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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_2 is also generated but its impact on the microbial activity remains unknown. In this study, the reversibility of dry AD inhibition by high H_2 partial pressure (P_{H2} of 1 bar) was investigated by adding CO_2 (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_2 was added, the methanogenic activity rapidly recovered within 3 days, from 0.41±0.1 to 3.77±0.8 and then 2.25±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

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duration of the inhibition period suggesting a different impact of the high P_{H2} on hydrogenotrophic and acetoclastic methanogens.

Keywords

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

1 Introduction

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

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

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

In particular, dissolved hydrogen accumulation rapidly makes the acetogenic reactions thermodynamically unfavourable causing higher VFAs accumulation, a subsequent decrease of the pH and finally methanogenesis inhibition (Guo et al., 2010). Under anaerobic conditions, H_2 is produced by acidogenic bacteria and is immediately consumed in combination with CO_2 by either homoacetogenic bacteria to produce acetate, or hydrogenotrophs to produce methane (Amani et al., 2010). This latter pathway represents about 30% of the CH_4 produced in anaerobic digestion (Amani et al., 2010). All these reactions are reversible in the AD process where acetate oxidation plays also a key role between methanogenic pathways (Karakashev et al., 2006). The local H_2 partial pressure can transitorily increase but must remain low enough to avoid inhibition of syntrophic acetogenic bacteria. When the hydrogen partial pressure (P_{H2}) is high, VFAs production increases, causing a decrease of the pH to lower value than 6 (Guo et al., 2010). Such variation in pH can impact biomass hydrolysis as well as the following steps of acidogenesis and methanogenesis (Siegert and Banks, 2005; Veeken et al., 2000). Indeed, the growth of methanogenic and acidogenic bacteria are strongly affected by the pH (Luo and Angelidaki, 2013).

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

However, the exact role of CO_2 on the bacterial activity in AD remains unclear. On the one hand, CO_2 has been reported as inhibitor of the production and degradation of VFAs, as previously shown by Hansson and Molin (1979) and Arslan et al. (2012) who worked on acetate and propionate accumulation at pH 7 and pH 4.5 and under 1 bar of CO_2, respectively. Consistently, it was elsewhere reported that an inhibitory impact of CO_2 on acetogenic and lactic acid bacteria at pH 5.3 (Kim et al., 2006) and on acetoclastic methanogens at pH 7 (Hansson and Molin, 1981). On the other hand, acidogenesis and more particularly H_2 production was shown to be improved by sparging CO_2 before fermentation (at 30 to 300 $ml_{CO2}.min^{-1}$) (Bru et al., 2012; Kim et al., 2006). Nonetheless, an inhibitory effect was observed when CO_2 was sparged at higher rate (500 $ml_{CO2}.min^{-1}$) (Bru et al., 2012). In contrast, Park et al. (2005) reported that fermentative H_2 production was improved by removing the CO_2. Since all experiments were carried out under different operating conditions and different

91 microbial communities, concluding on the exact impact of CO_2 on the different AD
92 microbial activities remains unclear.

93 The aim of this study was to evaluate the impact of adding CO_2 in mesophilic dry AD
94 when methanogenesis was artificially inhibited by high initial H_2 partial pressure in
95 headspace. Two inhibition durations (11 and 18 days) prior to CO_2 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 $NaHCO_3.g^{-1}$ of substrate) was used to keep
111 the pH at 8 all along the experiment (data not shown). A solution of trace elements
112 ($FeCl_2$ 2 $g \cdot L^{-1}$, $CoCl_2$ 0.5 $g \cdot L^{-1}$, $MnCl_2$ 0.1 $g \cdot L^{-1}$, $NiCl_2$ 0.1 $g \cdot L^{-1}$, $ZnCl_2$ 0.05 $g \cdot L^{-1}$,

H_3BO_3 0.05 g·L^{-1}, Na_2SeO_3 0.05 g·L^{-1}, $CuCl_2$ 0.04 g·L^{-1}, Na_2MoO_4 0.01 g·L^{-1}) was added (0.2 mL by flask) at start of the experiment, using the same protocol than Cazier et al. (2015). Initial TS content of the mixture (inoculum, wheat straw, sodium bicarbonate solution and trace elements) was fixed at 25% corresponding to the maximal TS value where no inhibitory effect was observed in dry AD (Abbassi-Guendouz et al., 2012; Motte et al., 2013)and to only investigate the reversibility of the inhibition only caused by high hydrogen partial pressure (Cazier et al. (2015)).

First, the mixture was introduced into a reactor with a working volume of 3 L operated during 10 days at 35°C under N_2 atmosphere to reach an active phase of methanogenesis and homogenize the substrate/inoculum medium. Then, 20 g of this pre-culture was put into the bottom of a 600 ml flask, which corresponded to a thin layer of less than 1 cm of substrate to limit the influence of the gas diffusion in the medium. The flasks were initially flushed with N_2 gas. Hydrogen was then added to reach an initial H_2 partial pressure of 996 ± 27 mbars, under a total pressure of 1 500 mbars. A control, with only N_2 in headspace, was also carried out. All the flasks were then incubated at 35°C for 32 days. In some of the flasks filled with hydrogen, 396 ± 44 mbars of CO_2 were added after 11 and 18 days of operation. Batch tests were carried out in triplicates for each condition. Flasks were sampled at day 0, 11, 18, 25 and 32 for further analysis of fermentative metabolite concentrations.

2.3 Analytical methods

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

137 CO_2 and the second column an RtMolsieve used for the detection of the O_2, H_2, N_2 and
138 CH_4. The carrier gas was argon at 350 kPa and under a flowrate of 31.8 $ml.min^{-1}$. 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 $mL.min^{-1}$ (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 $ml.min^{-1}$.

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). 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

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

163 *2.4 Data analysis*

164 R software (version 2.15.2) coupled with the package Rcmdr (version 1.8-4) was used
165 for statistical analysis of the experimental data, using variance analysis (ANOVA).
166 Non-significant p-values were fixed > 0.05 and significant p-values were fixed when <
167 0.05.

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

172 $$\text{TSD} = \text{Final State} - \text{Initial State} = \frac{A_{H_2,f}+A_{CH_4,f}+A_{met,f}+A_{GC}}{TSi} - \frac{A_{H_2,i}+A_{met,i}}{TSi} \quad \text{(Eq.1)}$$

173 where, $A_{H_2,f}$ is the amount of H_2 remaining at the end in the headspace, $A_{CH_4,f}$ the final
174 amount of accumulated CH_4, $A_{met,f}$ the final amount of metabolic products, A_{GC} the
175 total amount of gas (H_2 and CH_4) sampled for analyses, $A_{H_2,i}$ the initial amount of H_2
176 added and $A_{met,i}$ the initial amount of metabolites in the medium. Since all these
177 parameters are expressed in grams of COD, TSD corresponded to gram of COD per
178 gram of initial TS of wheat straw.

3 Results and discussion

3.1 Recovery of the methanogenic activity after CO_2 addition

Figure 1.a shows the cumulated production of CH_4 along reactor operation time. For the reactors carried out at high initial P_{H2} and, thus, operated under inhibitory conditions, CO_2 was added after 11 and 18 days of operation. The control corresponds to a reactor without initial addition of H_2 in headspace.

In the control reactor, a maximal and constant CH_4 production rate was observed after a lag phase of 5 days and reached a value of 2.7 ± 0.32 $ml_{CH4} \cdot g_{TS}^{-1} . day^{-1}$, equivalent to 2.98 $ml_{CH4} \cdot g_{VS}^{-1} \cdot day^{-1}$. This result is significantly lower than previous reported values of 12 $ml_{CH4} . g_{VS}^{-1} \cdot day^{-1}$ for wheat straw at 22% TS (Liew et al., 2012). Such difference resulted either from different microbial inoculum origins or from a TS content slightly higher in the present experiment (25% TS), considering that 28-30 % TS was previously reported as a threshold value prior inhibition of the methanogenic and acidogenic microbial activities (Abbassi-Guendouz et al. 2012; Motte et al. 2013).

In the reactors where H_2 was initially added, a small quantity of CH_4 accumulated the first day of experiment at low production rates of 0.96 ± 0.52 and 0.96 ± 0.42 $ml_{CH4} \cdot g_{TS}^{-1} \cdot day^{-1}$ (Table 1): this production rates correspond to the mean values of the triplicates used to evaluate the addition of CO_2 after 11 and 18 days of inhibition, respectively. Thereafter, CH_4 production was strongly inhibited due to the presence of high partial pressure of H_2 in the headspace, with average production rates of only 0.45 ± 0.1 and 0.38 ± 0.1 $ml_{CH4} \cdot g_{TS}^{-1} . day^{-1}$. In comparison, the control (only N_2) showed a methane production rate ten times higher at 2.7 ± 0.3 $ml_{CH4} \cdot g_{TS}^{-1} . day^{-1}$ for the same experimental time. Consequently, the amounts of cumulated methane after 11 and 18 days reached

only 4 ± 0.5 and 6 ± 1 $ml_{CH4} \cdot g_{TS}^{-1}$ in the inhibited reactors against 20 ± 4 and 39 ± 4 $ml_{CH4} \cdot g_{TS}^{-1}$ in the controls, respectively. It was concluded that methanogenesis was clearly inhibited in presence of high initial H_2 partial pressure in headspace (996 ± 27 mbars). The corresponding concentration of dissolved H_2 in the medium at 35°C was estimated at 0.58 ± 5 x 10^{-2} $mg_{H2} \cdot L^{-1}$. Consistently, a similar value was reported as a threshold H_2 concentration prior to wheat straw hydrolysis inhibition in AD by Cazier et al. (2015).

When CO_2 was added in reactor headspace, the methane rapidly accumulated within the first 3 days to reach values of 12 ± 1 and 10 ± 2 $ml_{CH4} \cdot g_{TS}^{-1}$ in the reactors where CO_2 was added at day 11 and 18, respectively. This first phase of CH_4 production was called "phase 1", as shown in Figure 1.b. During phase 1, H_2 and CO_2 were both rapidly consumed until total exhaustion of H_2 in headspace (data not shown). No significant acetate accumulation was observed during the first 7 days after CO_2 addition (Figure 2). It was therefore concluded that H_2 and CO_2 were most likely consumed by hydrogenotrophic methanogens to produce CH_4. Consistently, methane production rates in phase 1 were higher than in the controls, with 4 ± 0.75 $ml_{CH4} \cdot g_{TS}^{-1}.day^{-1}$ and 3.55 ± 0.87 $ml_{CH4} \cdot g_{TS}^{-1}.day^{-1}$ for CO_2 added at day 11 and 18, respectively, versus an average value of 2.7 ± 0.3 $ml_{CH4}.g_{TS}^{-1}.day^{-1}$ 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 CO_2 addition, suggesting that hydrogenotrophic methanogens were not inhibited at high P_{H2}, at least during the first 18 days. This is consistent with previous observations where the production of CH_4 by hydrogenotrophic methanogens

225 was previously shown to be favoured at high P_{H2} (> 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 ± 0.26 and 0.41 ±
230 0.27 $ml_{CH4} \cdot g_{TS}^{-1} \cdot day^{-1}$ 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 ± 1
236 $ml_{CH4} \cdot g_{TS}^{-1}$ and 11 ± 3 $ml_{CH4} \cdot g_{TS}^{-1}$ 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 ± 0.45 and 1.61 ± 0.23 $ml_{CH4} \cdot g_{TS}^{-1} . day^{-1}$ 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).

Interestingly, CH_4 production rates were slightly higher when CO_2 was added after 11 days of inhibition (4 ± 0.75 and 2.74 ± 0.45 $ml_{CH4} \cdot g_{TS}^{-1}.day^{-1}$ for phases 1 and 2, respectively) than 18 days (3.55 ± 0.87 and 1.77 ± 0.23 $ml_{CH4} \cdot g_{TS}^{-1}.day^{-1}$ for phases 1 and 2, respectively). Such a difference suggests a cumulative inhibitory effect of the time of exposure to H_2 on both hydrogenotrophic and acetoclastic methanogens.

3.2 Impact of the P_{H2} on other metabolic by-products dynamics

Figure 2 presents the accumulation of metabolic by-products (VFAs and methane), the remaining hydrogen in the controls and in the reactors carried out with high initial P_{H2}. In the controls, microbial metabolites, *i.e.* all VFAs, formate, succinate, and ethanol, transitorily accumulated after 11 and 18 days of operation (35 ± 2 and 39 ± 5 $mg_{COD}.g_{TS}^{-1}$ at day 11 and 18, respectively) likely because of the high TS content, and then decreased to 7 ± 2 $mg_{COD}.g_{TS}^{-1}$ at day 32, confirming the efficient methanogenic activity even at 25% TS.

In comparison, the amount of metabolites was higher in the reactors where H_2 was initially added. The concentrations of metabolites reached 58 ± 5 $mg_{COD}.g_{TS}^{-1}$ at day 11 and 82 ± 17 $mg_{COD}.g_{TS}^{-1}$ at day 18 prior to CO_2 addition (Figure 2). These values corresponded to a total concentration in metabolites of about 20 ± 2 $g \cdot L^{-1}$ at day 11, and 28 ± 6 $g \cdot L^{-1}$ at day 18, respectively. Such value is above the inhibitory limit of 20 $g \cdot L^{-1}$ as previously reported in wet AD processes (Siegert and Banks, 2005). Interestingly, most of the hydrogen consumed at day 11 and at day 18 (69 ± 2 $mg_{COD}.g_{TS}^{-1}$ and 72 ± 2 $mg_{COD}.g_{TS}^{-1}$) corresponds to the concentration of produced metabolites (51 ± 2 $mg_{COD}.g_{TS}^{-1}$ and 75 ± 17 $mg_{COD}.g_{TS}^{-1}$ at day 11 and 18 prior to CO_2 addition, respectively=. The small difference at day 11 between the hydrogen recoveries into metabolites (18 $mg_{COD}.g_{TS}^{-1}$) could correspond to the methane produced during this time

271 (17.5 ± 4 $mg_{COD}.g_{TS}^{-1}$). Such high concentration of metabolites might have been the
272 cause of the strong inhibition of methanogenic activity at high P_{H2}, prior to 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 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_{H2} was
277 mostly due to an increase of acetate, and, at a lower extent, butyrate and isobutyrate.
278 The acetate concentration increased from 2 ± 0 $mg_{COD}\cdot g_{TS}^{-1}$ to 35 ± 4 $mg_{COD}\cdot g_{TS}^{-1}$ at
279 day 11 and 43 ± 11 $mg_{COD}\cdot g_{TS}^{-1}$ at day 18, prior to CO_2 addition. Meanwhile, the
280 butyrate and isobutyrate concentration increased from 1.7 ± 0 $mg_{COD}\cdot g_{TS}^{-1}$ at start to 9 ±
281 0.2 $mg_{COD}\cdot g_{TS}^{-1}$ and 18 ± 4 $mg_{COD}\cdot g_{TS}^{-1}$ at day 11 and 18, respectively. Such increase of
282 acetate and butyrate concentrations under an atmosphere rich in 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_{H2} of 2 bars in comparison to only N_2.

286 When CO_2 was added after 11 days of inhibition, no metabolite degradation was
287 observed during the first 7 days after 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 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_2 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 ±
296 2.4 $mg_{COD}.g_{TS}^{-1}$ to 8 ± 6 $mg_{COD}\cdot g_{TS}^{-1}$ between day 7 and 14, respectively. Such
297 difference between the acetate concentration at day 0 and 14 after CO_2 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_4 by acetoclastic methanogens
300 (Pavlostathis and Giraldo-Gomez, 1991) or to the oxidation of acetate by acetate-
301 oxidizing bacteria into H_2 and CO_2 that are then converted to CH_4 by hydrogenotrophic
302 methanogens (Karakashev et al., 2006). A similar trend was observed when CO_2 was
303 added after 18 days with an acetate content that decreased from 30 ± 10 $mg_{COD}\cdot g_{TS}^{-1}$ at
304 day 7 to 18 ± 3 $mg_{COD}\cdot g_{TS}^{-1}$ at day 14 (Figure 2). Interestingly, acetate degradation from
305 day 7 to 14 (phase 2) was slower when CO_2 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_2 partial pressure. In other studies, a specific inhibitory effect was observed on the
308 growth of *Methanosarcina* sp. when H_2 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_4 producers in phase 1 since no significant
312 difference between the two times of exposure.

313 *3.3 Impact of the P_{H2} on the overall substrate degradation*

314 To estimate the impact of the P_{H2} on the global microbial activity, the overall substrate
315 degradation was calculated in $mg_{COD}\cdot g_{TS}^{-1}$ using Equation 1, which takes in
316 consideration the amount of H_2 initially added (Figure 3).

In the reactors where CO_2 was added at day 11 or day 18, the substrate degradation was very similar 14 days after CO_2 addition, with 55 ± 9 $mg_{COD} \cdot g_{TS}^{-1}$ and 54 ± 17 $mg_{COD} \cdot g_{TS}^{-1}$, respectively. Therefore, the impact of the time exposure on the methane production rate in phase 2, was probably not due to a persistent effect on the global microbial activity since the overall substrate degradation was the same after 14 days, but more likely to a transitory accumulation of metabolites due to a slower methanogenic activity, as shown in Figure 2. Nevertheless, when comparing these values to the control, the global substrate degradation was lower in the reactors operated at high initial P_{H2} for a close duration of operation. About 80 ± 5 $mg_{COD} \cdot g_{TS}^{-1}$ were reached at day 11 in the control that is substantially higher than in inhibited reactors. All these observations suggest that the high initial P_{H2} had very likely a persistent inhibitory effect on the hydrolytic activity of the consortium.

The exact mechanisms behind microbial hydrolysis are still uncertain and probably highly diverse when considering complex substrates. Two main mechanisms have been proposed in the AD model (ADM1): (1) the enzymes are directly secreted into the liquid phase by hydrolytic microorganisms with a direct effect on substrate hydrolysis that releases free sugars on the bulk phase or (2) the microorganisms attach on the substrate surface with the formation of a biofilm and produce enzymatic complexes to disrupt the organic material (Batstone et al., 2002). Recently, Cazier et al. (2015) reported an initial a strong inhibitory effect of high P_{H2} on the hydrolytic activity in dry AD. High P_{H2} could have either influenced the production or secretion of extracellular enzymes by retro-inhibition, or reduced the physiological activity of the 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_{H2} is high enough, hydrolysis may therefore be inhibited, especially if the local P_{H2} 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_{H2} 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_{H2} 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_{H2} before CO_2 addition, and 7 days (end of 'plateau' phase) and 14 days (end of phase 2) after CO_2 addition (Table 2).

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

In the reactor where CO_2 was added at day 11, the overall composition of the archaeal community did not significantly change. Indeed, the microbial community was composed of 90-91% *Methanobacteriales* and only 6-8% *Methanosarcinales* all along the experiment. Since hydrogenotrophic methanogens (*Methanobacteria* sp. and several *Methanosarcina* sp.) were present in much higher concentration that acetoclastic methanogens (*Methanosarcina* sp. only), hydrogenotrophic CH_4 production from H_2 and CO_2 was likely more efficient than from acetate. Such microbial community structure is in accordance with an absence of acetate accumulation during the first 7 days after CO_2 addition. After a time between 7 and 14 days necessary to reactivate acetotrophic pathways by *Methanosarcinales*, a subsequent decrease of acetate concentration was observed.

In comparison, the composition of the archaeal community was significantly different when CO_2 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_4 production
390 from acetate was most probably higher when CO_2 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_2 (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_{H2}. 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_2 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_2 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_{H2}).

4 Conclusion

In this study, inhibition of dry AD at high initial H_2 partial pressure was found to be reversible by adding CO_2 whatever the time of exposition to H_2. The reversibility occurred in two steps, with a very probable first consumption of H_2 and CO_2 by hydrogenotrophic methanogens followed by acetoclastic methanogen. Methanogenic performances depended then on the time of exposure to high P_{H2} with a persistent impact on AD kinetics. These results suggest that injecting CO_2 may represent a solution to improve solid-state AD at high TS content by avoiding local inhibition of H_2.

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6 Tables captions

Table 1 : Methanogenic activity performances (cumulated CH_4 production, CH_4 production rate) for the different phases, in the control (with no H_2 initially added) and for reactors with initial H_2 in headspace at a partial pressure of 996 ± 27 mbars and where CO_2 was added at day 11 and 18.

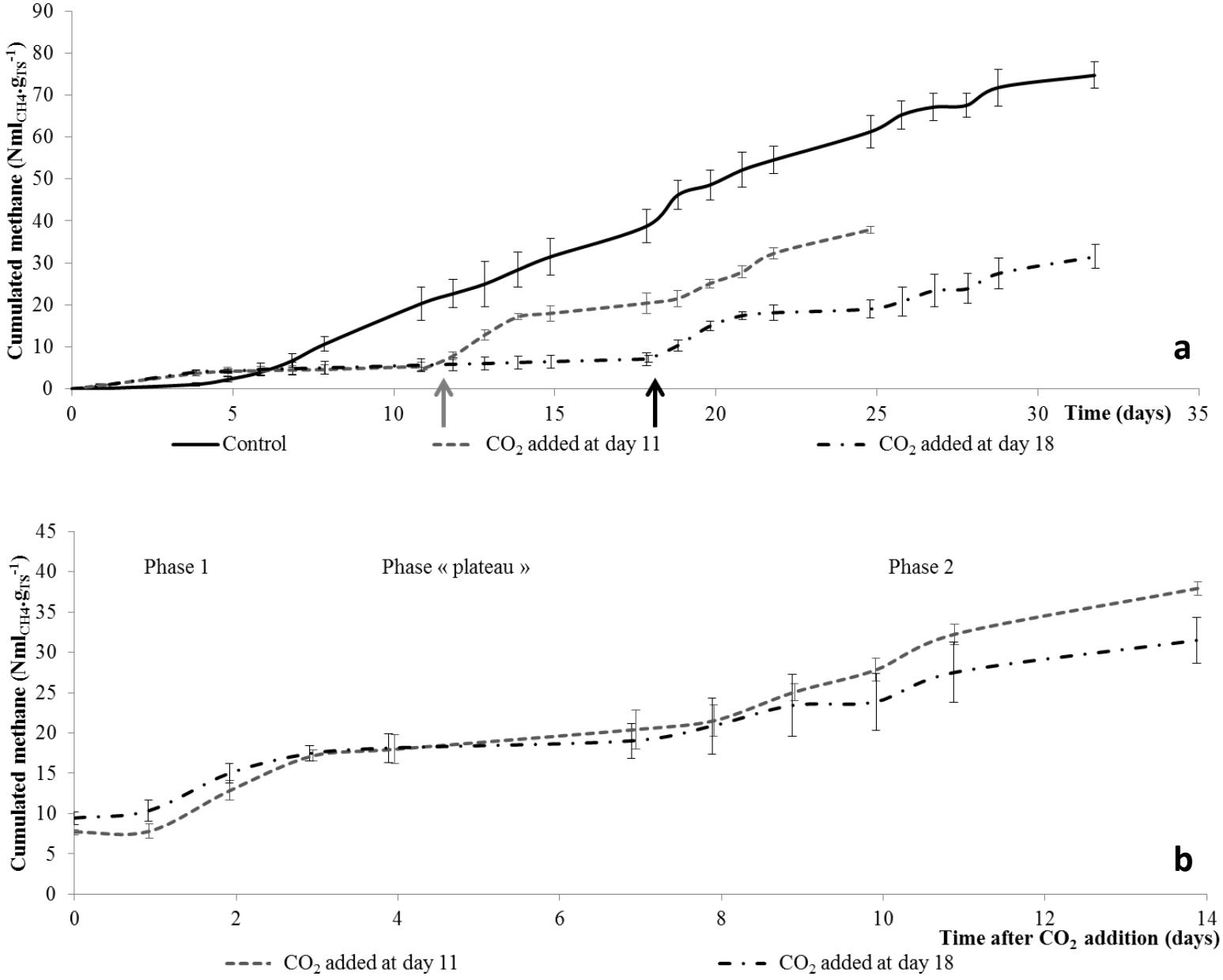
Table 2 : Phylum and class of *Archaea* and *Bacteria* presents in the control (without gas added) and when the CO_2 was added at 11 and 18 days in % (results of the sequencing) at 0, 11, 18, 25 and 32 days after the beginning of the experiment.

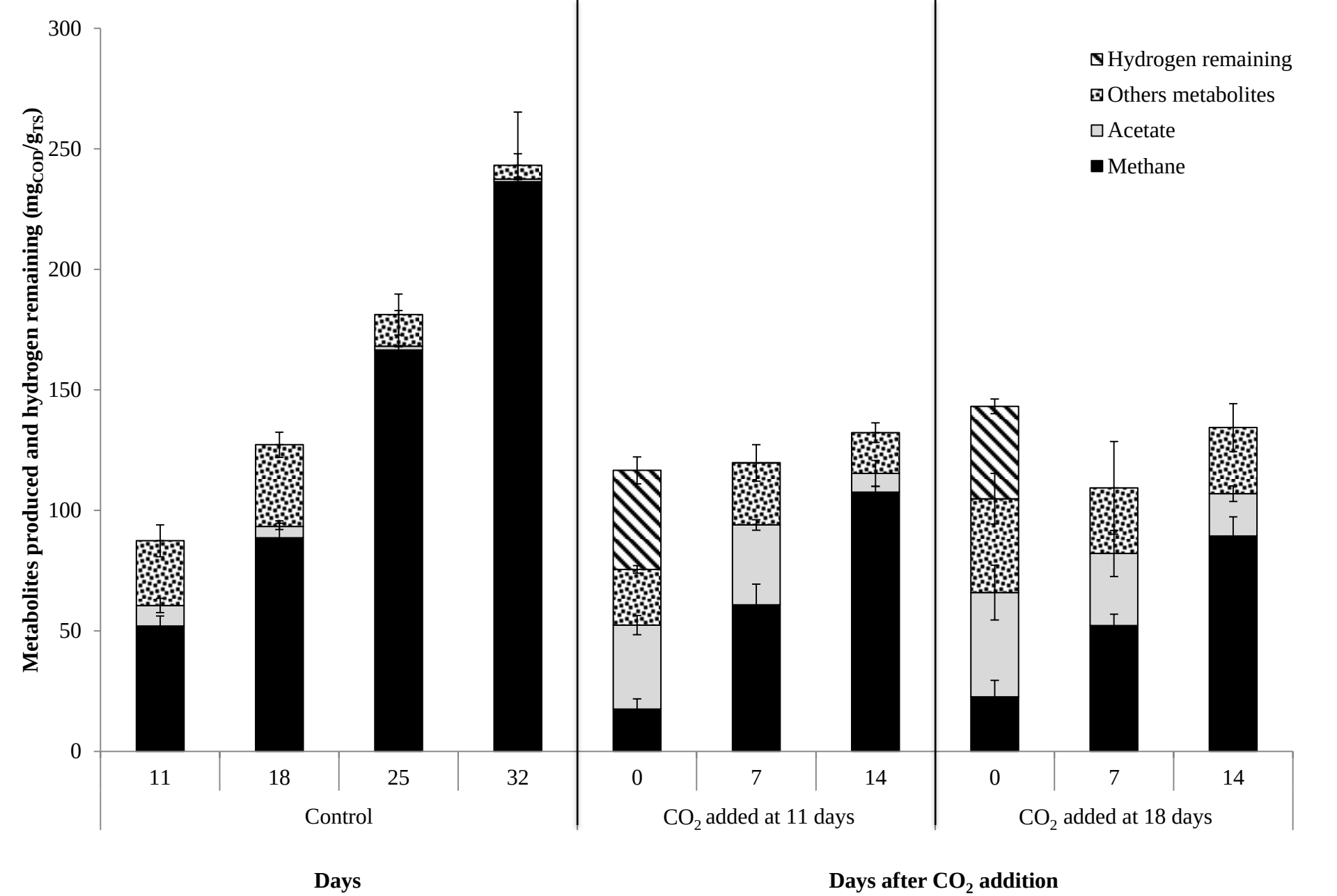
7 Figures captions

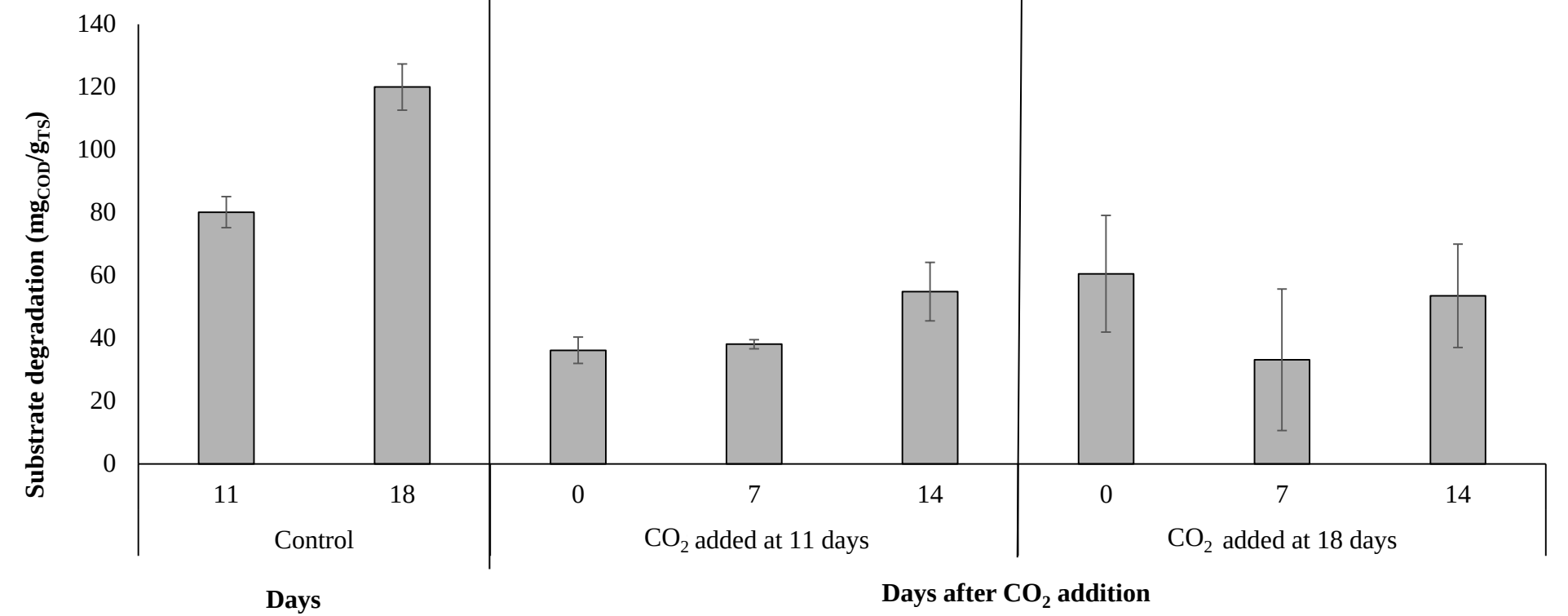
Figure 1: Cumulative methane production (in $mL_{CH4}.g_{TS}^{-1}$), for reactors with H_2 initially present in headspace and where CO_2 was added after 11 and 18 days of operation; according to (A) the time of reactor operation or (B) the normalized time after CO_2 addition . Tests were operated at pH 8, 25% TS and at 35°C.The grey and black arrows show the time when CO_2 was added at 11 and 18 days, respectively.

Figure 2: Metabolites production (in $mg_{COD}.g_{TS}^{-1}$) according to the time of operation in the control (only N_2 initially in headspace), and according to the time after CO_2 addition after 11 and 18 days of operation of the reactors running at high P_{H2}. All tests were carried out at pH 8, 25% TS and 35°C.

Figure 3: Substrate degradation in $mg_{COD}.g_{TS}^{-1}$, according to the time after adding CO_2 (CO_2 added at 11 and 18 days) and since the beginning (control)


Cumulated methane (Nml$_{CH4}$.g$_{TS}^{-1}$)
Time (days)
Control
CO$_2$ added at day 11
CO$_2$ added at day 18
a
Phase 1
Phase « plateau »
Phase 2
Time after CO$_2$ addition (days)
b


Hydrogen remaining
Others metabolites
Acetate
Methane
Metabolites produced and hydrogen remaining (mg$_{COD}$/g$_{TS}$)
300
250
200
150
100
50
0
11
18
25
32
0
7
14
0
7
14
Control
CO$_2$ added at 11 days
CO$_2$ added at 18 days
Days
Days after CO$_2$ addition


Substrate degradation (mg$_{COD}$/g$_{TS}$)
140
120
100
80
60
40
20
0
11
18
0
7
14
0
7
14
Control
CO$_2$ added at 11 days
CO$_2$ added at 18 days
Days
Days after CO$_2$ addition

		Phases	Time (days of operation)	Time since CO_2 addition (days)	Cumulated CH_4 produced per phase ($Nml.g_{TS}^{-1}$)	Average CH_4 production rate ($Nml.g_{TS}^{-1}.day^{-1}$)
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_2 added at 11 days	**Before CO_2 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_2 addition	CH_4 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_4 production phase 2	18 to 25	7 - 14	16 ± 1	2.74 ± 0.45
CO_2 added at 18 days	**Before CO_2 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_2 addition	CH_4 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_4 production phase 2	25 to 32	7 – 14	11 ± 3	1.77 ± 0.23

		Control			CO_2 added at day 11			CO_2 added at day 18		
Total operation time (days)		0	11	18	11	18	25	18	25	32
Time after CO_2 addition (days)		--	--	--	0	7	14	0	7	14
Class	***Order***									
				Archaea						
Methanobacteria	*Methanobacteriales*	91.6%	81.7%	78.1%	90.8%	89.6%	89.8%	83.4%	83.0%	79.8%
Methanomicrobia		5.8%	15.6%	17.0%	6.2%	6.9%	7.8%	13.4%	13.0%	17.3%
	Methanomicrobiales	*0%*	*1%*	*1%*	*0.2%*	*0.3%*	*0.2%*	*0%*	*1%*	*1%*
	Methanosarcinales	*6%*	*15%*	*16%*	*6%*	*7%*	*8%*	*13%*	*12%*	*17%*
Thermoplasmata		0.5%	0.1%	0.4%	0.5%	0.7%	0.6%	0.3%	0.5%	0.5%
				Bacteria						
Clostridia	*Clostridiales*	26.4%	29.5%	19.7%	31.0%	39.8%	46.8%	30.3%	41.9%	39.9%
Bacteroidia	*Bacteroidales*	29.2%	41.5%	46.9%	24.4%	20.5%	18.5%	31.9%	25.2%	20.9%
Spirochaetes	*Spirochaetales*	10.3%	15.0%	17.6%	10.5%	7.7%	15.5%	10.6%	6.1%	20.5%
Synergistia	*Synergistales*	5.6%	1.7%	2.1%	4.1%	3.9%	2.6%	5.3%	4.5%	3.0%
Anaerolineae	*Anaerolineales*	5.1%	1.3%	1.4%	7.7%	7.4%	3.3%	5.3%	5.2%	2.2%
Deltaproteobacteria	*Syntrophobacterales*	3.8%	1.4%	1.3%	2.4%	2.7%	1.2%	2.8%	4.3%	1.8%