Hybrid biogas upgrading in a two-stage thermophilic reactor

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The aim of this study is to propose a hybrid biogas upgrading configuration composed of two-stage thermophilic reactors. Hydrogen is directly injected in the first stage reactor. The output gas from the first reactor (in-situ biogas upgrade) is subsequently transferred to a second upflow reactor (ex-situ upgrade), in which enriched hydrogenotrophic culture is responsible for the hydrogenation of carbon dioxide to methane. The overall ob-jective of the work was to perform an initial methane enrichment in the in-situ reactor, avoiding deterioration of the process due to elevated pH levels, and subsequently, to complete the biogas upgrading process in the ex-situ chamber. The methane content in the first stage reactor reached on average 87% and the corresponding value in the second stage was 91%, with a maximum of 95%. A remarkable accumulation of volatile fatty acids was observed in the first reactor (in-situ) after 8 days of continuous hydrogen injection reaching a concentration of 5.6 g/L. Nevertheless, after an adaptation period, the system managed to recover and the volatile fatty acids decreased to 2.5 g/L. No pH drop was recorded during the period characterised by increased volatile fatty acids concentration mainly due to the consumption of the endogenous carbon dioxide by hydrogenotrophic metha-nogens. The effect of hydrogen injection on the microbial community in both reactors was analysed by 16S rRNA gene amplicon sequencing. The results demonstrated an increment in relative abundance of hydrogenotrophic methanogens and homoacetogens in the in-situ reactor, while the microbial community in the ex-situ chamber was simpler and dominated by hydrogenotrophic methanogens.

Keywords: Anaerobic digestion, Biogas upgrading, Hybrid configuration, Hydrogenotrophic methanogenesis, Power to gas

1. Introduction

The generation of electricity from renewable energy sources (RES) is fundamental for reducing polluting emissions from fossil fuels. One implication while designing and implementing RES systems is the potential excess electricity that can be generated under certain conditions (e.g. high wind peak loads), which contributes to the increment of market volatility and frequency of sudden drops in electricity prices [1]. Unfortunately, the direct storage of the surplus energy produced from RES is still economically unfavourable. Therefore, several alternative options have been demonstrated in the concept of "Power-to-X" for transforming excess RES into power, heat, and/or gas.

In the context of Power-to-Gas (P2G), the biological biogas upgrading via hydrogenotrophic methanogenesis opens new horizons due to the more efficient exploitation of RES by integrating two renewable sources, such as biogas and wind/eolic or photovoltaic power generation [2]. From the perspective of an energy smart-grid, P2G has the inherent advantage of exploiting the existing infrastructure of the natural gas grid. Currently, this is achievable mainly via a two-step process: (1) utilisation of excess renewable energy for water electrolysis and subsequent hydrogen (H_2) production [3] and (2) conversion of H_2 by means of biological reactions with external carbon monoxide (CO) and carbon dioxide (CO_2) sources into methane (CH_4) [1].

It is widely known that biogas is typically burned in a Combined Heat and Power (CHP) unit providing thermal energy and electricity. However, the high content of CO_2 in biogas reduces its energetic value, and therefore, its conversion to CH_4 will enable the development of carbon-negative renewable energy production [4]. In order to obtain biogas with natural gas standard quality, it is necessary to increase its calorific value by removing CO_2, thus obtaining a purified gas, which is so-called "biomethane" [2]. The upgrading process allows the transformation of more than 80% of the energy content of raw biogas into new energy, as the existing biomethanation technologies consume less than 20% of biogas energy for upgrade and compression [5]. The specific requirements of biomethane for injection into natural gas grids or for exploitation as a vehicle fuel varies among different countries and

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Nomenclature

Abbreviations

CHP	Combined Heat And Power
CSTR	Continuous Stirred Tank Reactor
HRT	hydraulic retention time
OLR	Organic Loading Rate
OTU	Operational Taxonomic Unit
P2G	Power-to-Gas
R1	reactor 1
R2	reactor 2
PCA	principal component analysis
RES	renewable energy sources
rRNA	ribosomal ribonucleic acid
SAOB	syntrophic acetate-oxidising bacteria
SRA	sequence read archive
TCD	thermal conductivity detector
TS	total solids
TKN	total Kjeldahl nitrogen
VFA	volatile fatty acids
VS	volatile solids

Chemical compounds

CH_4	methane
CO_2	carbon dioxide
H_2	hydrogen
HCL	hydrochloric acid
NH_4^+-N	ammonium nitrogen

are in the range of > 96% for CH_4, < 6% for CO_2 and < 3% for O_2 [6]. According to the IEA Bioenergy Task 37, more than 400 biogas upgrading plants are now in operation worldwide [7]. The commercial technologies implemented are mainly physically or chemically based [8]. In particular, these technologies include water scrubbing (38%), organic (25%) and chemical scrubbing (23%), physical adsorption (9%) and membrane separation (5%), while cryogenic technology is used in only 0.4% of the facilities [7]. However, the main disadvantages of these technologies, such as the high consumption of chemicals, pressure or energy, enabled new research work on less energetic or cost expensive and simpler solutions. In this context, biological biogas upgrading has attracted increasing attention over the last years.

The biological biogas upgrading process has been defined in three different concepts depending on where the H_2 is provided with respect to the anaerobic digestion process [1]. These alternatives are the *in-situ*, the *ex-situ* and the hybrid options. In the *in-situ* option, H_2 is injected directly into the biogas digester and is biologically coupled with the endogenous CO_2. In the *ex-situ* option, CO_2 from external sources (e.g. biogas, CO_2 storage and syngas) and H_2 are injected together inside a reactor containing enriched hydrogenotrophic cultures, resulting in their conversion to CH_4. Finally, in the hybrid option, *in-situ* and *ex-situ* processes are implemented together in biogas upgrading plants forming an integrated system [1].

Several previous studies demonstrated the feasibility of *in-situ* and *ex-situ* biogas upgrading achieving CH_4 content of 95% under various conditions [9–11]. However, it has been reported that especially during the *in-situ* process, there are some technical challenges related to increased pH due to the bicarbonate consumption, which caused inhibition of methanogenesis [12]. Moreover, increased H_2 partial pressure, as a result of H_2 addition, could affect the interplay of specific bacteria and methanogens. Thus, a direct injection of H_2 into the anaerobic reactor might inhibit the activity of syntrophic bacteria decreasing the anaerobic substrate degradation [13]. The *ex-situ* concept was indeed conceived to avoid inhibition of the core biogas production process so that H_2 and CO_2 conversion takes place in a separate chamber. The main bottleneck in the methanation process, which is common in both *in-situ* and *ex-situ* concepts, is the poor gas-liquid H_2 mass transfer [10] that can be alleviated by using more efficient gas dispersion systems [14] or reactor configurations [13].

Thus, the aim of the present work was to evaluate the performance of a novel hybrid biological biogas upgrading system with respect to the conversion efficiency and final methane content in the output gas. This study brings new knowledge on the changes occurring in each individual stage of the hybrid biogas upgrading process (i.e. *in-situ* and *ex-situ* stage) once they are coupled in an integrated biomethanation system. More specifically, it was assessed whether the hybrid technology is able to address important technical challenges related to increased pH during the *in-situ* application and dimensioning of the overall process by operating a considerably smaller separate reactor for the *ex-situ* application, if compared to the volume of the conventional biogas reactor. Particular attention was given to the reactor stability, and for that reason, monitoring of the main operating parameters, such as pH, volatile fatty acids (VFA) and methane yield was performed during the whole experimental work. In addition, in order to better

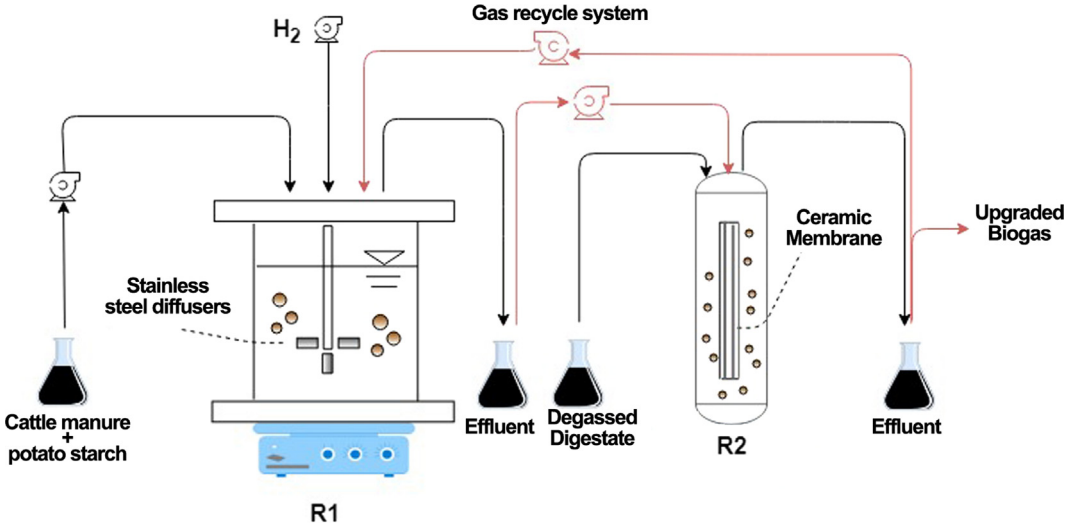

Fig. 1. Schematic diagram of hybrid system set-up.

Table 1
Characteristics of the used substrates. The feedstock mixture was composed of cattle manure and potato-starch 3:2 v/v.

Parameter	Diluted cattle manure	Diluted potato-starch	Mixture	Acidified digestate
pH	7.45	5.35	6.94	6.71
TS (g/L)	36.4 ± 1.0	41.6 ± 1.6	38.5 ± 1.2	45.0 ± 0.4
VS (g/L)	26.9 ± 1.1	27.4 ± 2.1	27.1 ± 1.5	20.2 ± 0.2
TKN (g/L)	1.63 ± 0.08	1.92 ± 0.01	1.74 ± 0.05	5.14 ± 0.04
NH_4^+-N (g/L)	0.89 ± 0.04	0.49 ± 0.01	0.73 ± 0.03	4.27 ± 0.05
VFA (g/L)	10.21 ± 0.33	0.045 ± 0.0	2.72 ± 0.12	0.94. ± 0.02

understand the structure of microbial communities populating the biogas upgrading systems, high-throughput sequencing of 16S rRNA gene amplicons was performed in samples from both reactors during various experimental time points.

2. Materials and methods

Cattle manure obtained from Snertinge biogas plant (Denmark) was preventively sieved through a 2 mm net. Up-concentrated potato-starch wastewater was obtained from Karup Kartoffelmelfabrik potato-starch processing factory (Denmark). Both substrates were stored at −20 °C, in 5 L tanks and thawed at 4 °C for 3 days, before usage.

2.1. Reactors' configuration and setup

The hybrid configuration was composed by a Continuous Stirred Tank Reactor (CSTR), denoted as R1 for the *in-situ* stage, and an upflow reactor, denoted as R2, for the *ex-situ* stage (Fig. 1). Both reactors were operating at thermophilic conditions (53 ± 1oC). The selection of an upflow column reactor was based on previous studies, which demonstrated that such a type of reactor can maximize the gas-liquid mass transfer rate by increasing the gas retention time [2]. The CSTR, which had a working volume of 3L and was operated at HRT of 15 days, was initially inoculated with thermophilic digestate obtained from Snertinge biogas plant (Denmark). Prior to the inoculation, the digestate was sieved using a net (2 mm) to remove large particles and to avoid clogging of the pump's tubes. The upflow reactor (850 mL working volume) was inoculated with 600 mL of undiluted degassed digestate and 250 mL of active enriched hydrogenotrophic inoculum obtained from an upgrading biogas reactor [15]. The purpose of the enriched culture was to provide active hydrogenotrophic methanogens, and thus, shorten the overall adaptation period. During the whole experiment, R1 was co-digesting cattle manure and potato-starch, while degassed digestate (30 mL/day, with an HRT of 28 days) was provided to the R2 in order to supply the microbial community with all the necessary nutrients. The whole experiment lasted 115 days and was divided into three periods. During Period I (days 0–36), the configuration was operated as a conventional anaerobic digester. During Period II (days 37–80), H_2 was directly injected in the first stage reactor and the output gas from the first reactor (*in-situ* biogas upgrade) was subsequently transferred to the second upflow reactor (*ex-situ* upgrade), in which enriched hydrogenotrophic culture is responsible for the hydrogenation of CO_2 to CH_4. In Period III (days 81–115), the injection of H_2 was stopped and the system worked with the same operating conditions as in Period I. The two reactors were connected by a gas recirculation system supported by a gas pump. The recirculation gas flow rate was fixed for all three periods at a rate of 82 mL/min. This flow rate value was based on a previous study, which demonstrated a positive effect on gas-liquid mass transfer rate enhancing H_2 availability for microorganisms [10]. The H_2 was injected into R1 using three stainless steel diffusers (2 μm pore size) while it was dispersed into R2 through a ceramic membrane.

2.2. Feedstock preparation

A mixture of cattle manure and potato-starch was used as influent feedstock for reactor R1. The feedstock mixture was composed of diluted cattle manure (1:1) and diluted potato-starch (1:7) in a mixing ratio of 3:2 v/v. Cattle manure and potato-starch were pre-diluted with water to obtain the same Volatile Solid (VS) content. In order to provide nutrients to the microorganisms populating the R2 reactor, completely degassed digestate obtained from Snertinge biogas plant (Denmark) was used as nutrient feedstock. The digestate, preventively filtered through a 2 mm net, was then stored at 55 °C at anaerobic conditions for a period of 3 months to ensure total degradation of the residual organic matter. In order to maintain pH values in the optimum values for methanogenesis, the digestate was acidified using hydrochloric acid (HCl) (i.e. the specific ratio of digestate, HCl 1 M and distilled water was 1:0.1:0.3). More specifically, the initial pH of the digestate (i.e. 8.61 ± 0.18) was reduced to 6.71 ± 0.04 after acidification. Table 1 presents the chemical composition of the used substrates.

2.3. Analytical methods and calculations

Total solids (TS), VS and pH were measured according to APHA standard methods for the examination of water and wastewater [16]. The methane content in the batch assay was determined using a gas-chromatograph (Shimadzu GC-8A, Tokyo-Japan). For the continuously fed reactors, the daily volume of effluent gas was recorded using an automated displacement gas metering system. The composition of gases CH_4, CO_2 and H_2 in the effluent of both reactors was determined using a gas chromatograph (Mikrolab, Aarhus A/S, Denmark), equipped with a thermal conductivity detector (TCD). Organic loading rate, H_2 gas feed, biogas rate and CH_4 production rate were all referred only to R1 volume. The VFA concentration was analyzed using a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan) following the procedure previously described [17]. All analyses were done in triplicate samples.

2.4. Microbial community analysis

Four samples (14 mL each) were taken from the R1 reactor and two samples (10 mL each) were taken from R2 for microbial analysis. In brief, for R1, the samples were corresponding to steady-state of Period I, 2 collection points during Period II and one collection point at the end of Period III. For R2, the two samples were obtained during Period II and Period III, respectively. Residual particles present in the samples were removed using a 100 μm nylon filter. Centrifugation of the filtered samples (10,000 rpm, at 4 °C for 10 min) was conducted to obtain around 2*g* of cell pellet. DNA extraction was performed using the PowerSoil® DNA Isolation Kit protocol (MO BIO Laboratories) with an additional initial cleaning step by Phenol:Chloroform:Isoamyl Alcohol 25:24:1 pH 8 (Sigma-Aldrich). The quality of the purified DNA was examined, and the DNA concentration was analysed using NanoDrop 2000 (ThermoFisher Scientific). 16S rRNA gene V4 hypervariable region was amplified with universal primers and was sequenced using Illumina MiSeq sequencing technology. The obtained reads were submitted to the NCBI sequence read archive database (SRA) [18] with accession number SRP126498, BioProject PRJNA421924 and with the following IDs: SAMN08160168 (R1 period I, corresponding to sample R1-1), SAMN08160169 (R1 period II1, corresponding to sample R1-2), SAMN08160170 (R1 period II2, corresponding to sample R1-3), SAMN08160171 (R1 period III, corresponding to sample R1-4), SAMN08160172 (R2 period II2, corresponding to sample R2-3), and SAMN08160173 (R2 period III, corresponding to sample R2-4).

The Operational Taxonomic Unit (OTU) profile, phylogeny tree, alpha diversity and beta diversity were analysed using CLC Workbench software (V.8.0.2) equipped with the Microbial genomics module plugin. The detailed procedure of the followed bioinformatic pipeline was previously described [19]. Principal component analysis (PCA)

based on ANOVA was performed using STAMP software to assess the dissimilarity among the samples and make a comparison between R1 and R2. The relative abundance of a given OTU in each sample was calculated based on the share of its sequenced reads towards the sequenced reads of the total community. Therefore, the OTUs were classified as high abundant (> 0.5% relative abundance) or low abundant microorganisms (between 0.01% and 0.5% of relative abundance). OTUs with relative abundance lower than 0.01% were discarded from the analysis. The discussion of the results is focused on the most abundant microbes in the community (> 0.5% of relative abundance). Low abundant microorganisms presenting statistically significant changes were exceptionally included in the discussion.

3. Results and discussion

During Period I, the system operated as a conventional biogas reactor co-digesting cattle manure and potato-starch with an Organic Loading Rate (OLR) fixed at 1.7 gVS/(L reactor/day) (Table 2). The methane yield of the feedstock at steady-state conditions (approximately after 3 HRTs) reached 211 mLCH_4/gVS (Table 2). The average CH_4 and CO_2 content in the biogas were 69% and 31% for R1 and 75% and 25% for R2, respectively. The higher CH_4 content in the secondary reactor can be associated with the enhanced dilution of CO_2 into the reactor's liquid phase due to the gas recirculation system.

3.1. Reactor performance during biogas upgrading process

The methane production rate increased upon H_2 addition and reached a 434 mLCH_4/L reactor/day, achieving a maximum CO_2 removal of 91% (on average 80%) (Fig. 2). The methane content in the output gas was on average 85% in the *in-situ* and 88% in the *ex-situ* reactor, while the concentrations of CO_2 were 13% and 10%, respectively. A small content of H_2 (i.e. approximately 2%) remained unconverted so that 98% of the injected H_2 was consumed. However, as it can be noticed in Fig. 2, the increment in methane production rate due to the hydrogenation of CO_2 did not last long and did not stabilize. The imbalanced process performance is clearly evidenced by the VFA accumulation (Fig. 3). Indeed, it was found that propionate and mainly acetate were the short-chain fatty acids whose concentration increased by 1.9 and 3 g/L in R1, respectively. Similarly, a slight VFA accumulation (from approximately 0.1 to 0.3 g/L) was also recorded in R2 (Fig. 3). The increment of VFA concentration in R1 and R2 indicates a strong inhibition of acetoclastic methanogenesis. As a consequence of the increased H_2 partial pressure, the system most probably shifted the metabolic pathway towards homoacetogenesis. This argument was additionally supported by the decrease of the methane production rate. The outcome of the present work is in agreement with previous studies, which reported increased acetate concentration upon H_2 addition both in fed-batch systems [20] and also in continuously fed reactor configurations [2].

As can be noticed from Fig. 3 after the peak of VFA, the gas quality remained constant until day 60, while the methane yield decreased reaching a new stable value, which was even lower than the corresponding one at steady-state conditions of the pre-H_2 period. Moreover, it was found that the acetate to propionate ratio was inversed in R1, with higher propionate concentration compared to acetate (Fig. 3). It has been previously suggested that acetate to propionate ratio can serve as an indicator for process performance [21]. In this context, when propionate exceeds acetate concentration above a certain threshold, an impending digester failure is indicated [22]. The system started to recover as soon as the concentration of propionate was decreased and was found to be lowered when compared to acetate. As will be further discussed, this is attributed to the fact that the H_2 imposed a selective pressure on the microbial community, shaping its structure into a new consortium that is able to metabolize the intermediate compounds of anaerobic digestion process. Thus, after a period that lasted one HRT, acetate and propionate concentrations were 1.4 and 1 g/L, respectively.

At day 71, it was noticed that the gas distribution system in R1 was clogged (no H_2 was injected). The system was immediately repaired; however, the process was slightly disturbed as shown by the VFA results (days 68–78). Indeed, there was a further VFA peak in R1 but with a remarkably lower concentration (2.2 and 0.95 gVFA/L of acetate and propionate, respectively) than those caused by the initial inhibition.

At the end of Period II, the gas composition of the hybrid system reached on average 91% of methane confirming a progressive adaptation of the system to the high H_2 partial pressure. Finally, at the end of Period II, the highest methane concentration was achieved (86% in R1 and 95% in R2). A direct consequence of the biogas upgrading process, due to the higher consumption of CO_2 in Period II, was a transient rise in pH levels (Fig. 3). More specifically, the pH values were on average 8.3 and 8.1 for R1 and R2, respectively (Table 2). In Period III, the injection of H_2 was stopped in order to allow the system to recover from the new VFA concentration stress. It can be noted that acetate and propionate reached concentration values comparable to the pre-H_2 period only after 15 days (i.e. one HRT) after the second peak of VFA. Since there is not any previous research reporting results for a hybrid system, a direct comparison of the obtained efficiency is not possible. Nevertheless, Table 3 presents the performance from *ex-situ* and *in-situ* systems having similarities with the current study (e.g. temperature conditions, same reactors type, etc.) in order to evaluate the efficiencies of the individual steps. It could be drawn that even if the biomethanation rate in previous systems was higher, the pH levels in the current system were maintained in a more favourable range for methanogenesis.

Table 2
Reactors' operations and performance under steady state conditions.

Reactor	Value	Period I		Period II		Period III	
		R1	R2	R1	R2	R1	R2
Organic Loading Rate	gVS/(L reactor/day)	1.68		1.68 ± 0.2		1.80	
Gas feed (H_2 100%)	mL/(L reactor/day)			550			
Gas recirculation	L/(L/hr)	0.79	0.8	0.67	0.67	0.92	0.92
Biogas rate	mL/(L reactor/day)		472.72 ± 47		400.4 ± 67		552.4 ± 144.5
CH_4 production rate*	mL/(L reactor/day)		355.1 ± 8		352 ± 53		426.9 ± 77.6
CH_4 yield	mL/gVS		211 ± 19		214 ± 63		198 ± 36
CH_4	%	69.2% ± 1	75.4% ± 1	86.4% ± 1	91% ± 2	71% ± 1	77% ± 4.1
CO_2	%	30.7% ± 1	24.3% ± 2	10.7% ± 3.6	7% ± 1	29% ± 1	23% ± 4.3
H_2	%	–	–	3.5% ± 1.5	2% ± 1	–	–
pH	–	8.35 ± 0.1	8.1 ± 0.1	8.6 ± 0.0	8.1 ± 0.0	8.09 ± 0.1	7.66 ± 0.2
Total VFA	g/L	0.2	0.03	2.7	0.1	0.6	0.1

* Methane production rate is calculated considering CH_4% from the *ex-situ* reactor.

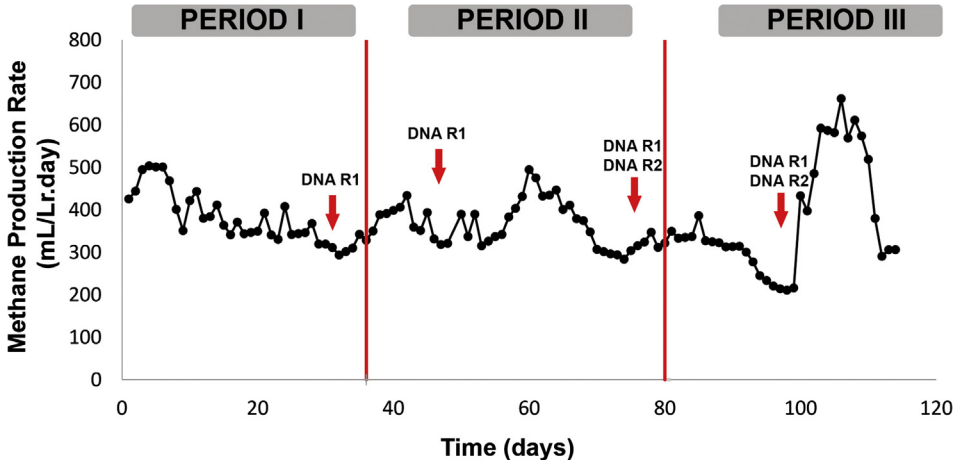

Fig. 2. Methane production rate during the different experimental periods, red arrows indicate DNA extraction for R1 and R2 respectively. CH_4 production rate is referred only to R1 reactor volume.

3.2. Microbial community composition

The microbiological composition of the two reactors should reflect distinct differences due to the divergent biogas upgrading methods (i.e. *in-situ* versus *ex-situ*). Thus, a greater microbial richness and diversity was expected in R1, taking into account the trophic chain of the anaerobic digestion process of the influent feedstock. More specifically, during the transition from Period I (conventional biogas production) to Period II (injection of H_2), it was hypothesised that an increment in relative abundance of hydrogenotrophic methanogens would occur in R1 with a concomitant decrease of the other members of the microbial community due to a potential inhibition caused by the high H_2 partial pressure. On the contrary, it was expected that the microbial community would be more specialised in R2 because of the initial inoculation (enriched with hydrogenotrophic methanogens [15]) and due to the fact that only gas fermentation was occurring.

Illumina sequencing generated more than 4.76 million of raw reads with average length of 250 bp. After filtering and merging by CLC Workbench, on average 63% of them were assigned to OTUs. The results of the 16S rRNA gene sequencing and diversity indexes are summarized in Table 4. Rarefaction curves, which are illustrated in the supplementary information (Fig. S1), showed that the sequencing depth was adequate to cover the sample richness in most of the replicates. Shannon indexes and numbers of OTUs of all samples from both reactors are illustrated in Fig. 4a. Samples obtained from the *in-situ* reactor were characterized by a higher diversity compared to the samples obtained from R2. Moreover, the samples that showed the highest diversity were R1-3 and R2-4. Fig. 4b presents the beta diversity displayed in PCA plot. The graphical representation revealed a relative distance in microbial diversity between the two reactors R1 and R2. In detail, all replicates can be divided into 2 different groups. Notably, the replicates from R1 were all clustered together except for one (purple dot), which clearly showed a higher similarity to R2 samples (red[2] and yellow dots) (Fig. 4b). One replicate of R2-4 was inconsistent with others most probably due to technical issues, and thus, was removed from the analysis.

The phylogenetic tree representing the global community for both reactors R1 and R2 is shown in the supplementary information (Fig. S2). Table 4 summarises the sequencing results with alpha diversity indexes and the threshold of OTUs. Bacterial population in both reactors covered on average 95% of the whole microbial community, whilst archaea accounted on average for 5%. The taxonomic classification of the microbial community showed that the most abundant phyla were *Firmicutes* (60%), *Proteobacteria* (10%), *Bacteroidetes* (8%), *Synergistetes* (8%), *Euryarchaeota* (3%), *Thermotogae* (3%) and *WWE1* (3%) (Fig. S3). Notably, only 35% of the OTUs were assigned at genus level (Fig. S4) indicating that a high share of the microbial community is composed of uncharacterized species. In particular, 40 OTUs represent the most abundant members and can be considered representative of all the samples. In Fig. 5, the relative abundance and fold change of the identified OTUs are represented for all collection points from the two reactors.

In Fig. 5a, it can be noted that all samples related to R1 showed a greater diversity when compared to the two samples from R2. Furthermore, the most distinct observation was that samples R1-1, R1-2 and R1-3 were all clustered together, while R1-4 is reported to be more similar to R2-3 and R2-4 in accordance with PCA results (Fig. 4). This outcome indicates that the changes in the microbial community in the *in-situ* reactor resulted in a new consortium that is more specialised in CO_2 and H_2 methanation even after stopping the H_2 provision.

The identified OTUs can be divided into 5 main clusters based on their behaviour in terms of increased or decreased relative abundance stimulated by H_2 injection (Fig. 5). For example, the first cluster includes microbes whose relative abundance in R1 was found to be increased during Period III (sample R1-4) compared to Period I, or microbes whose relative abundance was high in R2 and remained unchanged during the experimental periods. Therefore, it can be concluded that this cluster represents the group of microorganisms that are closely involved in the CO_2 hydrogenation process. In this context, the dominant methanogen of the community (i.e. relative abundance of 0.06% in Period I and 2.6% in Period III in R1 and stable 5% in R2) belonged to this cluster and was taxonomically assigned to genus *Methanothermobacter*. The dominance of hydrogenotrophic methanogens in biological biogas upgrading processes is in accordance with previous studies performed in upflow [10] and continuously stirred tank reactors [23]. Based on the results from the BLASTn search against 16S rRNA sequences (Bacteria and Archaea) database, *Methanothermobacter* sp. 7 was found to be 100% similar to *Methanothermobacter thermautotrophicus* and its abundance was enhanced by 45-fold from Period I to Period III in the R1 reactor. The significant increment of this specific hydrogenotroph is in agreement with a previous study on biogas upgrading systems [2].

Moreover, it was shown that the addition of H_2 in the reactors led to the promotion of specific metabolic pathways related to homoacetogenesis (Wood-Ljungdahl pathway) or syntrophic acetate oxidation (reverse Wood-Ljungdahl pathway). Thus, the increased H_2 partial pressure favoured the proliferation of homoacetogenic bacteria that are

[2] For interpretation of color in Fig. 4, the reader is referred to the web version of this article.

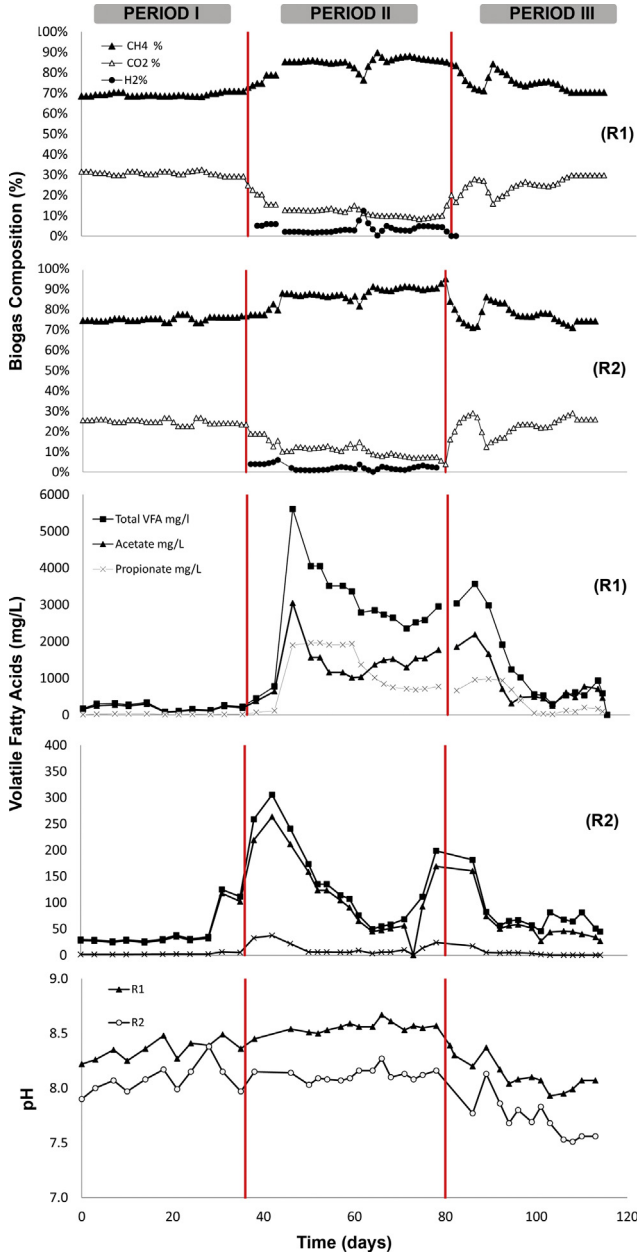

Fig. 3. Gas compositions, VFA and pH values for the *in-situ* (R1) and *ex-situ* (R2) biogas upgrading reactors during the whole experiment.

known to be able to grow on autotrophic and/or heterotrophic substrates and metabolize H_2 and CO_2 producing acetate [24]. On the contrary, the accumulation of acetate might in turn favour the growth of syntrophic acetate-oxidising bacteria (SAOB) that will oxidise acetate back to H_2 and CO_2 [25]. The presence of both bacteria (i.e. homoacetogens and SAOB) in the studied system could be attributed to the changes in operational conditions (i.e. periods with or without H_2 addition) that were shifting the thermodynamic equilibrium. Thus, the microbial analysis revealed the high abundance of species belonging to family *Thermoanaerobacteraceae*; members of *Thermoanaerobacteraceae* have been previously recognised as homoacetogens [26]. According to the results of the BLASTn search, the identified OTUs were possibly assigned to genus Moorella, which includes several species capable of performing homoacetogenic fermentation [27]. More specifically, *Thermoanaerobacteraceae* sp. 5 had 91% similarity to *Moorella humiferrea* or *Moorella stamsii* and *Thermoanaerobacteraceae* sp. 24 was found to be 91% similar to *Moorella humiferrea*. Nevertheless, the low sequence identity score compared to the threshold for genera classification (> 94.5%) demonstrated that these OTUs are most probably belonging to an unknown microbial species [28]. Both OTUs presented a statistically significant increase in their abundance of more than 8-fold and 459-fold, respectively (Fig. 6). Similarly, *Syntrophaceticus schinkii* 6 showed a statistically significant increase of 30-fold in Period III (sample R1-3) compared to Period I (sample R1-1) (Fig. 6a). *Syntrophaceticus schinkii* is a well-known syntrophic acetate-oxidizing bacteria previously isolated from sludge digesters [29].

One of the most dominant OTUs in both reactors was Clostridia sp. 1 (Fig. 5b), whose relative abundance was significantly increased upon long-term addition of H_2 in R2 (Fig. 6b). This species was assigned to the recently discovered order MBA08, belonging to Clostridia class with 90% similarity to *Hydrogenispora ethanolica*. The high abundance of this OTU, which is probably an anaerobic carbohydrate-fermenting bacterium, is clearly aligned with other studies on biological biogas upgrading systems [2]. However, the difficulty of assigning this OTU in lower taxonomic classification levels that are based on the existing public genomic databases (i.e. Greengenes and 16S ribosomal RNA sequences database of NCBI) highlights its importance as novel microbe residing in engineered anaerobic digestion ecosystems.

Finally, it is shown that there was a cluster of bacteria, whose relative abundance was significantly reduced in all the samples of R1, indicating that high H_2 partial pressure and VFA accumulation severely inhibited their growth. For example, *Trichococcus* sp. 3 with 100% similarity to *Trichococcus pasteurii* significantly decreased its relative abundance by approximately 165-fold from Period I to Period III (Fig. 6a). Members of genus *Trichococcus* are well known to be present in anaerobic reactors processing sludge and dairy manure. Moreover, they are characterized by a homofermentative metabolism leading to the production of lactic and acetic acids from several carbohydrate-rich substrates [30]. Another bacterium that presented high decrease among this cluster was *Pelotomaculum isophthalicicum* 37 showing 98% identity

Table 3
Comparison of *in-situ* and *ex-situ* upgrading processes.

Upgrading system	Reactor type	Temp. °C	Substrate (*in-situ*) Inoculum (*ex-situ*)	OLR g VS/(L reactor/day)	H_2 flow rate L/(L reactor/day)	CH_4%	CO_2%	pH	Reference
in-situ	CSTR	55	Cattle manure and potato-starch	1.7	0.41*	86.4	10.7	8.6	Current study
in-situ	CSTR	55	Cattle manure and whey	1.66	1.7	75	15	7.8	[11]
in-situ	CSTR	55	Cattle manure and whey	1.66	0.93–1.76	78.4–96.1	47–9	7.61–8.31	[14]
Ex-situ	Up-flow	55	Enriched Hydrogenotrophic culture			91	7	8.1	Current study
Ex-situ	Up-flow	55	Enriched Hydrogenotrophic inoculum		1–3.6**	89.5–96.3	14.5–0	8.03–8.81	[10]
Ex-situ	Up-flow in series	55	Anaerobic digestate		0.79**	98	50	8.5	[2]

* Value derived considering R1 + R2 volume.
** Values derived from H_2% in the feed gas mix and mix flow rate.

Table 4
Summary of sequencing results with alpha diversity indexes.

Sample	Replicates	Experimental Period	External H_2 addition	VFA concentration	Raw reads	Reads assigned to OTUs (%)	OTUs > 0.01%	OTUs > 0.5%	Diversity > 0.5% (%)
R1-1	3	I	No	Standard	222,597	56%	322	20	79%
R1-2	1	II	Yes	High	434,264	68%	392	16	75%
R1-3	3	II	Yes	High	315,084	57%	460	31	69%
R1-4	1	III	No	Standard	408,442	72%	357	27	73%
R2-3	3	II	Yes	High	359,785	66%	321	20	80%
R2-4	3	III	No	Standard	411,433	63%	391	25	75%

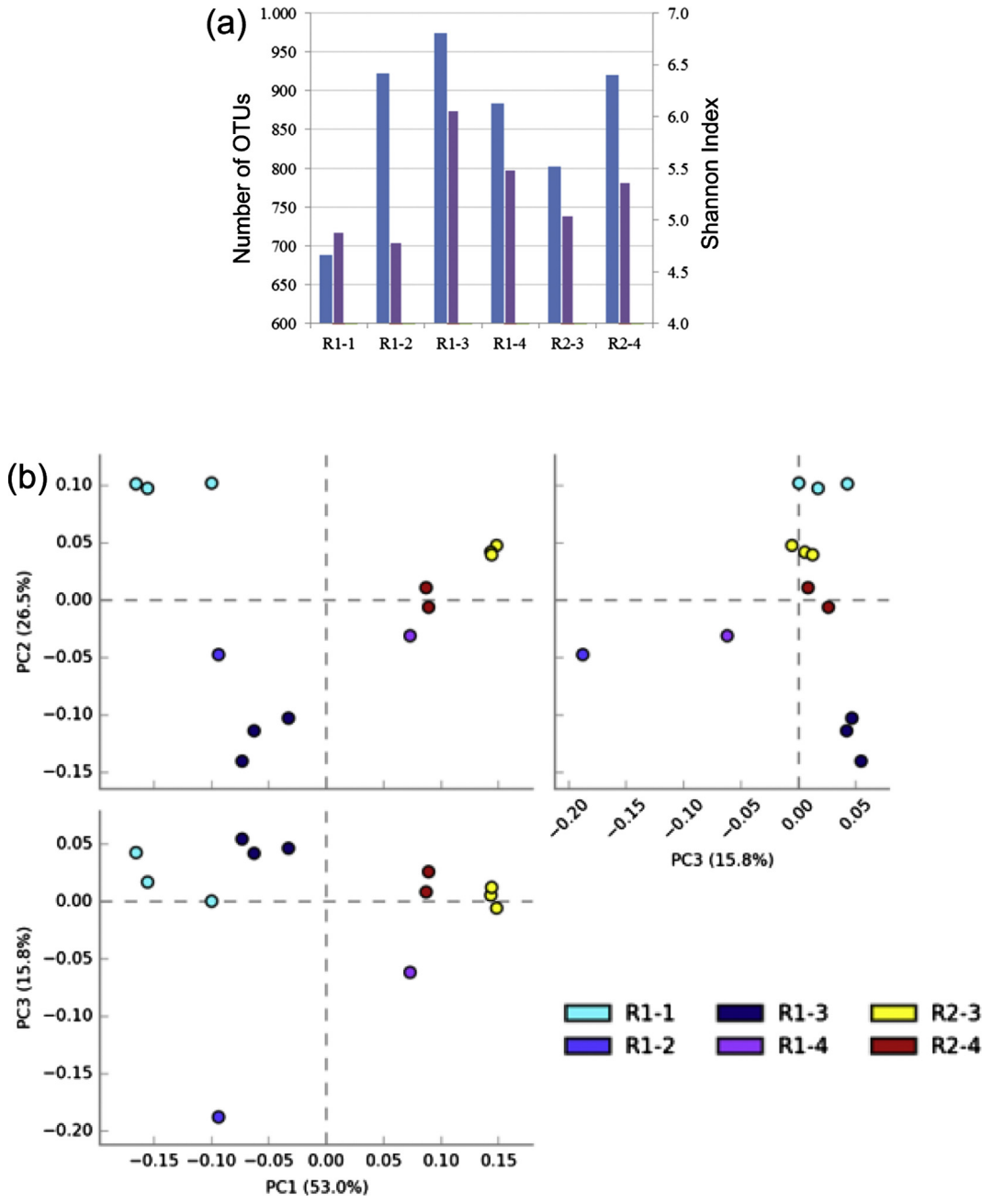

Fig. 4. (a) Alpha diversity histograms of OTUs and Shannon Index for all samples of both reactors and (b) beta diversity.

to *Pelotomaculum isophthalicicum*; this OTU was found to decrease its relative abundance by 84-fold upon H_2 addition.

3.3. Practical implications of the study

During the experiment, the close monitoring of the reactors' performance provided practical considerations regarding the biological aspects of upgrading technology, and other aspects that are more

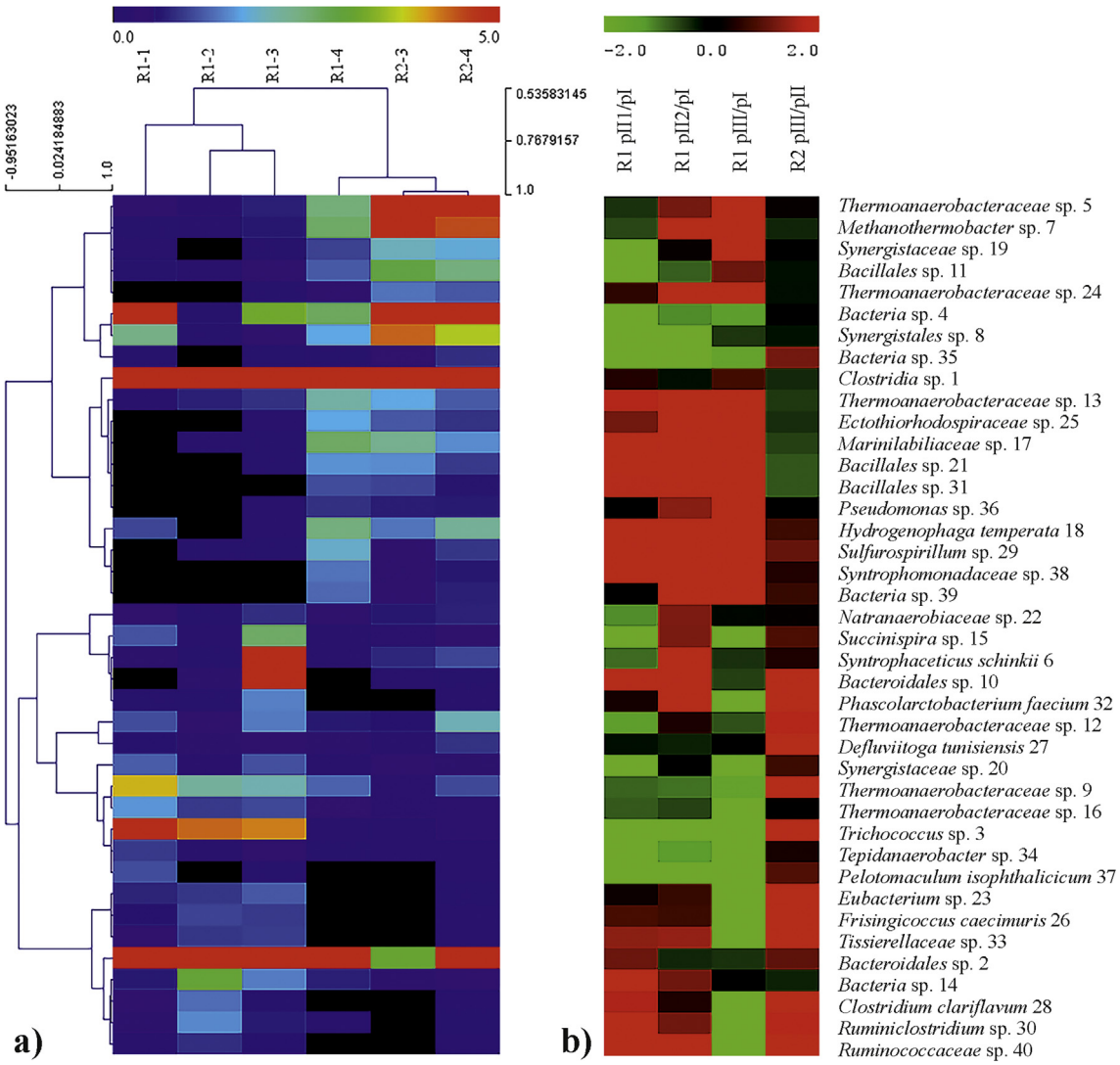

Fig. 5. Heat maps of relative abundance (%) (a), and fold change (log 2) (b) of the most abundant OTUs. Colour scales are shown on top of each panel. On the left panel, the most abundant microorganisms are shown in red colour and the less abundant in blue and black. On the right panel, the relative abundance increment in fold change is coloured by red, while the decrease in fold change is coloured in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

related to process operation. These implications can facilitate further studies on biological upgrading systems. In general, for a future scaling up of the hybrid upgrade system, the main challenge will be to improve the overall stability of the process. In particular, a close monitoring and control of the VFA is mandatory since it was shown that the system might be prone to accumulation of intermediate compounds that will in turn influence negatively the CH_4 production rate. Therefore, a period of biomass adaptation must be taken into account as the immediate addition of H_2 led to a considerable accumulation of VFA in R1, mainly in the form of acetate. Indeed, in the R2 reactor, which was inoculated with a microbial consortium previously adapted to H_2 addition, the corresponding concentration of VFA was remarkably lower. Moreover, the exploitation of mixed hydrogenotrophic cultures in biological methanation systems has been demonstrated to benefit the overall process in comparison with the use of pure cultures due to increased process robustness and cost reduction [1]. Particular attention should also be paid to the pH control, which is an essential parameter for the stability of the process. In the current study, a slight increase in pH in reactor R1 was observed, due to the *in-situ* removal of CO_2. The absorption of bicarbonate imbalances the buffer capacity of the system [11], and thus, leads to incremental increase of pH at levels that can exceed the optimum range for methanogenesis [31]. Another key parameter for the operation of all H_2-mediated upgrading technologies is the poor gas-liquid mass transfer [1]. This technical challenge can be solved by using more advanced gas dispersion systems [9] or by increasing the gas-liquid contact time using different reactor configurations (e.g. fed-batch reactor) [20]. In the current study, excellent results (i.e. 98% H_2 utilisation efficiency) were obtained using three steel diffusers in R1 (supported by magnetic stirring) and a ceramic membrane in R2. Moreover, it should be noted that the *ex-situ* reactor was supplemented with degassed digestate as nutrient source. For full-scale demonstration of the biomethanation concept, this nutrient source offers a great advantage in terms of limited consumption of chemical reagents, improving the overall cost balance.

4. Conclusions

The outcomes of the present work demonstrate the feasibility of the hybrid concept and also identify specific issues that need to be addressed for further process development. The methane content in the final output gas reached on average 91% (with a maximum of 95%). The CO_2 was decreased by 57% and 98% of the injected H_2 was utilized.

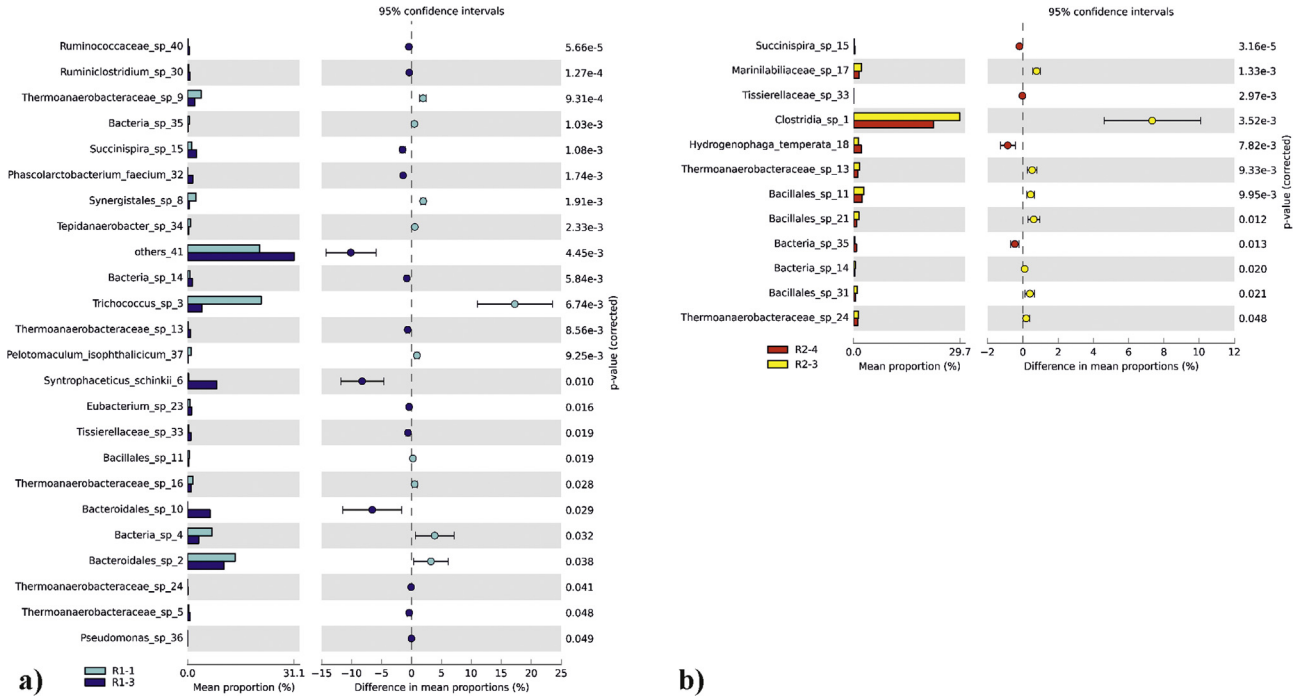

Fig. 6. Statistical comparison between different extraction point samples; (a) comparison between Period III (R1-3) and Period I (R1-1); (b) between Periods II (R2-3) and III (R2-4) of R2.

The removal and subsequent conversion of CO_2 to CH_4 slightly increased the pH, maintaining it within the range of optimal methanogenesis. Moreover, it was shown that the addition of exogenous H_2 in the system might cause an abrupt accumulation of intermediate compounds, such as acetate, limiting the enhancement of CH_4 production. Therefore, the outcomes of this study clearly underline the importance of monitoring the concentration of VFA to ensure a stable and efficient biomethanation process. Results from the microbial analysis showed that the most abundant microbes belong to uncharacterized taxa. This argument clearly demonstrates that the anaerobic digestion microbiome during hydrogen assisted methanogenesis is strongly stimulated to increase in diversity towards the hydrogenotrophic community contained in the *ex-situ* reactor. The findings of the present research work can be directly exploited by other researchers or biogas plant operators for developing strategies targeting the optimisation of biological biogas upgrading technologies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enconman.2018.04.074.

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