L operated  
121 during 10 days at 35°C under  $\text{N}_2$  atmosphere to reach an active phase of  
122 methanogenesis and homogenize the substrate/inoculum medium. Then, 20 g of this  
123 pre-culture was put into the bottom of a 600 ml flask, which corresponded to a thin  
124 layer of less than 1 cm of substrate to limit the influence of the gas diffusion in the  
125 medium. The flasks were initially flushed with  $\text{N}_2$  gas. Hydrogen was then added to  
126 reach an initial  $\text{H}_2$  partial pressure of  $996 \pm 27$  mbars, under a total pressure of 1 500  
127 mbars. A control, with only  $\text{N}_2$  in headspace, was also carried out. All the flasks were  
128 then incubated at 35°C for 32 days. In some of the flasks filled with hydrogen,  $396 \pm 44$   
129 mbars of  $\text{CO}_2$  were added after 11 and 18 days of operation. Batch tests were carried out  
130 in triplicates for each condition. Flasks were sampled at day 0, 11, 18, 25 and 32 for  
131 further analysis of fermentative metabolite concentrations.

### 132 2.3 Analytical methods

133 Biogas production volume was periodically estimated by measuring the total pressure  
134 and the biogas composition. The gas composition was determined using a gas  
135 chromatograph Perkin Clarus 580 composed of an injector heated at 250°C and two  
136 capillary columns heated at 60°C. The first column corresponded to an RtUbond for the

137 CO<sub>2</sub> and the second column an RtMolsieve used for the detection of the O<sub>2</sub>, H<sub>2</sub>, N<sub>2</sub> and  
138 CH<sub>4</sub>. The carrier gas was argon at 350 kPa and under a flowrate of 31.8 ml.min<sup>-1</sup>. The  
139 detection was ensured by a thermal conductivity detector kept at 150°C.

140 Metabolites were quantified by diluting 5 g of digestate in 20 g of deionized water for  
141 30 minutes. The mixture was then centrifuged during 20 min at 39 121 g and 4°C and  
142 filtrated at 0.2 µm with a nylon membrane using the same protocol than Cazier et al.  
143 (2015). VFAs were measured with a gas chromatograph Perkin Clarus 580 equipped  
144 with an Elite-FFAP crossbond® carbowax® 15 m column connected to a flame  
145 ionization detector at 280°C. Nitrogen was used as carrier gas under a flow rate of 6  
146 mL.min<sup>-1</sup> (Motte et al., 2013). Other metabolites than VFAs were quantified using high  
147 performance liquid chromatograph, e.g. lactic acid and ethanol. The chromatograph was  
148 composed of an automatic sampler (Water 717), a pre-column to filter residues (Micro  
149 guard cation H refill cartridges, Bio-Rad) and an Aminex HPX-87H column (300 mm  
150 on 7.8 mm, Bio-Rad). The carrier eluent was a sulfuric acid solution at 0.005 M under a  
151 fixed flowrate of 0.4 ml.min<sup>-1</sup>.

152 The microbial communities of *Archaea* and *Bacteria* were characterized after DNA  
153 extraction and amplification of the V3 region of the 16S rRNA according to the  
154 protocols of Braun et al. (2011) and Bru et al. (2012).

155 The PCR products were purified and sequenced, using the Illumina MiSeq System with  
156 2x300 bp paired-end chemistry used at the GenoToul sequencing centre  
157 ([www.genotoul.fr](http://www.genotoul.fr)). An average of 46 021 high quality sequences per sample for  
158 *Archaea* and for *Bacteria* were retained after assembly, de-multiplexing and cleaning  
159 with Mothur software version 1.33.2, as described by Schloss et al. (2009). SILVA



release 102 was used for alignment and taxonomic affiliation. Sequences are registered on NCBI database under the accession numbers KY229870 to KY229893 for archaea, and KY234504 – KY235143 for bacteria.

#### 2.4 Data analysis

R software (version 2.15.2) coupled with the package Rcmdr (version 1.8-4) was used for statistical analysis of the experimental data, using variance analysis (ANOVA). Non-significant p-values were fixed > 0.05 and significant p-values were fixed when < 0.05.

Total Substrate Degradation (TSD) was estimated from a theoretical Chemical Oxygen Demand (COD) mass balance between the start-up and the end of each experiment, as described elsewhere (Cazier et al., 2015). All calculation was expressed according to the initial TS content of wheat straw (TSi expressed in grams of dry solids), as follows:

$$\text{TSD} = \text{Final State} - \text{Initial State} = \frac{A_{\text{H}_2,\text{f}} + A_{\text{CH}_4,\text{f}} + A_{\text{met},\text{f}} + A_{\text{GC}}}{\text{TSi}} - \frac{A_{\text{H}_2,\text{i}} + A_{\text{met},\text{i}}}{\text{TSi}} \quad (\text{Eq.1})$$

where,  $A_{\text{H}_2,\text{f}}$  is the amount of  $\text{H}_2$  remaining at the end in the headspace,  $A_{\text{CH}_4,\text{f}}$  the final amount of accumulated  $\text{CH}_4$ ,  $A_{\text{met},\text{f}}$  the final amount of metabolic products,  $A_{\text{GC}}$  the total amount of gas ( $\text{H}_2$  and  $\text{CH}_4$ ) sampled for analyses,  $A_{\text{H}_2,\text{i}}$  the initial amount of  $\text{H}_2$  added and  $A_{\text{met},\text{i}}$  the initial amount of metabolites in the medium. Since all these parameters are expressed in grams of COD, TSD corresponded to gram of COD per gram of initial TS of wheat straw.

### 179 3 Results and discussion

#### 180 3.1 Recovery of the methanogenic activity after CO<sub>2</sub> addition

181 Figure 1.a shows the cumulated production of CH<sub>4</sub> along reactor operation time. For the  
182 reactors carried out at high initial  $P_{H_2}$  and, thus, operated under inhibitory conditions,  
183 CO<sub>2</sub> was added after 11 and 18 days of operation. The control corresponds to a reactor  
184 without initial addition of H<sub>2</sub> in headspace.

185 In the control reactor, a maximal and constant CH<sub>4</sub> production rate was observed after a  
186 lag phase of 5 days and reached a value of  $2.7 \pm 0.32 \text{ ml}_{CH_4} \cdot g_{TS}^{-1} \cdot \text{day}^{-1}$ , equivalent to  
187  $2.98 \text{ ml}_{CH_4} \cdot g_{VS}^{-1} \cdot \text{day}^{-1}$ . This result is significantly lower than previous reported values  
188 of  $12 \text{ ml}_{CH_4} \cdot g_{VS}^{-1} \cdot \text{day}^{-1}$  for wheat straw at 22% TS (Liew et al., 2012). Such difference  
189 resulted either from different microbial inoculum origins or from a TS content slightly  
190 higher in the present experiment (25% TS), considering that 28-30 % TS was previously  
191 reported as a threshold value prior inhibition of the methanogenic and acidogenic  
192 microbial activities (Abbassi-Guendouz et al. 2012; Motte et al. 2013).

193 In the reactors where H<sub>2</sub> was initially added, a small quantity of CH<sub>4</sub> accumulated the  
194 first day of experiment at low production rates of  $0.96 \pm 0.52$  and  $0.96 \pm 0.42 \text{ ml}_{CH_4} \cdot g_{TS}^{-1} \cdot \text{day}^{-1}$   
195 (Table 1): this production rates correspond to the mean values of the triplicates  
196 used to evaluate the addition of CO<sub>2</sub> after 11 and 18 days of inhibition, respectively.  
197 Thereafter, CH<sub>4</sub> production was strongly inhibited due to the presence of high partial  
198 pressure of H<sub>2</sub> in the headspace, with average production rates of only  $0.45 \pm 0.1$  and  
199  $0.38 \pm 0.1 \text{ ml}_{CH_4} \cdot g_{TS}^{-1} \cdot \text{day}^{-1}$ . In comparison, the control (only N<sub>2</sub>) showed a methane  
200 production rate ten times higher at  $2.7 \pm 0.3 \text{ ml}_{CH_4} \cdot g_{TS}^{-1} \cdot \text{day}^{-1}$  for the same experimental  
201 time. Consequently, the amounts of cumulated methane after 11 and 18 days reached

only  $4 \pm 0.5$  and  $6 \pm 1$   $\text{ml}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1}$  in the inhibited reactors against  $20 \pm 4$  and  $39 \pm 4$   $\text{ml}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1}$  in the controls, respectively. It was concluded that methanogenesis was clearly inhibited in presence of high initial  $\text{H}_2$  partial pressure in headspace ( $996 \pm 27$  mbars). The corresponding concentration of dissolved  $\text{H}_2$  in the medium at  $35^\circ\text{C}$  was estimated at  $0.58 \pm 5 \times 10^{-2}$   $\text{mg}_{\text{H}_2} \cdot \text{L}^{-1}$ . Consistently, a similar value was reported as a threshold  $\text{H}_2$  concentration prior to wheat straw hydrolysis inhibition in AD by Cazier et al. (2015).

When  $\text{CO}_2$  was added in reactor headspace, the methane rapidly accumulated within the first 3 days to reach values of  $12 \pm 1$  and  $10 \pm 2$   $\text{ml}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1}$  in the reactors where  $\text{CO}_2$  was added at day 11 and 18, respectively. This first phase of  $\text{CH}_4$  production was called “phase 1”, as shown in Figure 1.b. During phase 1,  $\text{H}_2$  and  $\text{CO}_2$  were both rapidly consumed until total exhaustion of  $\text{H}_2$  in headspace (data not shown). No significant acetate accumulation was observed during the first 7 days after  $\text{CO}_2$  addition (Figure 2). It was therefore concluded that  $\text{H}_2$  and  $\text{CO}_2$  were most likely consumed by hydrogenotrophic methanogens to produce  $\text{CH}_4$ . Consistently, methane production rates in phase 1 were higher than in the controls, with  $4 \pm 0.75$   $\text{ml}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1} \cdot \text{day}^{-1}$  and  $3.55 \pm 0.87$   $\text{ml}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1} \cdot \text{day}^{-1}$  for  $\text{CO}_2$  added at day 11 and 18, respectively, versus an average value of  $2.7 \pm 0.3$   $\text{ml}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1} \cdot \text{day}^{-1}$  in the controls (Table 1). Such observation strongly supports the fact that efficient hydrogenotrophic methanogenesis was the main methanogenic pathway during phase 1. Moreover, the methanogenic activity recovered immediately after  $\text{CO}_2$  addition, suggesting that hydrogenotrophic methanogens were not inhibited at high  $P_{\text{H}_2}$ , at least during the first 18 days. This is consistent with previous observations where the production of  $\text{CH}_4$  by hydrogenotrophic methanogens

225 was previously shown to be favoured at high  $P_{H_2}$  ( $> 5$  mbars) in anaerobic digestion  
226 systems (Demirel and Scherer, 2008; Schink, 1997).

227 After phase 1, a phase of 4 days, called ‘plateau’, was observed with only a small  
228 amount of  $CH_4$  that accumulated whatever the time of  $CO_2$  addition (Figure 1.b). The  
229  $CH_4$  production rate during the plateau phase was very low, *i.e.*  $0.82 \pm 0.26$  and  $0.41 \pm$   
230  $0.27 \text{ ml}_{CH_4} \cdot \text{g}_{TS}^{-1} \cdot \text{day}^{-1}$  when the  $CO_2$  was added at 11 and 18 days respectively (Table  
231 1). Since no  $H_2$  was present in headspace, this plateau phase corresponded probably to  
232 the time for the microbial community to readapt to favourable conditions for substrate  
233 degradation, as initially observed in the control, *i.e.* a lag phase of 4 days at the start of  
234 the experiment.

235 Afterwards, methane production increased to reach a cumulated methane yield of  $16 \pm 1$   
236  $\text{ml}_{CH_4} \cdot \text{g}_{TS}^{-1}$  and  $11 \pm 3 \text{ ml}_{CH_4} \cdot \text{g}_{TS}^{-1}$  in 7 days, when  $CO_2$  was added at day 11 and 18,  
237 respectively. This second production phase was denominated ‘phase 2’ (Figure 1.b). In  
238 phase 2, the methane production rates decreased by half when compared to phase 1  
239 (Table 1), with  $2.74 \pm 0.45$  and  $1.61 \pm 0.23 \text{ ml}_{CH_4} \cdot \text{g}_{TS}^{-1} \cdot \text{day}^{-1}$  when the  $CO_2$  was added  
240 at 11 and 18 days respectively. Since the  $CH_4$  production rates were substantially  
241 different during for the first and second phase, two different methanogenic pathways  
242 were likely involved. Indeed, it is well established that hydrogenotrophic  
243 methanogenesis is faster than the acetoclastic methane producing pathway (Pan et al.,  
244 2016). While  $CH_4$  production in phase 1 seemed to be mainly due to hydrogenotrophic  
245 methanogens, methanogenesis was most probably resulting from the degradation of  
246 acetate by acetoclastic methanogens in phase 2 (Demirel and Scherer, 2008).

247 Interestingly, CH<sub>4</sub> production rates were slightly higher when CO<sub>2</sub> was added after 11  
248 days of inhibition ( $4 \pm 0.75$  and  $2.74 \pm 0.45$  ml<sub>CH<sub>4</sub></sub>·g<sub>TS</sub><sup>-1</sup>·day<sup>-1</sup> for phases 1 and 2,  
249 respectively) than 18 days ( $3.55 \pm 0.87$  and  $1.77 \pm 0.23$  ml<sub>CH<sub>4</sub></sub>·g<sub>TS</sub><sup>-1</sup>·day<sup>-1</sup> for phases 1  
250 and 2, respectively). Such a difference suggests a cumulative inhibitory effect of the  
251 time of exposure to H<sub>2</sub> on both hydrogenotrophic and acetoclastic methanogens.

### 252 3.2 Impact of the $P_{H_2}$ on other metabolic by-products dynamics

253 Figure 2 presents the accumulation of metabolic by-products (VFAs and methane), the  
254 remaining hydrogen in the controls and in the reactors carried out with high initial  $P_{H_2}$ .  
255 In the controls, microbial metabolites, *i.e.* all VFAs, formate, succinate, and ethanol,  
256 transitorily accumulated after 11 and 18 days of operation ( $35 \pm 2$  and  $39 \pm 5$   
257 mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> at day 11 and 18, respectively) likely because of the high TS content, and  
258 then decreased to  $7 \pm 2$  mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> at day 32, confirming the efficient methanogenic  
259 activity even at 25% TS.

260 In comparison, the amount of metabolites was higher in the reactors where H<sub>2</sub> was  
261 initially added. The concentrations of metabolites reached  $58 \pm 5$  mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> at day 11  
262 and  $82 \pm 17$  mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> at day 18 prior to CO<sub>2</sub> addition (Figure 2). These values  
263 corresponded to a total concentration in metabolites of about  $20 \pm 2$  g·L<sup>-1</sup> at day 11, and  
264  $28 \pm 6$  g·L<sup>-1</sup> at day 18, respectively. Such value is above the inhibitory limit of 20 g·L<sup>-1</sup>  
265 as previously reported in wet AD processes (Siegert and Banks, 2005). Interestingly,  
266 most of the hydrogen consumed at day 11 and at day 18 ( $69 \pm 2$  mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> and  $72 \pm 2$   
267 mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup>) corresponds to the concentration of produced metabolites ( $51 \pm 2$   
268 mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> and  $75 \pm 17$  mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup> at day 11 and 18 prior to CO<sub>2</sub> addition,  
269 respectively. The small difference at day 11 between the hydrogen recoveries into  
270 metabolites ( $18$  mg<sub>COD</sub>·g<sub>TS</sub><sup>-1</sup>) could correspond to the methane produced during this time

271  $(17.5 \pm 4 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1})$ . Such high concentration of metabolites might have been the  
272 cause of the strong inhibition of methanogenic activity at high  $P_{\text{H}_2}$ , prior to  $\text{CO}_2$   
273 addition. Since VFAs did not accumulate during this period, it can also be concluded  
274 that hydrolysis and/or acidogenesis may also have been inhibited under these  
275 conditions, prior to  $\text{CO}_2$  addition, as previously reported by Cazier et al. (2015).

276 Furthermore, the increase in the total amount of metabolites at high initial  $P_{\text{H}_2}$  was  
277 mostly due to an increase of acetate, and, at a lower extent, butyrate and isobutyrate.  
278 The acetate concentration increased from  $2 \pm 0 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  to  $35 \pm 4 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  at  
279 day 11 and  $43 \pm 11 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  at day 18, prior to  $\text{CO}_2$  addition. Meanwhile, the  
280 butyrate and isobutyrate concentration increased from  $1.7 \pm 0 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  at start to  $9 \pm$   
281  $0.2 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  and  $18 \pm 4 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  at day 11 and 18, respectively. Such increase of  
282 acetate and butyrate concentrations under an atmosphere rich in  $\text{H}_2$  was previously  
283 observed during the anaerobic conversion of carbohydrates-rich wastes (Arslan et al.,  
284 2012). These authors reported an increase of 31% and 51% of acetate and butyrate  
285 production, respectively, under a  $P_{\text{H}_2}$  of 2 bars in comparison to only  $\text{N}_2$ .

286 When  $\text{CO}_2$  was added after 11 days of inhibition, no metabolite degradation was  
287 observed during the first 7 days after  $\text{CO}_2$  addition (phase 1), confirming the assumption  
288 of a dominant hydrogenotrophic pathway producing methane (Figure 2). The decrease  
289 of the total COD concentration between the day of addition of  $\text{CO}_2$  (day 11) and 7 days  
290 after, was probably due to the fact that the analysis of metabolites and acetate was only  
291 done in one sacrificed replicate and not all replicates. Therefore, ANOVA was used to  
292 statistically compare the results for each.

293 7 days after CO<sub>2</sub> addition, a decrease of the total metabolites concentration was  
 294 observed, and was mostly due to acetate consumption. This observation is consistent  
 295 with the recovery of the methanogenic activity. The acetate content decreased from  $33 \pm$   
 296  $2.4 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  to  $8 \pm 6 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  between day 7 and 14, respectively. Such  
 297 difference between the acetate concentration at day 0 and 14 after CO<sub>2</sub> addition was  
 298 statistically significant (ANOVA, p-value <0.05). The decrease of acetate concentration  
 299 was likely due to the conversion of acetate into CH<sub>4</sub> by acetoclastic methanogens  
 300 (Pavlostathis and Giraldo-Gomez, 1991) or to the oxidation of acetate by acetate-  
 301 oxidizing bacteria into H<sub>2</sub> and CO<sub>2</sub> that are then converted to CH<sub>4</sub> by hydrogenotrophic  
 302 methanogens (Karakashev et al., 2006). A similar trend was observed when CO<sub>2</sub> was  
 303 added after 18 days with an acetate content that decreased from  $30 \pm 10 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  at  
 304 day 7 to  $18 \pm 3 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  at day 14 (Figure 2). Interestingly, acetate degradation from  
 305 day 7 to 14 (phase 2) was slower when CO<sub>2</sub> was added after 18 days of inhibition. This  
 306 result was likely due to the time of exposure of acetate-degrading methanogens at high  
 307 H<sub>2</sub> partial pressure. In other studies, a specific inhibitory effect was observed on the  
 308 growth of *Methanosarcina* sp. when H<sub>2</sub> partial pressure was increased from 2.5 to 20  
 309 mbars and a specific effect on acetate degradation was observed (Ahring et al., 1991).  
 310 Such observation is also supporting the fact that hydrogenotrophic methanogens were  
 311 most probably the most efficient CH<sub>4</sub> producers in phase 1 since no significant  
 312 difference between the two times of exposure.

### 313 3.3 Impact of the $P_{\text{H}_2}$ on the overall substrate degradation

314 To estimate the impact of the  $P_{\text{H}_2}$  on the global microbial activity, the overall substrate  
 315 degradation was calculated in  $\text{mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  using Equation 1, which takes in  
 316 consideration the amount of H<sub>2</sub> initially added (Figure 3).

317 In the reactors where CO<sub>2</sub> was added at day 11 or day 18, the substrate degradation was  
318 very similar 14 days after CO<sub>2</sub> addition, with  $55 \pm 9 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  and  $54 \pm 17$   
319  $\text{mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$ , respectively. Therefore, the impact of the time exposure on the methane  
320 production rate in phase 2, was probably not due to a persistent effect on the global  
321 microbial activity since the overall substrate degradation was the same after 14 days, but  
322 more likely to a transitory accumulation of metabolites due to a slower methanogenic  
323 activity, as shown in Figure 2. Nevertheless, when comparing these values to the  
324 control, the global substrate degradation was lower in the reactors operated at high  
325 initial  $P_{H_2}$  for a close duration of operation. About  $80 \pm 5 \text{ mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$  were reached at  
326 day 11 in the control that is substantially higher than in inhibited reactors. All these  
327 observations suggest that the high initial  $P_{H_2}$  had very likely a persistent inhibitory  
328 effect on the hydrolytic activity of the consortium.

329

330 The exact mechanisms behind microbial hydrolysis are still uncertain and probably  
331 highly diverse when considering complex substrates. Two main mechanisms have been  
332 proposed in the AD model (ADM1): (1) the enzymes are directly secreted into the  
333 liquid phase by hydrolytic microorganisms with a direct effect on substrate hydrolysis  
334 that releases free sugars on the bulk phase or (2) the microorganisms attach on the  
335 substrate surface with the formation of a biofilm and produce enzymatic complexes to  
336 disrupt the organic material (Batstone et al., 2002). Recently, Cazier et al. (2015)  
337 reported an initial a strong inhibitory effect of high  $P_{H_2}$  on the hydrolytic activity in dry  
338 AD. High  $P_{H_2}$  could have either influenced the production or secretion of extracellular  
339 enzymes by retro-inhibition, or reduced the physiological activity of the  
340 microorganisms. Similarly to the present study, these experiments were carried out with



thin layer of substrate to reduce gas transfer limitation and investigate the local effect of  $H_2$  partial pressure. In dry AD reactors, the effect of gas transfer limitation must also to be considered. Since diffusion coefficients decrease when TS contents increase (Bollon et al., 2013), dissolved gas diffusion and gas-liquid transfer may become a limiting factor (Abbassi-Guendouz et al., 2012), with a local accumulation of  $H_2$  and  $CO_2$  in the medium. With a substrate rich in carbohydrates and at high TS content, hydrolysis and acidogenesis is highly favoured with a rapid production of VFAs,  $CO_2$  and  $H_2$ . That could lead to a local accumulation of  $H_2$  since  $CO_2$  could be dissolved in carbonates at high pH. Therefore, if the local  $P_{H_2}$  is high enough, hydrolysis may therefore be inhibited, especially if the local  $P_{H_2}$  is low. The results of the present study suggest that the addition of  $CO_2$  in dry AD digester may improve the methanogenic performances not only by increasing the gas-transfer kinetics, but also by reducing the local  $P_{H_2}$  through  $H_2$  consumption. Adding  $CO_2$  may also present inhibitory effects on AD performances if the medium is not properly buffered since  $CO_2$  could decrease the pH down to 6 that has a strong inhibitory effect on methanogens (Ward et al., 2008). However, this is unlikely to occur since tests operated under similar conditions with only  $CO_2$  added in headspace (no  $H_2$ ) were carried out and no impact on dry AD performances was observed (data not shown).

### 3.4 Impact of the $P_{H_2}$ on microbial community dynamics

The compositions in *Archaea* and *Bacteria* of the microbial communities were determined in the control reactor, and in the reactors containing a high  $P_{H_2}$  before  $CO_2$  addition, and 7 days (end of 'plateau' phase) and 14 days (end of phase 2) after  $CO_2$  addition (Table 2).

364 First, the composition in *Archaea* in the inoculum (day 0) was mainly dominated by  
365 hydrogenotrophic methanogens (*Methanobacteriales*: 91.6%) followed by mostly  
366 acetoclastic methanogens (*Methanosarcinales*: 5.8%), as already described by Amani et  
367 al. (2010). Interestingly, in the control, the relative proportion of acetoclastic  
368 methanogens increased over the experimental time to reach 17 % after 18 days. Such  
369 variation in the type of methanogens was already reported in dry AD (31% TS) during  
370 the start-up period followed by a stabilization period, for a semi-continuous  
371 thermophilic reactor treating the organic fraction of municipal solid waste (Montero et  
372 al. 2008). A higher proportion in *Methanosarcinales* sp. might indicate that an efficient  
373 microbial process occurred in the controls, as previously suggested in dry AD by  
374 Abbassi-Guendouz (2013).

375 In the reactor where CO<sub>2</sub> was added at day 11, the overall composition of the archaeal  
376 community did not significantly change. Indeed, the microbial community was  
377 composed of 90-91% *Methanobacteriales* and only 6-8% *Methanosarcinales* all along  
378 the experiment. Since hydrogenotrophic methanogens (*Methanobacteria* sp. and several  
379 *Methanosarcina* sp.) were present in much higher concentration than acetoclastic  
380 methanogens (*Methanosarcina* sp. only), hydrogenotrophic CH<sub>4</sub> production from H<sub>2</sub>  
381 and CO<sub>2</sub> was likely more efficient than from acetate. Such microbial community  
382 structure is in accordance with an absence of acetate accumulation during the first 7  
383 days after CO<sub>2</sub> addition. After a time between 7 and 14 days necessary to reactivate  
384 acetotrophic pathways by *Methanosarcinales*, a subsequent decrease of acetate  
385 concentration was observed.

386 In comparison, the composition of the archaeal community was significantly different  
387 when CO<sub>2</sub> was added at day 18 (Table 2). In that case, the percentage of

388 *Methanosarcinales* amongst *Archaea* was not only initially higher but also increased  
389 from 12% to 17% at the end of the experiment. This result suggests that CH<sub>4</sub> production  
390 from acetate was most probably higher when CO<sub>2</sub> was added after 18 days than after 11  
391 days. As reported elsewhere, acetate metabolism in *Methanosarcinales* starts to be  
392 inhibited with only 2.5 mbars of H<sub>2</sub> (Ahring et al., 1991). Therefore, a higher  
393 composition in *Methanosarcinales* supports a higher persistence of the inhibitory effect  
394 of the initial high  $P_{H_2}$ . Nonetheless the final increase in *Methanosarcinales* relative  
395 abundance might indicate a recovery of efficient methanogenesis in these conditions.

396 Characterization of the bacterial community showed clear differences between control  
397 reactors and inhibited reactors (either 11 or 18 days). Although all other clusters of  
398 bacteria remained in similar proportion, the relative abundance of *Clostridiales*  
399 increased in inhibited reactors at a similar extent from 30-31% to 40-42% during the  
400 first 7 days after CO<sub>2</sub> addition. Meanwhile, the proportion of *Bacteroidales* decreased  
401 from 24-32% to 19-21% in inhibited reactors even though their relative abundance  
402 reached up to 47% after 18 days in the control. Since many members of *Clostridiales*  
403 are involved in hydrolytic and acidogenic activities in AD, a reactivation of the  
404 hydrolytic activity after H<sub>2</sub> inhibition seemed have to be carried out by members of the  
405 *Clostridiales* order. The imbalance between *Bacteroidales* and *Clostridiales* orders  
406 might have resulted from a differential sensitivity to inhibitor exposure. Consistently,  
407 Abbassi-Guendouz et al. (2013) reported a *Clostridium* sp. enrichment when dry AD of  
408 cardboard was inhibited by metabolite accumulation suggesting a higher resistance of  
409 these microorganisms to detrimental conditions of growth (low pH, high  $P_{H_2}$ ).

#### 410   **4   Conclusion**

411   In this study, inhibition of dry AD at high initial H<sub>2</sub> partial pressure was found to be  
412   reversible by adding CO<sub>2</sub> whatever the time of exposition to H<sub>2</sub>. The reversibility  
413   occurred in two steps, with a very probable first consumption of H<sub>2</sub> and CO<sub>2</sub> by  
414   hydrogenotrophic methanogens followed by acetoclastic methanogen. Methanogenic  
415   performances depended then on the time of exposure to high  $P_{H_2}$  with a persistent  
416   impact on AD kinetics. These results suggest that injecting CO<sub>2</sub> may represent a  
417   solution to improve solid-state AD at high TS content by avoiding local inhibition of  
418   H<sub>2</sub>.

419

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500



501   **6   Tables captions**

502   **Table 1 :** Methanogenic activity performances (cumulated CH<sub>4</sub> production, CH<sub>4</sub>  
503   production rate) for the different phases, in the control (with no H<sub>2</sub> initially added) and  
504   for reactors with initial H<sub>2</sub> in headspace at a partial pressure of  $996 \pm 27$  mbars and  
505   where CO<sub>2</sub> was added at day 11 and 18.

506   **Table 2 :** Phylum and class of *Archaea* and *Bacteria* presents in the control (without  
507   gas added) and when the CO<sub>2</sub> was added at 11 and 18 days in % (results of the  
508   sequencing) at 0, 11, 18, 25 and 32 days after the beginning of the experiment.

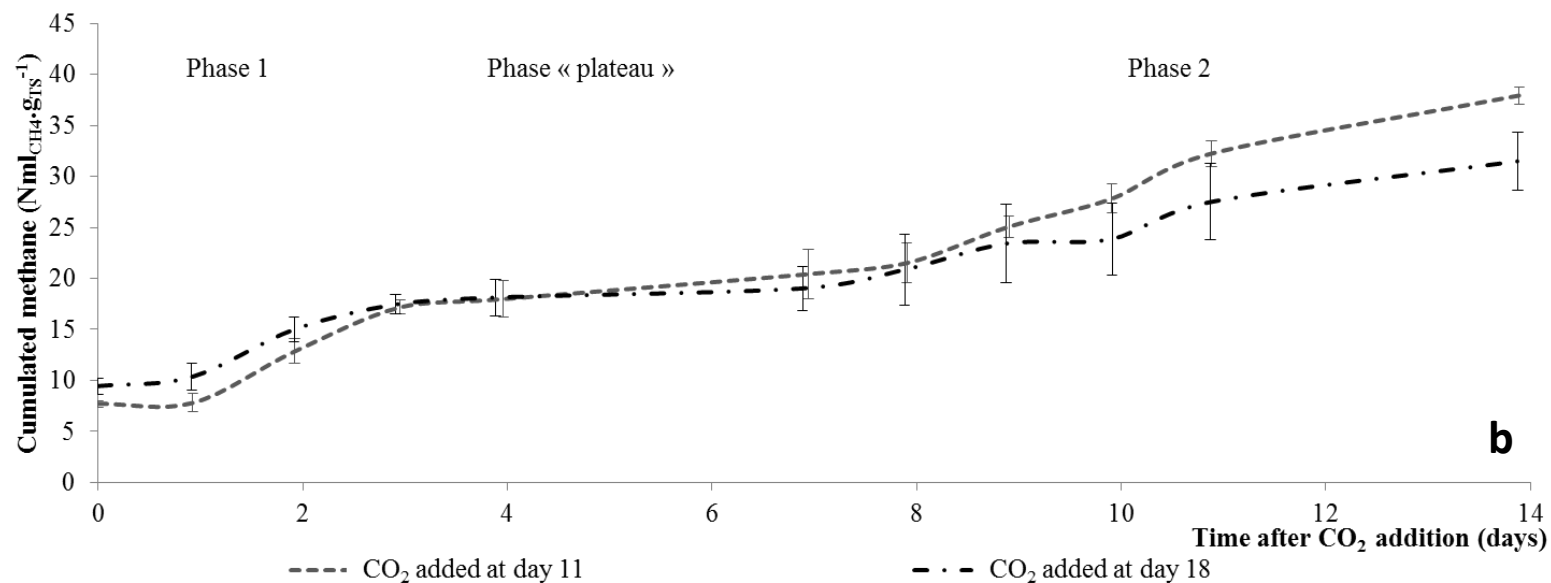
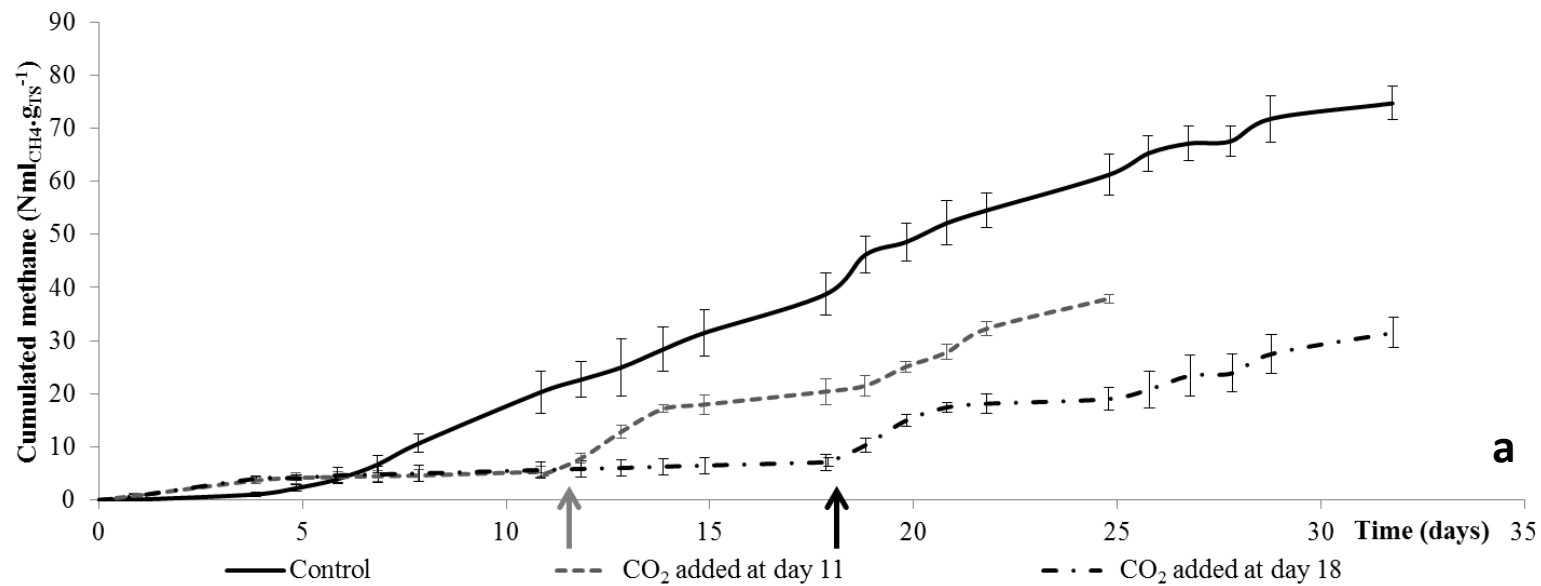
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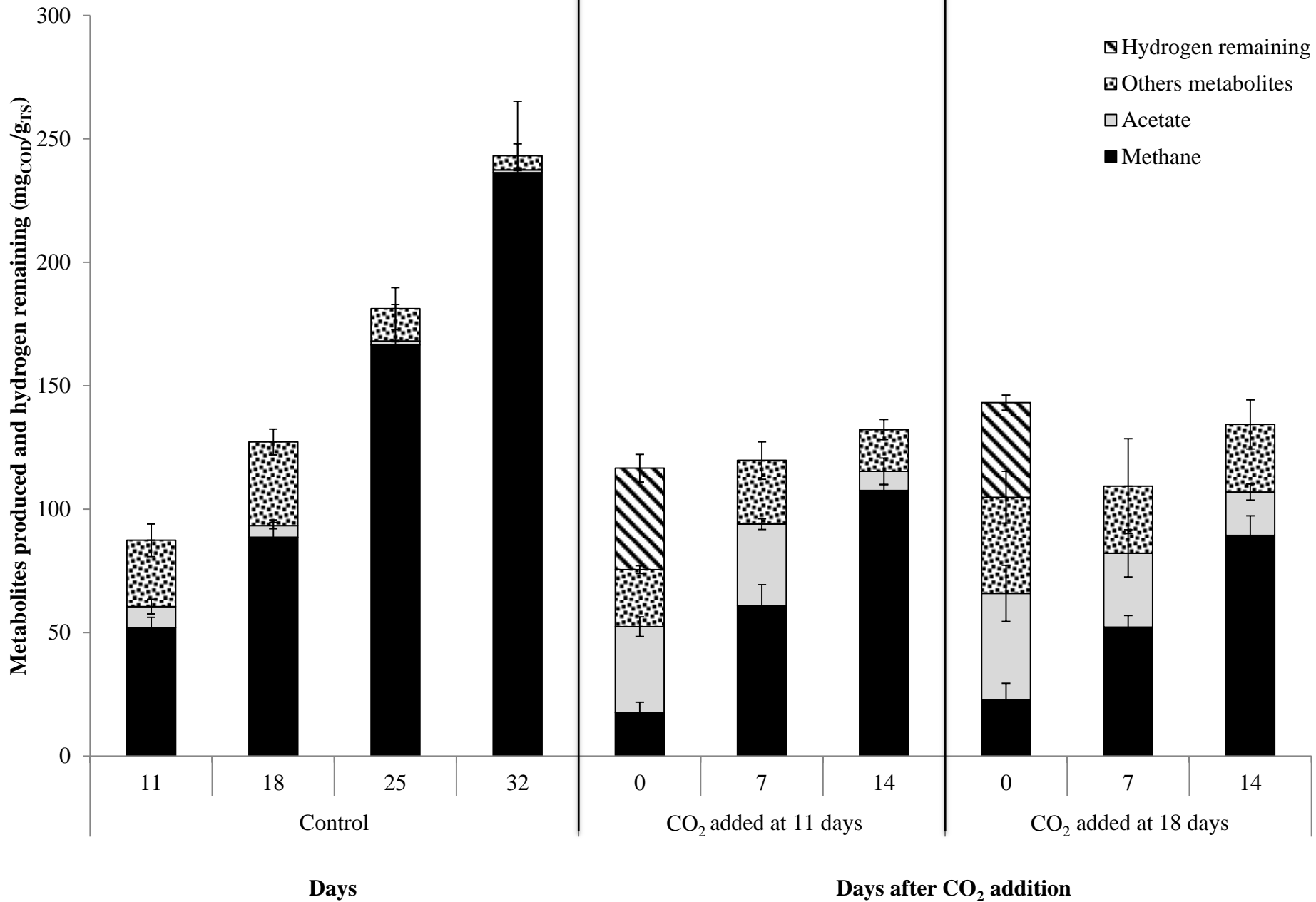
## 7 Figures captions

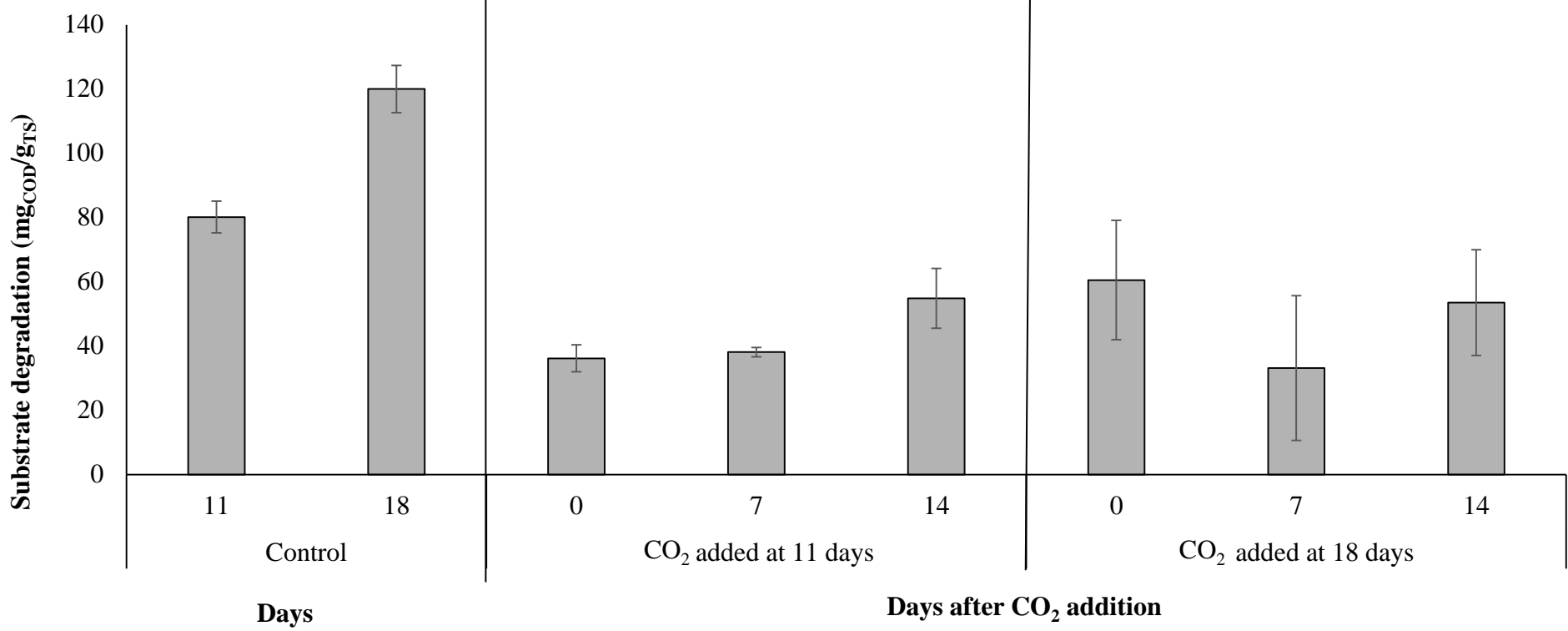
**Figure 1:** Cumulative methane production (in  $\text{mL}_{\text{CH}_4} \cdot \text{g}_{\text{TS}}^{-1}$ ), for reactors with  $\text{H}_2$  initially present in headspace and where  $\text{CO}_2$  was added after 11 and 18 days of operation; according to (A) the time of reactor operation or (B) the normalized time after  $\text{CO}_2$  addition . Tests were operated at pH 8, 25% TS and at  $35^\circ\text{C}$ . The grey and black arrows show the time when  $\text{CO}_2$  was added at 11 and 18 days, respectively.

**Figure 2:** Metabolites production (in  $\text{mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$ ) according to the time of operation in the control (only  $\text{N}_2$  initially in headspace), and according to the time after  $\text{CO}_2$  addition after 11 and 18 days of operation of the reactors running at high  $P_{\text{H}_2}$ . All tests were carried out at pH 8, 25% TS and  $35^\circ\text{C}$ .

**Figure 3:** Substrate degradation in  $\text{mg}_{\text{COD}} \cdot \text{g}_{\text{TS}}^{-1}$ , according to the time after adding  $\text{CO}_2$  ( $\text{CO}_2$  added at 11 and 18 days) and since the beginning (control)







		Phases	Time (days of operation)	Time since CO <sub>2</sub> addition (days)	Cumulated CH <sub>4</sub> produced per phase (Nml.g <sub>TS</sub> <sup>-1</sup> )	Average CH <sub>4</sub> production rate (Nml.g <sub>TS</sub> <sup>-1</sup> .day <sup>-1</sup> )
Control		Lag phase	0 to 5		2 ± 0.6	0.45 ± 0.13
		Exponential phase	5 to 32		72 ± 3	2.7 ± 0.32
CO <sub>2</sub> added at 11 days	Before CO <sub>2</sub> addition	Start phase	0 to 1		0.8 ± 0.4	0.96± 0.52
		Inhibition phase	1 to 11		4 ± 0.5	0.45 ± 0.14
	After CO <sub>2</sub> addition	CH <sub>4</sub> production phase 1	11 to 14	0 – 3	12 ± 1	4 ± 0.75
		Lag phase	14 to 18	3 – 7	4 ± 2.4	0.82 ± 0.27
		CH <sub>4</sub> production phase 2	18 to 25	7 - 14	16 ± 1	2.74 ± 0.45
CO <sub>2</sub> added at 18 days	Before CO <sub>2</sub> addition	Start phase	0 to 1		0.8 ± 3	0.96 ± 0.42
		Inhibition phase	1 to 18		6 ± 1	0.38 ± 0.1
	After CO <sub>2</sub> addition	CH <sub>4</sub> production phase 1	18 to 21	0 – 3	10 ± 2	3.55 ± 0.87
		Lag phase	21 to 25	3 – 7	3 ± 2.1	0.41± 0.27
		CH <sub>4</sub> production phase 2	25 to 32	7 – 14	11 ± 3	1.77 ± 0.23

Total operation time (days)		Control			CO <sub>2</sub> added at day 11			CO <sub>2</sub> added at day 18		
Time after CO <sub>2</sub> addition (days)		0	11	18	11	18	25	18	25	32
<i>Class</i>	<i>Order</i>									
<i>Archaea</i>										
<i>Methanobacteria</i>	<i>Methanobacteriales</i>	91.6%	81.7%	78.1%	90.8%	89.6%	89.8%	83.4%	83.0%	79.8%
<i>Methanomicrobia</i>		5.8%	15.6%	17.0%	6.2%	6.9%	7.8%	13.4%	13.0%	17.3%
	<i>Methanomicrobiales</i>	0%	1%	1%	0.2%	0.3%	0.2%	0%	1%	1%
	<i>Methanosarcinales</i>	6%	15%	16%	6%	7%	8%	13%	12%	17%
<i>Thermoplasmata</i>		0.5%	0.1%	0.4%	0.5%	0.7%	0.6%	0.3%	0.5%	0.5%
<i>Bacteria</i>										
<i>Clostridia</i>	<i>Clostridiales</i>	26.4%	29.5%	19.7%	31.0%	39.8%	46.8%	30.3%	41.9%	39.9%
<i>Bacteroidia</i>	<i>Bacteroidales</i>	29.2%	41.5%	46.9%	24.4%	20.5%	18.5%	31.9%	25.2%	20.9%
<i>Spirochaetes</i>	<i>Spirochaetales</i>	10.3%	15.0%	17.6%	10.5%	7.7%	15.5%	10.6%	6.1%	20.5%
<i>Synergistia</i>	<i>Synergistales</i>	5.6%	1.7%	2.1%	4.1%	3.9%	2.6%	5.3%	4.5%	3.0%
<i>Anaerolineae</i>	<i>Anaerolineales</i>	5.1%	1.3%	1.4%	7.7%	7.4%	3.3%	5.3%	5.2%	2.2%
<i>Deltaproteobacteria</i>	<i>Syntrophobacterales</i>	3.8%	1.4%	1.3%	2.4%	2.7%	1.2%	2.8%	4.3%	1.8%