Microbial community redundance in biomethanation systems lead to faster recovery of methane production rates after starvation

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

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ABSTRACT

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

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

KEYWORDS

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

HIGHLIGHTS

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

1. INTRODUCTION

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

catalysers for producing CH_4 from CO_2 and H_2. Using microorganisms allows to proceed at milder conditions (temperature between 35°C and 65°C, atmospheric pressures) than catalytic processes, with a smaller carbon footprint besides the potential of transforming CO_2 into CH_4 or other interesting biomolecules (Fu et al., 2021). Biomethanation can be applied in two configurations: (i) the *in-situ* biomethanation configuration, which consists in the direct injection of H_2 into an anaerobic digester treating organic substrate resources, to convert the CO_2 from the biogas into CH_4, resulting in an upgrade of the CH_4 content and (ii) the *ex-situ* biomethanation configuration, where biogas and H_2 are concomitantly injected into a dedicated reactor, separated from the anaerobic digester, in which the biogas upgrade takes place (Lecker et al., 2017). Besides, biogas, external CO_2 provided by other sources can be injected into the *in-situ* or *ex-situ* reactors, such as syngas or from other industrial gas streams (Lecker et al., 2017; Rittmann et al., 2015; Tao et al., 2020). In both process, the production of CH_4 is carried out by hydrogenotrophic methanogens, which have capacity to directly reduce CO_2 into CH_4 using H_2 as electron donor (Zabranska and Pokorna, 2018). These microorganisms are ubiquitous in anaerobic digesters.

Anaerobic digestion (AD) is a microbial process, which consists of successive microbial steps to degrade organic substrates into a biogas mainly composed of CH_4 and CO_2 (Aryal et al., 2018). First, hydrolytic microorganisms depolymerise the complex organic matter, as polysaccharides or proteins, into their smaller units which are further fermented into volatile fatty acids (VFA), alcohols, H_2 and CO_2 by acidogenic bacteria. These molecules are then used as substrates by acetogenic bacteria and methanogenic archaea which syntrophically degrade them into CH_4 and CO_2 (Merlin Christy et al., 2014). A stable methane production is the result of a well-balanced process between the different microbial populations and the products (or substrates) generated in each step of the AD pathway. The direct H_2 injection into the AD reactor during *in-situ* biomethanation can cause a disturbance of the microbial community as acetogens are extremely sensitive to dissolved H_2 (Schink, 1997). Excess H_2 may lead to VFA accumulation and pH drop, which can negatively affect the methanogenic activity and eventually cause a process failure (Cuff et al., 2020). Nonetheless, because of

their high diversity, the microbial AD ecosystems have the ability to handle disturbances (Carballa et al., 2015). Anaerobic digesters are often exposed to different types of disturbances such as starvation or overloads (Hwang et al., 2010; Regueiro et al., 2015), which deeply affect the microbial community. During the recovery period, the reactors are in general less performant or present unstable CH_4 production rates (de Jonge et al., 2017; Hwang et al., 2010). For instance, Wang et al. (2019) have observed a recovery period of 10 days after 59-days of starvation period, while de Jonge et al. (2017) have reported an instability of the AD process during a 45-day recovery period after a starvation of 55 days. Hwang et al. (2010) have observed that the total methane production after a 4-month starvation period was 1.7-fold lower than before starvation. De Jonge et al. (2017), Hwang et al. (2010) and Wang et al. (2019) have reported alterations in the microbial community composition and abundance, mostly affecting the syntrophic bacteria and methanogens interactions. Therefore, the observed recovery periods during conventional AD could be related to the fact that methane production in AD depends on the coordination of several microbial groups (de Jonge et al., 2017; Hwang et al., 2010). Meanwhile, during *ex-situ* and *in-situ* biomethanation, the methane production mostly depends on one microbial group, the hydrogenotrophic methanogens, whose direct substrate is added at no limiting conditions (Savvas et al., 2018).

Hydrogenotrophic methanogens have been reported to dominate the archaeal microbial community during *in-situ* and *ex-situ* biomethanation processes when achieving high-CH_4-content biogas. For instance, Wang et al. (2013) have reported the dominance of *Methanoculleus* sp. during *in-situ* biomethanation, when producing a biogas with a methane content of 98.8%, while Logroño et al. (2021) have found *Methanobacterium* sp. to be dominant during *ex-situ* biomethanation, when achieving 97.56 % CH_4 content in their biogas. Besides, these authors have observed that the persistence of *Methanobacterium* sp. was crucial for recovering the efficiency of the process after a 14-day starvation period (Logroño et al., 2021). On the other hand, H_2 can be also consumed by homoacetogens to produce acetate, which will be detrimental for CH_4 production. Although, the association

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

Therefore, the aim of this work was to evaluate and characterize the performance recovery and response of the microbial community of *in-situ* biomethanation reactors and *ex-situ* biomethanation reactors after being exposed to a short (1 week) and a long (4 weeks) starvation period.

2. MATERIALS and METHODS

2.1. Operational conditions

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

2.2. Operational strategy

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

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

Figure 1 – Operational diagram

2.3. Analytical methods

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

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

2.4. Calculations

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

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

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

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

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

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

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

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

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

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

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

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

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

2.5. Microbial community analysis

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

2.5.1. Sequencing of Bacterial and Archaeal Communities

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

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

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

2.6. Statistical analysis

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

3. RESULTS and DISCUSSION

3.1. Reactor performances before and after starvation

3.1.1. Methane production

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

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

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

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

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

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

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

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

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

3.1.2. Substrate consumption and metabolite production

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

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

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

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

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

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

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

3.2. Microbial community analysis

3.2.1. Bacterial community composition

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

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

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

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

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

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

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

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

3.2.2. Archaeal community composition

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

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

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

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

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

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

3.3. Reactors performances vs. microbial community composition

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

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

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

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

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

4. CONCLUSION

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

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

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