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Improved organic matter biodegradation through pulsed H_2 injections during *in situ* biomethanation

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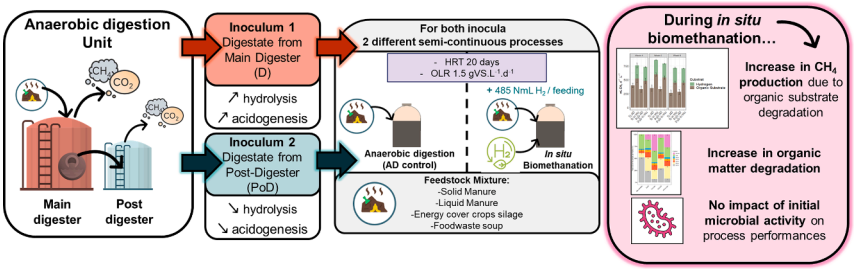
HIGHLIGHTS

- Improvement of the organic substrate biodegradation was associated to H_2 injections.
- Non-dominant hydrogenotrophic methanogens became predominant with H_2 injections.
- CH_4 production was similar regardless of initial microbial activity levels.
- CH_4 production from organic matter increased of a least 626 NmL with H_2 injections.

GRAPHICAL ABSTRACT



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ABSTRACT

During *in situ* biomethanation, microbial communities can convert complex Organic Matter (OM) and H_2 into CH_4. OM biodegradation was compared between Anaerobic Digestion (AD) and *in situ* biomethanation, in semi-continuous processes, using two inocula from the digester (D) and the post-digester (PoD) of an AD plant. The impact of H_2 on OM degradation was assessed using a fractionation method. Operational parameters included 20 days of hydraulic retention time and 1.5 $g_{VS}.L^{-1}.d^{-1}$ of organic loading rate. During *in situ* biomethanation, 485 NmL of H_2 were injected for each feeding (3 times a week). Maximum organic COD removal was 0.6 gCOD in AD control and at least 1.6 gCOD for *in situ* biomethanation. Therefore, COD removal was 2.5 times higher with H_2 injections. These results bring out the potential of H_2 injections during AD, not only for CO_2 consumption but also for better OM degradation.

1. Introduction

The biomethane production industry, relying on the treatment of organic waste by Anaerobic Digestion (AD), plays a pivotal role in the global effort to decarbonize the energetic mix (REPowerEU, 2022). This current interest is evident, with biomethane production expected to expand worldwide from 3.5 to over 11 billion cubic meters between 2018 and 2022. Several project developments are driving this industry, mostly located in Europe and North America (currently representing altogether more than 90 % of the global biomethane production), with a significant anticipated contribution expected from Brazil and India between 2022 and 2026 (IEA, 2023).

Subsequently, the deployment and optimization of AD are key factors in unlocking the potential of organic matter as an energy source. AD

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is considered a mature industrial process. Conventional optimization strategies are based on both process management itself and applied treatment to substrates. In addition, the potential for using H_2 in *ex situ* biomethanation has been explored, allowing the purification of biogas through the activity of hydrogenotrophic archaea, either in pure or mixed culture, within a dedicated reactor (Angelidaki et al., 2018). However, as hydrogenotrophic archaea are also present in the microbial community of the AD process, an alternative approach involves direct injection of H_2 into an AD process, leading to what is called *in situ* biomethanation (Rafrafi et al., 2021). For instance, the previous work of Luo et al. (2012) demonstrated that H_2 supplementation in AD reactors could enhance CH_4 production because H_2 and CO_2 are utilized by the microbial community to produce CH_4. Current H_2 production is mostly based on H_2 steam reforming, derived from the oil industry (Dincer and Acar, 2015). However, several technologies are considered to make H_2 production sustainable, such as water electrolysis coupled with intermittent renewable electricity production (Power-to-Gas processes) (Thema et al., 2019) and biological processes for H_2 production (Dincer and Acar, 2015).

H_2 is a well-known intermediate molecule for CH_4 production during the methanogenesis step of AD (Zhu et al., 2020). The introduction of exogenous H_2 during such a process was shown to negatively impact methanogenesis. Indeed, the accumulation of Volatile Fatty Acids (VFAs) due to both homoacetogenic activity stimulation and acetoclastic methanogenesis inhibition in the presence of high H_2 partial pressure has already been reported in the literature (Agneessens et al., 2018; Luo and Angelidaki, 2013). Conversely, no consensus is currently reached in the scientific community regarding the impact of exogenous H_2 addition on the organic matter degradation steps of AD (*e.g.*, hydrolysis, acidogenesis, acetogenesis). Indeed, it was previously demonstrated by Cazier et al., (2019) that H_2, at a partial pressure of 1 bar, could inhibit hydrolysis. However, Bassani et al., (2015) and Treu et al., (2018) both suggested that the development of syntrophic interactions between bacteria and archaea was promoted by H_2 injection during AD. These two studies suggested that the organic matter degradation process and, therefore, bacterial activities could be positively impacted by H_2 addition during *in situ* biomethanation. The development of this positive effect during *in situ* biomethanation was not exposed in the literature. In addition, the possible impact of H_2 on bacterial activity could be variable, depending on the initial activity of the community. Indeed, several authors reported that the microbial composition of one inocula was impacting its adaptation capacity to H_2 injections. (Agneessens et al., 2018; Braga Nan et al., 2020).

For that purpose, it appeared to be necessary to evaluate the impact of H_2 on communities with close composition but high and low levels of hydrolytic activity. As a result, two inocula from the main and post-digester of the same AD unit were selected for this experiment. Post-digesters are conceived to receive digestate from the main digester, to collect the remaining methane from that digestate, before its storage in dedicated tanks (Boe et al., 2009). Therefore, in conventional AD units, main digesters are expected to exhibit high organic matter degradation activity, as they received complex organic matter as feeding (*e.g.,* waste from agricultural and food industries) while post-digesters are expected to expose less active microbial consortium. Consequently, this study aimed to evaluate the effect of H_2 on hydrolytic, acidogenic, and acetogenic activities, but also the response of microbial communities to complex organic feedstock input under high H_2 concentrations in the gas phase.

2. Materials and methods

2.1. Inocula and feedstock preparations

Two inocula corresponding to the main and post-digester of the same AD units were used at a concentration of 15 $g_{VS}.L^{-1}$ during that experiment. They were sampled on the AD unit less than 5 days before that experiment. These inocula were selected due to their rapid response toward H_2 injection, which was observed in previous work (Mahieux et al., 2024). Both were sieved through a 20-mesh sieve to remove a part of their residual organic matter and decrease their endogenous methane production.

The organic feeding consisted of a mixture of 2 % liquid pig manure, 29 % freeze-dried solid cow manure, 28 % rye silage (which was mortar ground), and 42 % food waste soup, all expressed in terms of Volatile Solid (VS). The food waste soup was prepared following a protocol already described elsewhere (Noguer et al., 2022). All feedstocks were used less than 7 days after their collection. The food waste soup was prepared the day of the mix preparation. The mixture was prepared and then diluted with distilled water to obtain a 30 $g_{VS}.L^{-1}$ feeding solution and divided into aliquots before being stored at −20 °C.

The Total Solids (TS) and Volatile Solids (VS) contents of sieved inocula and feedstocks were measured as described in APHA standard methods (APHA, 2017). The correction of the TS and VS content of rye silage was performed following the method described in the work of Van Vlierberghe et al. (2022) and using the Porter and Murray equation. The biodegradable Chemical Oxygen Demand (COD) of the substrate was 0.024 $gCOD.g^{-1}$. This COD content was evaluated using near-infrared spectroscopy, as already described elsewhere (Zennaro et al., 2022).

2.2. Operational setup

The culture medium was composed of 20 mL of phosphate buffer (0.5 M, pH 7.12 adjusted using NaOH), 2 mL of an oligo-element solution, inoculum at a concentration of 15 $g_{VS}.L^{-1}$, 0.7 g_{VS} of feedstock mixture. The medium was filled to 200 mL with distilled water. The composition of buffer and oligo-element solutions was similar to those used by Braga Nan et al. (2020).

For the experiment, several modified 1 L SchottFlask® with a working volume of 200 mL were used. These bottles were flushed for 15 min with nitrogen at the beginning of the experiment to ensure anaerobic conditions. Feeding was operated for 48 days in semi-continuous conditions by setting a Hydraulic Retention Time (HRT) of 20 days and an organic loading rate of 1.5 $g_{VS}.L^{-1}.d^{-1}$ for the feedstock mix feeding.

Two conditions were assessed in this study: the Anaerobic Digestion condition (AD control condition), which solely received feedstock mixture, and the *in situ* biomethanation condition (*in situ* condition) (see supplementary material). In the latter, after one start-up week under similar feeding conditions as AD control, H_2 was additionally introduced rapidly from 1 to 1.45 bar. This gas injection corresponded to an addition of 485 NmL of H_2 after each organic feeding. Both gas and organic feeding were operated three times per week. Organic feeding was conducted by adding 23.3 g of feedstock mix (equivalent to 0.7 g_{VS}) and removing the same amount of culture media. The AD and *in situ* conditions were evaluated in triplicate and quadruplicate, respectively. Quadruplicates were chosen for the *in situ* condition to ensure a robust evaluation of this condition, as H_2 injection could trigger more variability for CH_4 and VFA production. Both conditions were incubated on a mechanical shaking table to ensure continuous stirring in a mesophilic chamber at 35 °C during the entire experiment.

2.3. Analytical methods

Physical-chemical analyses were performed on the Bio2E platform (Bio2E, 2018). Gas composition was analyzed three times per week before and after feeding by gas chromatography using GC Perkin Elmer model Clarus 580 equipped with a Thermal Conductivity Detector (TCD detector) as previously described by Mahieux et al. (2024). Before gas analysis, SchottFlask® inner gas pressure was measured using a manometer Keller LEO 2 (KELLER AG, Winterthur, Switzerland).

Once a week, a liquid medium was sampled to determine the concentrations of VFA and ammonium. For these analyses, liquid samples

underwent centrifugation for 15 min at 12,100 g, and the resulting supernatant was filtered using a 0.22 μm nylon filter (Fisherbrand®). Both the centrifugation pellet and the filtered supernatant were stored at −20 °C before their analysis. VFA concentration measurements were performed by gas chromatography using a Clarus 580 gas chromatograph (GC, Perkin Elmer), as reported by Noguer et al. (2022). The gas carrier was N_2 at 6 mL.min^{-1}. The GC was equipped with an Alltech-FFAP EC™1000 column coupled to a Flame Ionization Detector (FID) working at 280 °C. An Elite-FFAP cross-bond® carbowax® capillary column (15 m length, 0.53 mm diameter) was installed on the GC. Ammonium concentration was measured by automated spectrophotometric titration using Gallery™ instrument (Thermofischer Scientific) according to the manufacturer's instructions.

2.4. Fractionation method of organic matter

The fractionation method was conducted following the procedures outlined in Jimenez et al. (2015). The feedstock mixture used to supply the reactors and the digestate at the end of the experiment for the four conditions were analyzed. Initially, samples were centrifuged for 20 min at 18,600 g. The retrieved supernatant was assigned as the Dissolved Organic Matter fraction (DOM). Following centrifugation, TS and VS contents of sample pellets were determined before and after freeze-drying and grinding (1 mm). Then, 1 g of the resulting sample pellet was first subjected to automated sequential extraction using Dionex™ ASE™ 350. Different fractions of the sample were extracted sequentially using a 10 mM $CaCl_2$ solution, a 10 mM NaOH solution, and a 100 mM NaOH solution to determine the extractable Soluble from Particulate Organic Matter (SPOM), the Readily Extractable Organic Matter (REOM), and the Slowly Extractable Organic Matter (SEOM), respectively. Finally, a 72 % (v/v) H_2SO_4 solution was used as an extractant to manually determine the Poorly Extractable Organic Matter (PEOM). The remaining organic matter corresponded to the Non-Extractable Organic Matter (NEOM).

Chemical Oxygen Demand (COD) was measured using 2 mL of each fraction solution using Aqualytic® Vario COD kits (0–1500 mg O_2.L^{-1}) based on ultraviolet (UV) absorbance, which was read on a MultiDirect spectrophotometer from Aqualytic®. Total COD (mg O_2.g$_{TS}^{-1}$) was estimated in duplicate. For that purpose, 0.25 g of freeze-dried and ground (1 mm) raw samples were dissolved in 10 mL of a 99 % (v/v) H_2SO_4 solution for 24 h. Those solutions were diluted in demineralized water before COD measurement. Fractions of COD (mgO_2.L^{-1}) were assessed with 2 mL of each extracted solution. The difference in total COD observed between the raw substrate and all assessed conditions after two HRTs corresponded to the Degraded Organic Matter (DEGOM). This fraction allowed the evaluation of the portion of organic matter efficiently degraded through AD in all conditions.

2.5. Microbial community analysis

2.5.1. Sequencing strategy

Reactors were sampled for microbial analysis on the inocula (Week 0), at the beginning of H_2 injection in the *in situ* condition (Week 1), after one HRT (Week 3), and after two HRT (Week 6) during the semi-continuous process for both conditions.

The DNA extraction and quality control, as well as the two different PCRs that were conducted to analyze separately bacteria and archaea that were present in samples, were operated on following the previous work of Braga Nan et al. (2020). The sequencing reaction was performed with an Illumina sequencer (2 × 300 pb paired-end run) at the GenoToul Platform (Toulouse, France). Sequencing results were analyzed with bioinformatic tools. Mothur version 1.48.0 was used for sequence cleaning, assembly and control. Sequence matching and taxonomic assignment were performed on SILVA version 132. The Operational Taxonomic Units (OTUs) were created considering a 3 % dissimilarity level for both archaeal communities and bacterial communities. Results were registered in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) under the BioProject accession number PRJNA956344, with SRA accession numbers SAMN34423353 to SAMN34423370 for bacteria and SAMN34423253 to SAMN34423270 for archaea.

2.5.2. Microbial quantification

Bacteria and Archaea quantification was assessed using quantitative Polymerase Chain Reaction (qPCR). A BioRad CFX96 Real-Time Systems C1000 Touch Thermal Cycler (Bio-Rad Laboratories, USA) was used to perform qPCR programs. Primers used for bacterial and archaeal quantification, as well as the qPCR protocols, were previously described by Mahieux et al. (2024).

2.5.3. Data treatment for visualization of microbial communities

Graphical visualizations were obtained using R software version 4.1.3. Phyloseq, microbiome, and Fantaxtic R packages were used to obtain visual representations of the microbial communities (McMurdie and Holmes, 2013; Teunisse, 2022).

2.6. Estimation of the methane production rate associated with organic matter degradation

All gas volumes were calculated based on normalized data (NmL), considering atmospheric pressure and a temperature of 0 °C. The mean total CH_4 production rate (CH_4 total, expressed in NmL CH_4.L^{-1}.d^{-1}) was calculated for each week of the experiment, using Eq (1), where CH_4(i) represents the total CH_4 production in the assessed condition at the time i (ti) of the experiment.

$$CH_4total = [CH_4(i+1) - CH_4(i)]/[t_{i+1} - t_i] \quad (1)$$

Since no VFA accumulation was observed during the experiment, it was assumed that all the injected hydrogen was converted into CH_4. Subsequently, the mean CH_4 production rate associated with H_2 consumption (CH_4 biomethanation, expressed in NmL CH_4.L^{-1}.d^{-1}) was calculated as described in Eq. (2), where H_2(i) represents the total H_2 consumption observed at t_i, taking into account the stoichiometric reaction between CO_2 and H_2 to form CH_4.

$$CH_4biomethanation = [H_2(i+1)/4 - H_2(i)/4]/[t_{i+1} - t_i] \quad (2)$$

Finally, the CH_4 production associated with organic matter degradation (CH_4 biodegradation expressed in NmL CH_4.L^{-1}.d^{-1}) in the *in situ* reactors was determined by retrieving from the total CH_4 production the portion of the CH_4 produced through biomethanation at each time of the experiment, as shown in Eq (3).

$$CH_4biodegradation(i) = CH_4total(i) - CH_4biomethanation(i) \quad (3)$$

A COD mass balance was performed for each week of the experiment and allowed to confirm the robustness of the obtained results (see supplementary materials).

3. Results and discussions

3.1. Assessment of process overall performances for both inocula

To assess the effect of adding H_2 on the degradation of organic matter, experiments were conducted in semi-continuous feeding mode in *in situ* biomethanation conditions and in AD control conditions (without H_2 injections). Furthermore, to characterize the impact of H_2 on bacterial activity, two different inocula were used: one inoculum, expected to exhibit high bacterial activity, was obtained from the main digester (referred to as D), and the other, expected to expose lower bacterial activity, was sampled from the post-digester (referred to as PoD) of the same AD unit. The performances of *in situ* biomethanation and AD reactors were evaluated through the production rates of CH_4 and

CO_2, as well as the consumption rates of H_2. These rates were averaged over each week of the experiment, considering that reactor feeding occurred three times per week. Moreover, three experimental steps were considered: week 0, without H_2 injection, so-called the start-up of the experiment; the first HRT (weeks 1 to 3) as an adaptation phase for *in situ* biomethanation; and the second HRT (weeks 4 to 6) as a steady state.

The ratio between the injected H_2 and the produced CO_2 is considered an important parameter for the stability of *in situ* biomethanation processes (Angelidaki et al., 2018). For the *in situ* biomethanation reactors using inoculum D, the average apparent H_2:CO_2 ratio observed after each H_2 injection was 3.1 ± 0.6:1 H_2:CO_2 mol:mol while the real H_2:CO_2 ratio, considering the total H_2 consumed and the total CO_2 production, was 2.7 ± 0.1:1 H_2:CO_2 mol:mol. Similar ratios were observed in the *in situ* reactor using the inoculum sampled from the post-digester (inoculum PoD). In comparison with the literature, this ratio was lower than the stoichiometric 4:1 H_2:CO_2 molar ratio of the methanation reaction, which was considered optimal for *in situ* biomethanation (Wahid et al., 2019). These low ratios were chosen to avoid CO_2 depletion during *in situ* biomethanation. This setup limited the strong impact of H_2 injection on pH, which could have led to VFA accumulation and process inhibition. Indeed, acetate accumulation is more likely to occur during *in situ* biomethanation in response to CO_2 depletion in a medium, as previously shown by Agneessens et al. (2018) work. Specifically, these authors showed that CO_2 depletion from the gas phase, and the resulting increase in pH could lead to methanogenesis inhibition and homoacetogenesis stimulation, leading to an increase of acetate concentrations in the media.

The total CH_4 production performances were described, (Fig. 1) while separating the part of the CH_4 coming from H_2 or OM consumption. The inocula obtained from the main digester (inoculum D) and the post-digester (inoculum PoD) of the same AD unit exhibited different initial behaviors. For the Week 0, during which neither the AD control nor *in situ* condition received H_2, inoculum D showed a high CH_4 production rate, while for the inoculum PoD, the production was very limited (606 ± 41 *vs* 137 ± 4 NmL $CH_4.L^{-1}.d^{-1}$ for inocula D and PoD, respectively). A part of the observed production could be associated with the remaining organic matter degradation using inoculum D, as reactors using that inoculum displayed a CH_4 production rate almost five times higher than those using the inoculum PoD. In addition, the CH_4 production rate for inoculum D was higher than the theoretical CH_4 production rate that could have been obtained with the fed substrate (413 NmL $CH_4.L^{-1}.d^{-1}$ expected from the substrate, considering its COD content), reinforcing the association of the additional CH_4 production with endogenous organic matter conversion. These results also suggest higher initial microbial activity in inoculum D than in inoculum PoD, considering their respective initial CH_4 production performances during week 0. In addition, this first week of organic matter feeding (without H_2 addition in the *in situ* reactor) was associated with large VFA production regarding inoculum PoD, reaching 2.5 ± 0.2 $g.L^{-1}$ of acetate and 0.6 ± 0.3 $g.L^{-1}$ of propionate in all reactors inoculated with PoD at the beginning of week 1. This rapid accumulation of VFA suggests that the organic matter feeding induced an important environmental stress on the microbial community of the inoculum at the beginning of the experiment. Conversely, no significant VFA production was observed for reactors corresponding to inoculum D at the beginning of week 1.

Considering first the reactors inoculated with PoD, an improvement in the total CH_4 production rate was observed during the first part of the experiment under AD control conditions (from 116 ± 14 during Week 0 to 442 ± 109 NmL $CH_4.L^{-1}.d^{-1}$ during Week 2) as well as in the *in situ* condition (from 137 ± 4 during Week 0 to 658 ± 49 NmL $CH_4.L^{-1}.d^{-1}$ during Week 2). These results seemed to confirm an enhancement in the microbial consortia activity associated with that inoculum, both with and without H_2 addition. Conversely, using inoculum D, a stable total CH_4 production rate was observed in both AD control and *in situ* conditions from Week 1 to Week 3, which corresponds to the first HRT with H_2 addition in the *in situ* reactor (721 ± 13 and 379 ± 26 NmL $CH_4.L^{-1}.d^{-1}$ in average from Week 1 to 3, for the *in situ* condition and the AD control, respectively). The rapid stabilization of biomethanation was attributed to the low H_2:CO_2 ratio and the use of easily degradable substrates, such as food waste and rye silage (42 % and 28 % of the VS concentration in the feeding solution, respectively).

During the second HRT phase of the experiment, from Week 4 to Week 6, the CH_4 production rate remained stable in both AD control and *in situ* conditions (Fig. 1). This stability allowed considering this period as the steady state of the semi-continuous process. Limited VFA production was observed (Fig. 2) for that period. During this period, the CH_4 and VFA production patterns of inoculum D and PoD were almost identical. This similarity indicates that, after an initial gain of activity for inoculum PoD during the first HRT, both inocula exhibited comparable performance in AD and *in situ* biomethanation. Similar performances in the AD control were expected for these two inocula, as they were sampled on the same AD plant (close initial similar microbial communities) and fed similarly. Such similarity was not expected in the *in situ* performances of both inocula. Considering that H_2 injection was previously reported as triggering VFA production for non-adapted inocula (Agneessens et al., 2017), it could have been expected that H_2 injection had negatively impacted CH_4 production performance of both inocula and especially of inoculum PoD, which initially exhibited lower efficiency in CH_4 production compared to inoculum D.

Regarding the end of the process, propionic acid production, reaching a final concentration of 1 $g.L^{-1}$, was observed in all conditions, Fig. 2. This accumulation of VFA coincided with a decrease in

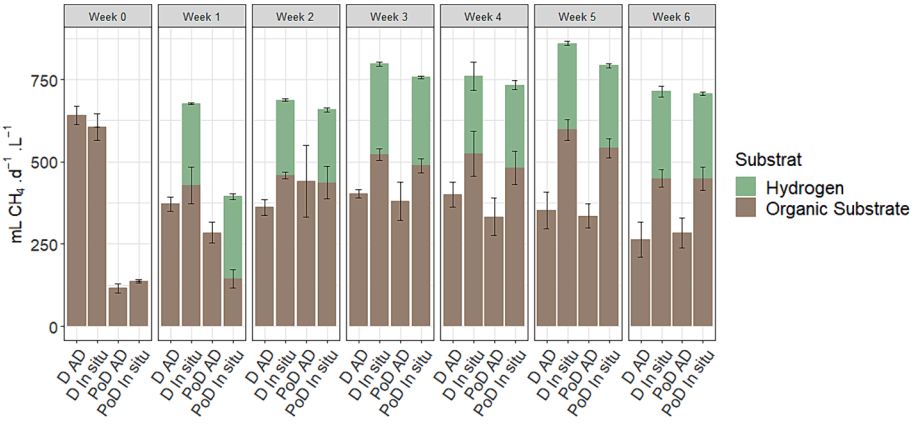

Fig. 1. Total methane production rate during the experiment (NmL $CH_4.d^{-1}$). All conditions were assessed in triplicates. D: AD Digestate of Digester as inoculum in anaerobic digestion process; D *in situ*: Digestate of Digester as inoculum in *in situ* biomethanation process; PoD AD: Digestate of Post-digester as inoculum in anaerobic digestion process; PoD *in situ*: Digestate of Post-digester as inoculum in *in situ* biomethanation process.

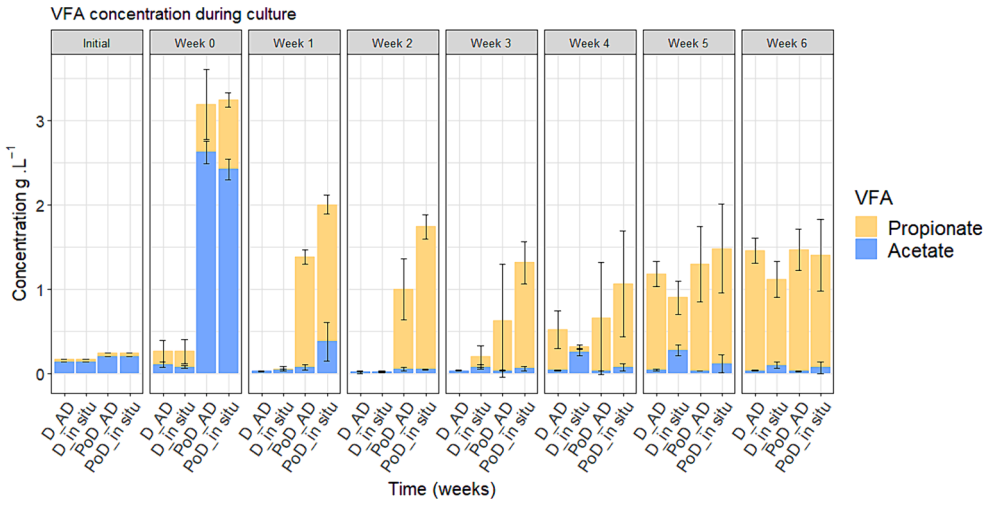

Fig. 2. VFA concentrations during the experiment (g.L^{-1}). All conditions were assessed in triplicates. D AD: Digestate of Digester as inoculum in anaerobic digestion process; D *in situ*: Digestate of Digester as inoculum in *in situ* biomethanation process; PoD AD: Digestate of Post-digester as inoculum in anaerobic digestion process; PoD *in situ*: Digestate of Post-digester as inoculum in *in situ* biomethanation process.

ammoniacal nitrogen concentration and a decline in pH in all conditions (Table 1). Initially, the inocula exhibited high N-NH_4^+ concentrations (i.e., 928 ± 19 and 1,270 ± 36 mg N–NH_4^+.L^{-1} for inoculum D and PoD, respectively). Subsequently, these inocula were considered adapted to relatively high free ammonia concentrations. Therefore, the decrease in N–NH_4^+ concentration could have altered the media buffering capacity, resulting in a higher impact of VFA concentration on pH in this experiment (Capson-Tojo et al., 2020).

During the present experiment, no residual H_2 was detected in the gas phase, supporting that all added H_2 during one feeding time was consumed before the next feeding. Moreover, for both inocula, the VFA production observed after the beginning of H_2 injections in the *in situ* conditions was similar to the one observed in AD controls (Fig. 2). Therefore, the VFA production was not associated with H_2 injections, and it was considered that all the consumed H_2 was converted into CH_4. As a result, the remaining CH_4 production was associated with organic matter conversion.

Table 1
Monitoring of pH and N-NH_3^+ (mg.L^{-1}). All conditions were assessed in triplicates. D AD: Digestate of Digester as inoculum in anaerobic digestion process; D *in situ*: Digestate of Digester as inoculum in *in situ* biomethanation process; PoD AD: Digestate of Post-digester as inoculum in anaerobic digestion process; PoD *in situ*: Digestate of Post-digester as inoculum in *in situ* biomethanation process.

	D AD		D *in situ*		PoD AD		PoD *in situ*	
	pH	N-NH_4^+ (mg. L^{-1})	pH	N-NH_4^+ (mg. L^{-1})	pH	N-NH_4^+ (mg. L^{-1})	pH	N-NH_4^+ (mg. L^{-1})
T0	7.8 ± 0.1	928 ± 19	7.8 ± 0.0	928 ± 19	7.8 ± 0.1	1270 ± 36	7.8 ± 0.1	1270 ± 36
Week 0	7.5 ± 0.3	1078 ± 114	7.5 ± 0.3	1011 ± 18	7.6 ± 0.3	1185 ± 84	7.5 ± 0.3	1113 ± 126
Week 1	7.3 ± 0.2	516 ± 16	7.4 ± 0.1	518 ± 45	7.1 ± 0.2	574 ± 26	7.2 ± 0.1	580 ± 20
Week 2	7.1 ± 0.2	572 ± 9	7.4 ± 0.1	568 ± 18	7.0 ± 0.1	653 ± 40	7.3 ± 0.2	652 ± 41
Week 3	7.0 ± 0.1	379 ± 7	7.4 ± 0.1	347 ± 23	7.0 ± 0.1	477 ± 19	7.5 ± 0.2	428 ± 28
Week 4	6.9 ± 0.1	341 ± 16	7.4 ± 0.1	321 ± 14	6.9 ± 0.1	354 ± 26	7.3 ± 0.2	367 ± 27
Week 5	6.8 ± 0.1	314 ± 17	7.2 ± 0.2	254 ± 17	6.7 ± 0.2	325 ± 13	7.1 ± 0.2	293 ± 30
Week 6	6.6 ± 0.1	264 ± 11	7.0 ± 0.1	213 ± 20	6.5 ± 0.2	286 ± 7	6.9 ± 0.1	225 ± 53

3.2. *Influence of hydrogen on the organic matter conversion in methane*

As detailed in Section 3.6, a mass balance was performed, taking into account that CH_4 was produced from both the injected H_2 (referred to as *CH_4 biomethanation*) and the degradation of organic matter (referred to as *CH4 biodegradation*). Both *CH_4 biomethanation* and *CH_4 biodegradation* were depicted in Fig. 1 for all conditions.

During the first weeks of operation, the results suggested that the inocula was adapting to the conditions of *in situ* biomethanation and anaerobic digestion. For the PoD inoculum during Week 1, the CH_4 production rate associated with organic matter degradation was higher in the AD condition compared to the *in situ* condition (285 ± 32 *vs* 144 ± 28 NmL CH_4.L^{-1}.d^{-1} for AD control and *in situ* conditions, respectively). However, this difference rapidly disappeared, as observed during Week 3 (442 ± 109 *vs* 437 ± 50 NmL CH_4.L^{-1}.d^{-1}, respectively, for AD control and *in situ* conditions, using the inoculum PoD). The capacity of both conditions to achieve similar methane production performances suggests that pulsed H_2 addition did not have a significant negative impact on organic matter degradation activity during this transitional phase. A similar pattern was also noted for reactors utilizing inoculum D. These results highlighted that, irrespective of the initial biodegradation activity of the consortia, pulsed H_2 injection did not inhibit organic matter degradation and even enabled a gain in biodegradation activity with the PoD inoculum. H_2 is a known inhibitor of various metabolic reaction chains that occur during the acidogenesis step (Bundhoo and Mohee, 2016) and has also been identified as an inhibitor of hydrolysis in specific conditions (Cazier et al., 2019) under high H_2 partial pressure (>0.5 bar). However, the present experiment showed that microbial consortia initially not adapted to H_2 injection could perform these activities without a significant negative impact of H_2. These results are consistent with the VFA consumption of the PoD inoculum in Fig. 2, where the VFAs produced during Week 0 (acclimation week, without H_2 addition) were rapidly consumed in the subsequent weeks, even with pulsed injections of H_2.

Remarkably, the degradation of organic matter was enhanced under *in situ* conditions when the inocula were acclimated to the operational conditions. For instance, at the beginning of the second HRT (Week 3),

with H_2 injection for the *in situ* condition, it was observed that CH_4 resulting from organic matter degradation was higher than in the AD control, regardless of the inocula used (403 ± 13 *vs* 522 ± 19 NmL CH_4.L^{-1}.d^{-1} in AD control *vs in situ* condition, considering inoculum D and 380 ± 57 *vs* 489 ± 22 NmL CH_4.L^{-1}.d^{-1} in AD control *vs in situ* condition, considering inoculum PoD). This improvement persisted until the end of the second HRT (average production from Week 3 to 6 of 349 ± 75 *vs* 523 ± 65 NmL CH_4.L^{-1}.d^{-1} in AD control *vs in situ* condition, considering inoculum D, and 333 ± 64 *vs* 490 ± 47 NmL CH_4.L^{-1}.d^{-1} in AD control *vs in situ* condition, considering inoculum PoD), suggesting that H_2, instead of inhibiting the first steps of AD, positively impacted the organic matter degradation during *in situ* biomethanation.

The improvement of CH_4 production rate associated with organic matter degradation could be linked to the increase in pH due to H_2 addition, as indicated in Table 1 (7.0 ± 0.1 *vs* 7.4 ± 0.1 in AD control *vs in situ* condition for inoculum D, and 7.0 ± 0.1 *vs* 7.5 ± 0.2 in AD control *vs in situ* condition for inoculum PoD during Week 3, which corresponds to the end of HRT 1). Indeed, the used substrate contained food waste and energy cover crops, potentially explaining the decrease in pH in AD control reactors during the experiment (from 7.0 ± 0.1 to 6.6 ± 0.1 and from 7.0 ± 0.1 to 6.5 ± 0.2, from Week 3 to Week 6, for AD control reactors using inoculum D and PoD during the second HRT, respectively). In contrast, the pH remained approximately 0.5 points higher in *in situ* conditions all throughout the experiments. As VFA concentrations were similar in all conditions, the pH difference between the AD and *in situ* reactors was associated with the difference in CO_2 content in the biogas for these two conditions (45 % *vs* 85 % of CO_2 retrieved in the gas phase on average for both inocula in the AD control *vs in situ* conditions, respectively, at the end of the experiment; see supplementary materials). Indeed, due to the injection of H_2, additional consumption of dissolved CO_2 occurred through the methanation reaction. As a result, the equilibrium between dissolved HCO_3^- and aqueous CO_2 led to the consumption of H^+ proton, promoting an increase in pH in the *in situ* reactors compared to the AD control. As a result, higher pH conditions could have increased microbial biodegradation activity, leading to an improvement in associated CH_4 production performances (Díaz et al., 2020; Luo and Angelidaki, 2013).

3.3. Enhancement of organic matter degradation and accessibility by hydrogen injection

Methane production rates clearly showed an improvement in the degradation of organic matter during the *in situ* biomethanation process, regardless of the inoculum used. To reinforce this observation and further explore the biodegradation of the organic matter in the *in situ* and AD control reactors, the fractionation method proposed by Jimenez et al. (2015) was employed. This method was selected as it had previously been used to efficiently characterize AD hydrolysis (Jimenez et al., 2020) and simulate AD digestate bioaccessibility (Fernández-Domínguez et al., 2021) through the six fractions characterized by different accessibility of the Organic Matter (OM) (Dissolved OM (DOM), Soluble Particular OM (SPOM), Readily Extractible OM (REOM), Slowly Extractible OM (SEOM), Poorly Extractible OM (PEOM), Non Extractible OM (NEOM)), ordered from the most to the less accessible fraction. The difference in the total COD observed between the raw substrate and all assessed conditions after two HRT corresponded to the Degraded Organic Matter (DEGOM), as detailed in Section 3.4. This fraction allowed the evaluation of the portion of organic matter efficiently degraded through AD in all conditions. Fig. 3 presents the results of the fractionation analyses for the initial substrate and the digestate at the end of the experiments for all conditions (AD control and *in situ* biomethanation reactors inoculated with D and PoD).

Considering the substrate, the fractionation analysis revealed that 48 % of it was accessible, considering DOM, SPOM, SEOM, REOM, and PEOM as accessible fractions. This composition is consistent with the substrate used in this study, which was composed of solid and liquid

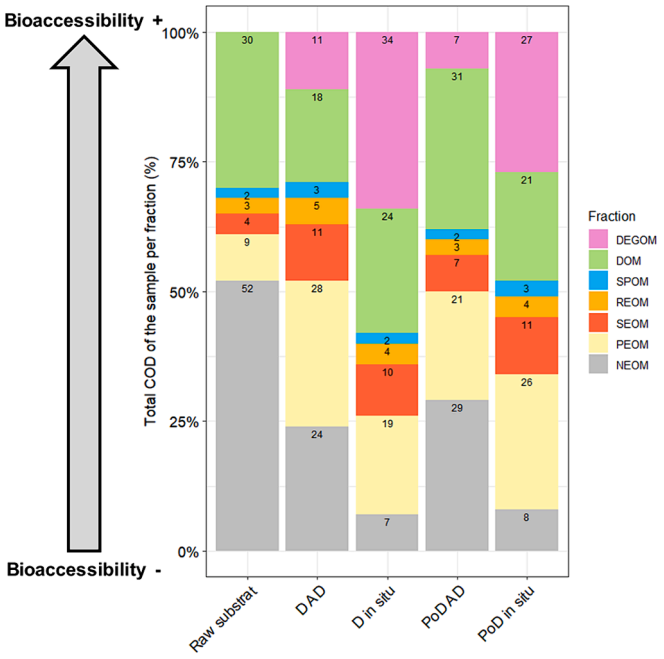

Fig. 3. Bioaccessibility and conversion of the organic matter between the beginning and the end of the experiment. Fractions were ordered from down to top part of the barplot by increasing bioaccessibility level. DEGOM Degraded organic matter; DOM Dissolved organic matter; SPOM soluble particular organic matter; REOM readily extractable organic matter; SEOM slowly extractable organic matter; PEOM poorly extractable organic matter; NEOM non extractable organic matter. D AD:Digestate of Digester as inoculum in anaerobic digestion process; D *in situ*: Digestate of Digester as inoculum in *in situ* biomethanation process; PoD AD: Digestate of Post-digester as inoculum in anaerobic digestion process; PoD *in situ*: Digestate of Post-digester as inoculum in *in situ* biomethanation process.

manure, energy covered crop silage, and food waste soup. Consistently, Fernández-Domínguez et al. (2021) reported an accessible portion of 46 % for a mixture of pig slurry and biowaste, which is close to the used substrate mixture.

Considering the degradation of the organic matter in all assessed conditions, Fig. 3 clearly shows that, regardless of the initial inoculum, the addition of H_2 significantly increases the DEGOM fraction. This result indicates that more organic matter was degraded under *in situ* biomethanation conditions compared to the AD control without external H_2. The distribution of organic matter fractions was consequently influenced by the addition of H_2, suggesting an increase in the hydrolysis of organic matter and in DEGOM in the case of *in situ* biomethanation.

Moreover, this influence on the degradation of organic matter was also observed with the other fractions. Indeed, while the SEOM and REOM fractions remained similar in the raw substrate and the digested media at the end of the experiment for all conditions, differences were observed for the DOM, SEOM, PEOM, and NEOM fractions.

The DOM fraction is considered to contain the part of the biomass that is already dissolved in water. This fraction was reduced by 10 % in all conditions in comparison with the raw substrate, except for the AD control condition using PoD as inoculum. For the latter, the DOM fraction was close to that of the substrate (29 % and 31 % for the raw substrate and the AD control, respectively). The DOM fraction has been reported to be deeply impacted by hydrolysis and organic matter solubilization phenomena during anaerobic digestion (Jimenez et al., 2020). As a result, the DOM could have been impacted by different microbial activities during the studied processes (Fernández-Domínguez et al., 2023). Indeed, solubilization of organic matter primarily extracted in other fractions (such as PEOM and SEOM) or microbial growth and its associated metabolite production could both have a strong impact on the

DOM fraction. Therefore, no clear links between the evolution of the DOM fraction and the improvement of organic matter degradation through H_2 could be made regarding the difference of 10 % between the DOM fraction of the AD control condition and the *in situ* condition using inoculum PoD. Similarly, the increase in the SEOM fraction observed for all assessed conditions could not lead to a stated conclusion. Indeed, the increase in this fraction could result from plural organic matter conversion processes that might have differed in AD control and *in situ* biomethanation conditions.

Clearer results could be obtained through the evolution of the NEOM fraction. Indeed, the NEOM fraction is primarily associated with lignin-like molecules (Jimenez et al., 2015). This fraction exhibited a reduction in all conditions compared to the raw substrate. Moreover, the NEOM conversion was at least doubled in the *in situ* condition compared to the AD control condition *(e.g.,* 28 % *vs* 10 % of the total COD of the sample corresponding to NEOM in the AD control *vs in situ* condition, using the inoculum D). The decrease in the NEOM portion could be attributed to the biodegradation activity of the microbial community. Indeed, through hydrolysis, the organic matter of the NEOM could have become more accessible and be extracted into the PEOM fraction, as the sum of the PEOM and NEOM fractions in all final digestates was close to the NEOM fraction of the substrate (Fig. 3). Following this transformation scheme, the organic matter contained in the PEOM fraction could have become more accessible for biodegradation, thus being associated with more accessible fractions (*i.e.*, REOM, SEOM, SPOM, or DOM), facilitating, in this case, their conversion for CH_4 production. Subsequently, these results reinforce the statement of an improvement in organic matter conversion in the *in situ* biomethanation condition in comparison with the AD process.

To ensure the robustness of these results, which were surprising considering the inhibitory effect of H_2 on AD metabolism, the DEGOM fraction analyzed at the end of the experiment was compared to the methane produced during the experiment (from week 0 to week 6). In the case of inoculum D, it was estimated 11 % and 34 % of the 30 $g.L^{-1}$ of total COD in the substrate were consumed under AD control and *in situ* biomethanation conditions, respectively, representing an increase of 1.4 gCOD removal between the two conditions, for a 200 mL working volume. As the experiment lasted 2 HRT, 400 NmL of substrate were used, doubling the observed difference in COD removal (2.8 gCOD). The CH_4 production associated to OM degradation corresponded to 10.7 and 14.1 gCOD under AD control and *in situ* conditions, respectively, representing an increase of 3.4 gCOD.. Similar results were obtained for reactors using the PoD inoculum, with a 2.4 gCOD removal increase (considering 400 mL of influent) and a 2.1 gCOD increase in CH_4 production between the control and *in situ* biomethanation conditions. Therefore, the combined analysis of the evolution of the DEGOM fraction and methane production clearly supports an enhancement of the organic matter biodegradation activity performed by the microbial community within H_2 addition in the reactor. As no other study using such a method to investigate the impact of H_2 on *in situ* biomethanation was retrieved, it was not possible to compare the present analysis with relevant literature.

3.4. Hydrogen-induced changes in microbial communities

The relative abundance of bacterial communities was monitored throughout the experiments (Fig. 4), by analyzing the initial inocula and the digested media sampled on week 1 (*i.e.*, end of the start-up phase), week 3 (after one HRT), and week 6 (after two HRT). However, only minor changes were observed regarding the structure of the bacterial community, whatever the conditions. For both inocula, the dominant phyla were *Bacteroidetes* and *Firmicutes*, which are common phyla of bacteria found in AD process communities (Carballa et al., 2015). The proportions of these two phyla remained stable from the beginning to the end of the experiment in AD conditions. In contrast, for the *in situ* condition, a slight increase in the proportions of *Bacteroidetes* and a concomitant decrease in *Firmicutes* were observed.

Regarding the relative abundance at family level, the decrease in proportion of a family from the MBA03 order and the growth of bacteria from the *Enterococcaceae* family were mainly attributed to the operational conditions, as these changes in the bacterial communities were observed for all conditions. Conversely, the *Rikenellaceae* family appeared to be more predominant in the *in situ* condition compared to the AD control. The retrieved members of the *Rikenellaceae* family were mostly attributed to the genus *vadinBC27* (wastewater sludge group; see supplementary material). Members of this genus were previously described as amino acid degraders in anaerobic processes (Guo et al., 2014). In addition, it was previously suggested that *vadinBC27* (wastewater sludge group) could degrade amino acids through syntrophic interaction with hydrogenotrophic methanogens (Li et al., 2015).

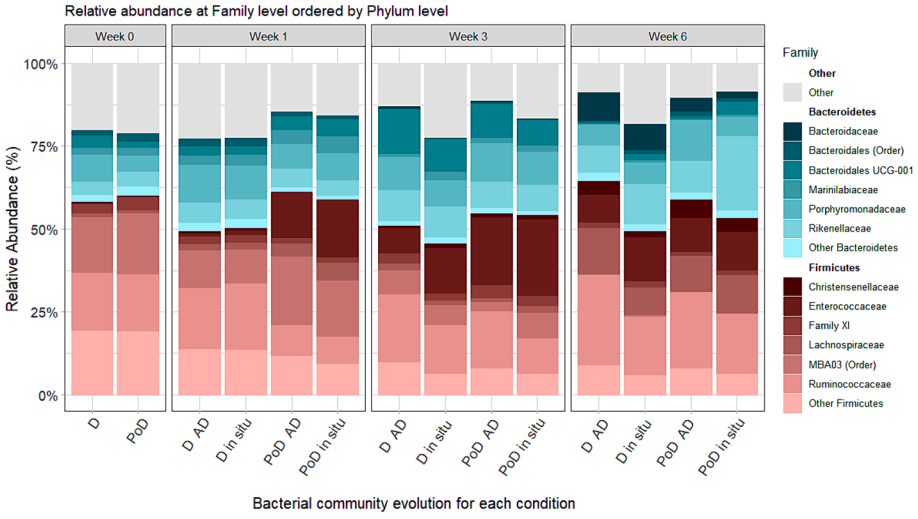

Fig. 4. Evolution of bacterial communities in assessed conditions. Relative abundance of bacterial communities at Family level, classified by Phylum at the beginning of the experiment, at the start of H_2 injections (Week 1) after one HRT (Week 3) and after two HRT (Week 6). The 6 dominant Families retrieved per Phylum are displayed while other families are summed up in "Other" categories for each Order; Displayed Phyla represented more than 5 % of the overall bacterial community; D AD: Digestate of Digester as inoculum in anaerobic digestion conditions; D *in situ:* Digestate of Digester as inoculum in *in situ* biomethanation conditions; PoD AD: Digestate of Post-digester as inoculum in anaerobic digestion conditions; PoD *in situ:* Digestate of Post-digester as inoculum in *in situ* biomethanation conditions.

Microorganisms from the *Ruminococcaceae* family were more abundant in AD control conditions than with H_2 injections. The effect of H_2 on that family remains uncertain, as these microorganisms are considered cellulolytic bacteria, promoting VFA and H_2 production through lignocellulosic biomass such as energy cover crops (Wojcieszak et al., 2017). Overall, no clear impact of H_2 on the bacterial community structure of the inocula could be stated from these results.

The lack of change in the main structure of the bacterial communities, despite the improved degradation of organic matter, may suggest a rapid adaptation of bacterial activity in the presence of H_2, as bacteria from *Bacteroidetes* and *Firmicutes* phyla are known for their diverse metabolic activities during AD (Campanaro et al., 2016; Treu et al., 2018; Westerholm et al., 2019). Besides, H_2 might impact only a minor part of the community, which may not be observable through the analysis of the overall bacterial community. For instance, syntrophic bacteria responsible for VFA degradation and H_2 production constitute a small portion of the AD community (Stams et al., 2012). These bacteria may be directly stimulated by the addition of H_2, either through the dissolved H_2 concentration or indirectly through the impact of H_2 on archaea structure and activity.

This hypothesis is supported by the high selective pressure exerted by H_2 on the archaeal communities in the *in situ* conditions compared to the AD control (Fig. 5). As already reported in the literature (Agneessens et al., 2017; Thapa et al., 2022), the injection of H_2 over several HRT led to substantial structural changes in the archaeal communities. In the present experiments, whatever the inoculum, the community composition was comprised of over 85 % archaea from the *Methanocorpusculum* genus at the end of the *in situ* experiment (i.e., after two HRT). Members of the *Methanocorpusculum* genus are known hydrogenotrophic methanogens (HM), exhibiting relatively rapid doubling times ranging from 8 to 14 h for identified species using H_2 and CO_2 as substrates (Zellner et al., 1989a; Zhao et al., 1989). Despite the low relative abundance of the *Methanocorpusculum* genus of less than 1 % in the inocula, it effectively outcompeted members of the *Methanobacterium* genus (also identified as HM) for H_2 consumption, with the latter representing over 40 % of the initial archaeal community in both inocula. The literature indicates that members of *Methanobacterium*, growing at mesophilic temperatures, have doubling times ranging from 5 h to 48 h when using H_2 and CO_2 as substrates (Garcia et al., 2000). In the present experiment, the strong development of the *Methanocorpusculum* genus suggests that its identified members in the community exhibited faster growth using exogenous H_2 than the *Methanobacterium* genus that were initially dominant in the inoculum. In addition, members of *Methanobacterium* genus reported optimal growth temperature of 38.3 ± 3.5 °C, considering 19 species; (see supplementary material) (Garcia et al., 2000). In comparison, species from *Methanocorpusculum* genus reported optimal growth temperature of 35.2 ± 2.7 °C, considering 5 species, (see supplementary material) (Zellner et al., 1989b), which may have favored them during the experiment.

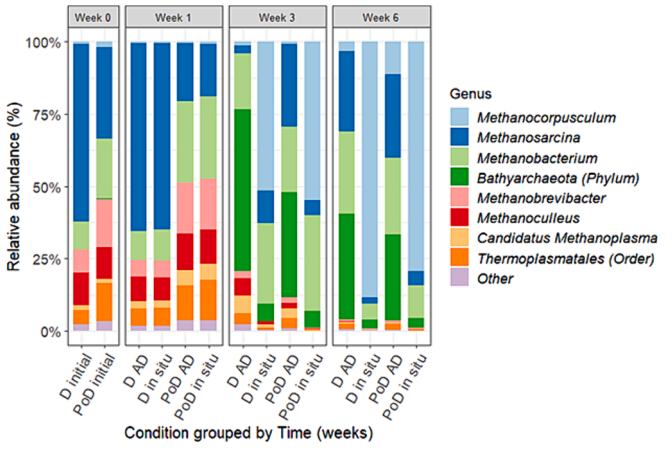

Fig. 5. Evolution of archaeal communities in assessed conditions. Relative abundance of archaeal communities at genus level at the beginning of the experiment, at the start of H_2 injections (Week 1) after one HRT (Week 3) and after two HRT (Week 6); D AD: Digestate of Digester as inoculum in anaerobic digestion conditions; D *in situ*: Digestate of Digester as inoculum in *in situ* biomethanation conditions; PoD AD: Digestate of Post-digester as inoculum in anaerobic digestion conditions; PoD *in situ*: Digestate of Post-digester as inoculum in *in situ* biomethanation conditions.

The growth of the *Methanocorpusculum* genus members increased the proportion of archaea in the community. Quantifying bacteria and archaea using qPCR revealed that the archaea/bacteria ratio (A/B ratio) (16S rDNA gene copy / 16S rDNA gene copy) increased from 2 % to 10 % and from 2 % to 9 % during the semi-continuous *in situ* biomethanation process using inoculum D and PoD, respectively. This increase in the archaeal population was not observed in AD control, where the A/B ratio remained stable at 2 % throughout the experiment for both inocula. The steadiness of the archaeal proportion in the AD control communities aligned with the balanced archaeal communities obtained in this process, indicating its stability under the control condition. Specifically, AD control communities were composed of three main genera of archaea, each representing approximately 30 % of the community at the end of the experiment: members of the *Methanobacterium* genus (HM), members of the *Methanosarcina* genus, and a genus of members of the *Bathyarchaeotha* phylum. While the metabolic activity of the *Bathyarchaeota* phylum members remains uncertain, members of *Methanosarcina* are known for their versatile methanogenic activity, with the capacity to produce CH_4 either from acetate or H_2 and CO_2 (De Vrieze et al., 2012; Evans et al., 2015; Maus et al., 2018). This composition of the archaeal communities in AD control was consistent with the archaeal communities of AD consortia observed in other studies (Braga Nan et al., 2020), suggesting stable methanogenesis under these conditions, despite changes in the community between the beginning and the end of the experiment.

However, the H_2 pulsed injection strategy used in the present experiment may have mitigated the known inhibitory effect of H_2 on the AD reaction chain, allowing an enrichment in hydrogenotrophic methanogens. As these species became dominant in the population, the overall H_2 uptake capacity of the community could have increased, reaching values close to the H_2 gas/liquid mass transfer rate of the experimental system. Subsequently, H_2 dissolved concentration could have been drastically reduced, limiting its effect on H_2 sensitive metabolisms.

At the same time, in the presence of an H_2-adapted archaeal population and with repeated H_2 additions, new syntrophic interactions might have been promoted, as previously reported by Basile et al. (2020). Additionally, the development of syntrophic interaction during *in situ* biomethanation of similar substrates was suggested in previous research (Treu et al., 2019). This synergy among microbial communities could have improved the AD reaction chain. Indeed, the H_2 uptake rate of the microbial community has been shown to impact metabolisms such as syntrophic acetate oxidation and VFA consumption pathways (Capson-Tojo et al., 2021). In the present experiment, the increased H_2 uptake rate, associated with the growth of hydrogenotrophic methanogens, could have indirectly improved acetogenic metabolisms, leading to improved OM biodegradation.

4. Conclusions

This study highlights that OM biodegradation was 2.5 times higher due to H_2 injections This result was corroborated with the increase of least 626 NmL in CH_4 production associated to OM biodegradation with H_2 injections. Similar improvements in biodegradation activity were observed regardless of the initial microbial activity of the inocula used,. Further investigations are required to elucidate the impact of H_2 on the AD reaction chain. These findings offer new perspectives for the

development of *in situ* biomethanation, as it could enhance CH_4 production through both improved organic matter conversion and the CO_2 transformation into CH_4.

CRediT authorship contribution statement

M. Mahieux: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Q. Aemig:** Writing – review & editing, Validation, Supervision, Methodology, Conceptualization. **C. Richard:** Writing – review & editing, Validation, Supervision, Investigation, Conceptualization. **J-P. Delgenès:** Writing – review & editing, Validation, Supervision. **M. Juge:** Project administration, Funding acquisition. **E. Trably:** Writing – review & editing, Validation, Supervision, Conceptualization. **R. Escudié:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2024.131101.

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