
**POLITECNICO DI MILANO**

**Department of Civil and Environmental Engineering, DICA**

**Doctoral programme in Environmental and Infrastructure Engineering, IAI**

# BIOLOGICAL $H_2$-MEDIATED IN-SITU BIOGAS UPGRADING

Doctoral Dissertation of: Viola Corbellini

Supervisor: Prof. Malpei Francesca

Tutor: Prof. Malpei Francesca

Chair of the Doctoral Program: Prof. Guadagnini Alberto

**XXXI Cycle (2015-2018)**

# Acknowledgments

At the end of this challenging experience, there are so many people I would like to thank, each one for a specific contribution.

First of all, the person who guided on my doctoral studies as a supervisor and tutor, Professor Francesca Malpei. Thank you for giving me the opportunity to grow both from a professional and a personal point of view during these years. Your punctual advice have allowed me to improve relevant aspects of my professional figure.

Likewise, I would thank all DICA professors, especially Professor Elena Ficara and PhD Davide Scaglione for having opened my sight to the wonderful and fascinating world of scientific research, from my early Master's degree. I would also express my special thanks to Professor Roberto Canziani for having encouraged me along the way, and for his valuable advice. Moreover, a mention of acknowledgment goes to the Director of the Dica Department, Professor Guadagnini with whom I had the honor of cooperating as PhD's candidate's representative. Thank you for allowing me to take part in all the engaging initiatives dedicated to the candidates.

A deep thank to all the uncountable mates met over the years: Tommaso, Giulia, Luigia, Andrea, Alida, Arianna but in a special way, Davide Beatrice and Matteo …without your laughs and support it would have been definitely harder. Thanks also to Nadia for technical support in the lab, and to Lia and Giovanna for your essential secretarial assistance.

A special thanks to professors Angelidaki and Kougias who very kindly welcomed me for a research period stay by Bioenergy group at DTU. Ilaria, Alessandra, and Maria thank you so much for having shared with me such an amazing period in Copenhagen. Heartiest thanks also to Marianne Sandberg, my dearest Danish friend with whom I shared a crazy experience I will never forget of diving, in winter, into the North Sea. Finally, thank my parents and my brothers who have participated (very actively!) in the formation of my temper, and giving me the grit I need every day. Lastly, thank you, Alessandro, for being with me with your deep love from the very first moment of this path.

# Summary

# Preface

This PhD thesis, entitled "Biological $H_2$-mediated *in-situ* biogas upgrading" comprises the research project carried out at the Department of Civil and Environmental Engineering at Politecnico di Milano from November 2015 till November 2018. Full Professor Francesca Malpei was in the role of Supervisor and Tutor. Full Professor Guadagnini was in the role of IAI Doctoral programme coordinator and Full Professor Roberto Canziani was in the role of IAI Doctoral programme vice-coordinator.

## Objective

The general objective of this work was the study of the innovative *in-situ* biological biogas upgrading via the enhancement of the hydrogenotrophic methanogenesis. More in detail, the project developed the proposal of a protocol for the hydrogenotrophic methanogenesis specific activity (SHMA) measurement that was evaluated as a useful tool for monitoring activity during the continuous upgrading process. Furthermore, a novel rapid start-up strategy to obtain the enrichment of hydrogenotrophic biomass *in-situ*, i.e. within the complex anaerobic biomass consortium was design and assessed. Thereafter, during a research period abroad at the Technical University of Denmark (DTU), an experimental trial was performed aiming at testing a novel set-up concept of the biological upgrading process, called "hybrid". Finally, a further long trial was conducted assessing the optimal operating conditions of the biological *in-situ* biogas upgrading process for a scaling-up purpose. The last two studies have been enriched with microbial investigations through 16rRNA analysis aimed at identifying the changes occurred within the anaerobic consortium aimed to deepen the comprehension of the biochemical implications of the process.

## Outline

This thesis is composed as follows: a first general introduction and state of the art review; then the work is developed through a collection of articles; finally, a chapter with general conclusions and perspectives is presented. Titles of the papers that constitute this thesis, and conference presentations are listed below:

I. Towards a standard method to measure the specific hydrogenotrophic methanogenic activity (SHMA) **paper in preparation;**

II. Corbellini, V., Catenacci A., Malpei F. (2019) "Hydrogenotrophic biogas upgrading integrated into WWTPs: enrichment strategy", **paper published on** Water Science and Technology https://doi.org/10.2166/wst.2019.096**;**

III. Corbellini, V., Kougias, P. G., Treu, L., Bassani, I., Malpei, F., and Angelidaki, I. (2018) "Hybrid biogas upgrading in a two-stage thermophilic reactor". **Paper published on** Energy Conversion and Management,168. https://doi.org/10.1016/j.enconman.2018.04.074;

IV. Biological biogas upgrading via in-situ hydrogenotrophic methanogenesis from sewage sludge: continuous operation and microbial investigations **paper in preparation;**

V. Corbellini V., Franzetti A., Malpei F., Microbial analysis of the *in-situ* biological biogas upgrading reactors, **paper in preparation.**

## *Presentations*

### *Oral presentations*

- **Corbellini V.**, Kougias P.G., Treu L., Bassani I., Malpei F., Angelidaki I. Hybrid biogas upgrading in a two-stage thermophilic reactor. 12th International Conference on Sustainable Development of Energy, Water and Environment Systems SDEWES, 4-8 October 2017, Dubrovnik, Croatia.
- **Corbellini V.,** and Malpei F. Upgrade del biogas per via biologica: prospettive. Il biometano prodotto dagli impianti di depurazione, Tecnologie innovative delle nuove "bioraffinerie" e prime esperienze aziendali". FAST (Federazione delle Associazioni Scientifiche e Tecniche), 23 October 2017, Milan, Italy.
- **Corbellini V.** & Malpei F.: "Biological biogas upgrading to biomethane: a novel route to be integrated in WWTP. International conference Sludge Management In Circular Economy, SMICE2018, 23-25 May 2018, Rome, Italy.

### *Poster presentations*

- **Corbellini V.**, Faravelli T., and Malpei F. Hydrogenotrophic methanogenesis: a promising upgrade bio-technology for WasteWater Treatment Plant". 4th International Conference on Renewable Energy Gas Technology, REGATEC, 22/23 May 2017, Pacengo (Verona), Italy.

# Abstract

In the current context of global energy demand increasing, and developing countries population expansions, renewable energy generation has to grow due to its key role in reducing global greenhouse gas emissions and offering enormous potential for replacing fossil fuels. Furthermore, for the fact that some renewable sources naturally fluctuating allow an intermittent production, it is essential to have a system able to converting the electricity produced into a form of storable energy such as methane. Furthermore, the recently proposed Power-to-Gas (P2G) technology consists of the highly smart integration of such naturally fluctuant renewable sources off-peaks to generate hydrogen via water electrolysis that can be further oxidized to methane and water. A quite recent process intensively investigated to achieve $H_2$ methanation is the biological reaction of $H_2$ and with external $CO_2$ sources into $CH_4$. This technology represents at the same time also an innovative option method for biogas upgrading which seems to be cheaper/lower energivoros if compared to currently available biogas upgrading technologies on the market.

The main goal of this PhD thesis was the study of the innovative biological upgrading process by means of *in-situ* hydrogen injections mainly focused on the biogas from sewage sludge. The biological upgrading biogas process is based on the exploitation of a well-known archaeal methanogenic metabolism of the hydrogenotrophic methanogens, autotrophs that utilize $CO_2$ for growth purpose and hydrogen as an electron donor, producing bio-methane. The novelty relies on the enhancement and optimizing of the process by means of exogenous $H_2$ injection to achieve biogas upgrading into biomethane. The use of this process for this specific purpose so far has been studied in two different set-ups, distinguished by where the $H_2$ is provided with respect to the anaerobic digestion process: *in-situ* option, in which $H_2$ is delivered directly inside the biogas digester and there biologically coupled with the endogenous $CO_2$ produced; the *ex-situ* option, in which $CO_2$ from external sources (e.g. biogas, $CO_2$ storage, syngas) and $H_2$ are injected together inside a reactor containing selected hydrogenotrophic cultures, resulting in their conversion to $CH_4$. Previous studies demonstrated the feasibility of *in-situ* and *ex-situ* biogas upgrading achieving $CH_4$ content of 95% under various conditions (Luo & Angelidaki 2013; Bassani et al., 2017; Kougias et al., 2017). However, since $H_2$ plays a key role in the whole anaerobic trophic chain, its exogenous addition *in-situ* may affect the normal running of the uncountable simultaneous reactions. It has been reported that especially during the *in-situ* process one technical challenge is the increased pH due to the bicarbonate consumption, which may cause inhibition of methanogenesis. 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 biological

methanation process, common in both *in-situ* and *ex-situ* concepts, is the poor gas-liquid $H_2$ mass transfer that can be alleviated by using more efficient gas dispersion systems or reactor configurations (Rachbauer et al., 2016; Luo et al., 2012).

In this dissertation a first study was aimed to provide fundamental elements to propose a simple protocol to measure the specific hydrogenotrophic methanogenic activity (SHMA), moving towards a standard activity measurement method not yet defined among the scientific community. Manometric batch tests assessing the specific hydrogenotrophic methanogenic activity of three anaerobic sludges taken from municipal full-scale digesters were assessed. Two different experimental set-ups were utilized for comparison: an automatic manometric device and a manual measurement system. Statistical analysis was carried out aimed at assessing the reproducibility of replicates for each sample of sludge performed with the two different apparatus. The SHMA standard measure will support the implementation of the process at full-scale in evaluating the initial substrate loads that can be treated (Souto at al., 2012) and could allow to carry out toxicity tests with respect to certain substrates (Lema at al., 1991). The activity determinations were conducted at substrate concentration well above Ks value to ensure to operate in the zero order kinetics conditions. Both apparatus allowed measuring the SHMA, further investigations are needed to test the reproducibility of another automatic system coupled with bottles with greater pressure-tightness. A noteworthy major result is that the statistical non-reproducibility may not lead to an error in the estimation of the kinetics; vice versa statistically reproducible replicas can lead to errors in the estimation of the kinetic parameter.

In order to establish and perform a stable process of *in-situ* biogas upgrading, a further experimentation was focused on a novel rapid enrichment strategy capable of limiting the organic degradation unbalance and allowing a fast start-up phase of the *in-situ* biogas upgrading reactors, at pilot or full-scale. This fundamental theme has been perused with 2+1 control lab-scale CSTRs filled with anaerobic sludge collected from a full-scale WWTP. The experimentation lasted 50 days and was divided into 5 phases: the anaerobic digestion start-up followed by four $H_2$ injection phases ($H_2/CO_2$ ranging from 1:1 to 4:1 on a molar basis). Despite a temporary slight increase in the total concentration of volatile fatty acids during phase II (2.56 $gHac \cdot L^{-1}$), and in phase III a mild pH increment indicating the expected $CO_2$ depletion (anyway below 7.4), the strategy proposed was effective and allow to achieve a very short process start-up methodology. In the last phase, in the biogas, the methane content of about 80% was achieved, thus suggesting that the use of $H_2/CO_2$ above the stoichiometric value could further improve the biological biogas upgrading.

To explore new process configuration, an innovative biological upgrading set-up, called Hybrid, was designed and evaluated in a continuous experiment lasted 4 months at DTU University

(Denmark). This innovative design exploits the combination of the *in-situ* and the *ex-situ* processes in a combined configuration. The system consists of a double-stage reactor composed of a CSTR, working as a conventional anaerobic digester and where the $H_2$ is injected (*in-situ* biogas upgrading), and an up-flow reactor, receiving the upgraded biogas from the CSTR, together with the unutilized $H_2$. The overall objective of the work was to perform 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 $CH_4$ 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 (VFA) was observed in the first reactor (*in-situ*) after 8 days of continuous $H_2$ injection reaching a concentration of 5.6 gVFA/L. Nevertheless, after an adaptation period of one hydraulic retention time (HRT), the system started to recover from the stress and the VFA decreased to 2.5 g/L. No pH drop was recorded during the period characterized by increased VFA concentration mainly due to the consumption of the endogenous $CO_2$ by hydrogenotrophic methanogenesis. Indeed, the bicarbonate contained in the liquid phase of the biogas reactor was coupled with the injected $H_2$, and thus the pH was maintained within the range for optimal methanogenesis (i.e. slightly increased from 8.3 to 8.5) despite the high VFA accumulation. The effect of $H_2$ injection on the microbial community in both reactors was analyzed by 16S rRNA gene amplicon sequencing. The results demonstrated an increment in the relative abundance of hydrogenotrophic methanogens and homoacetogens in *in-situ* reactors, while the microbial community in the *ex-situ* chamber was more simple dominated by hydrogenotrophic methanogens.

Finally, a further experiment was designed and operated to elucidated $H_2/CO_2$ ratio correlation on the $CH_4$% in the output gas process stability, VS% destruction as indicator of the running of substrate degradation, microbiological evolution analysis during the enrichment and the continuous process operations. The biological *in-situ* biogas upgrading from sewage sludge and in continuous-mode was investigated thus during a period of more than 7 months. 2 parallel Continuous-flow Stirred Tank Reactors (CSTRs V=11L) were fed on a mixture of sewage sludge at mesophilic conditions at fixed organic loading rate (OLR) of (1.5 gCOD $Lr^{-1}d^{-1}$), $H_2$ gas injections progressively increased from 0:5:1 to 7:1 ($H_2/CO_2$ ratio) with pH controlled to 7.4. Maximum methane content of 83% and a minimum of 5% of $CO_2$ and 91% of $H_2$ utilization were achieved at 7:1 $H_2/CO_2$ ratio. A noteworthy ethanol accumulation, during the very first $H_2$ Phase ($H_2/CO_2$ of 0.5:1) occurred (up to 2.5-3 gCOD $L^{-1}$). Nonetheless, maintaining the $H_2$ feeding, ethanol was rapidly depleted, thus indicating the system was able to withstand the new operative conditions. A significant alkalinity reduction due to $CO_2$ depletion in the liquid phase of 50% and 17% in R1 and

R2 was registered. Also in this work, the effect of $H_2$ injection on the microbial community in both reactors was analyzed by 16S rRNA gene amplicon sequencing. Results revealed a new shape of the core microbial community able in co-operating to the parallel organic substrate degradation and $CO_2$ conversion to extra methane. More in detail, the anaerobic consortia presented a slight variation of the bacterial community in which homoacetogens were detected, and to an archaeal community mostly composed by hydrogenotrophic methanogenic species and only one acetoclastic methanogenic species.

# 1. Introduction

## 1.1. Renewable energy background

According to IPCC, (2011), economic development is strongly correlated with both increasing energy use and growth of GHG emissions. Renewable energy sources (RES) have the valuable ability to decouple that correlation, permitting sustainable development (SD). The last statistical review of world energy consumption reported a slight increase of 2.2% in 2017 respect to 2016, and of about 1.7% above its 10-years average (British Petroleum, June 2018). The major contribution of the energy world consumption came from developing countries, such as India and China that largely utilize fossils fuels, accounting for about 80% of that growth. In these countries, renewables represent for both only 3% of their overall energy consumption. Moreover, it is disconcerting, considering also that latest United Nations (2017) projections on population growth reported an overall 9.8 billion people by 2050 and that in particular China and India will reach respectively 1.4 and 1.3 billion inhabitants.

Moreover, also in the most developed areas like European Union countries, the dominant fuel remains oil, even if the share of renewables in total power (heat and electricity) generation slowly increased from 7% in 2016 to 8% in 2017. In this world energy context, greenhouse gases (GHGs) emissions reached about 49.3 $GtCO_2$ equivalents in 2016, with China as the most important contributor (**Figure 1**).

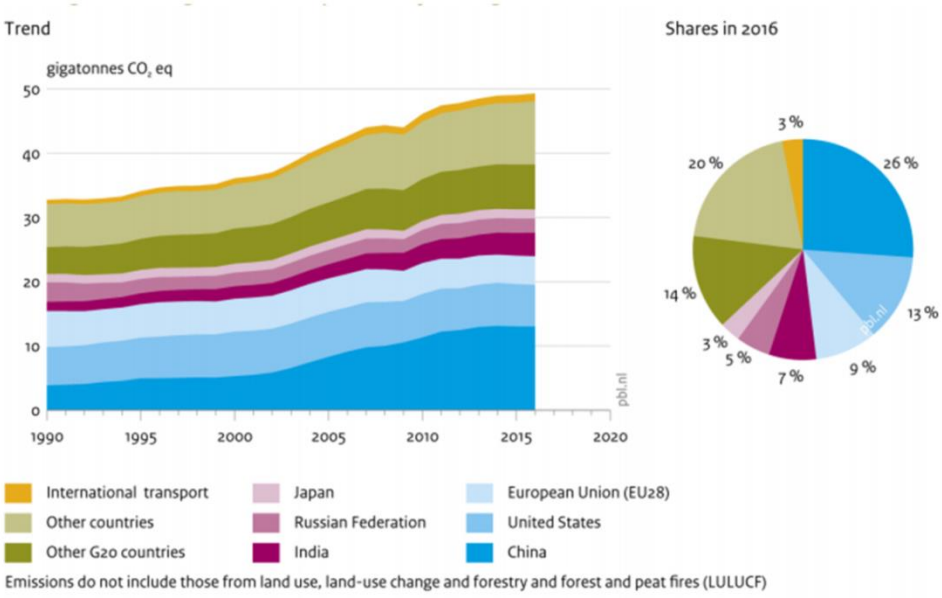



**Figure 1. The 20 largest economies $CO_2$eq emission trends on the left, and share in 2016 on the right (Oliver et al., 2017).**

The increment on the renewable energy sector is mostly due to the expansion of wind power, solar power and solid biofuels (including renewable wastes). Despite the fact that hydropower remained the largest source for renewable electricity generation (in 2016 36.9 % of the total), electricity generated from wind turbines and from solar dramatically increase of 3.7-folds and 44.4 folds from 2006 to 2016 (**Figure 2**). The shares of wind and solar power in the total quantity of electricity generated from renewable energy sources rose to 31.8 % and 11.6 % in 2016, respectively (Eurostat, 2016). The share of gross final energy consumption from renewable sources reached 17% in the European Union (EU) in 2016, doubling the share of 2004 (8.5%). Furthermore, among the 28 EU Member States, eleven members already reached their national 2020 targets; Italy included (17.4 out of a 17% target). Projections show that most countries will exceed the target of 20% in 2020 (Scarlat et al., 2018).

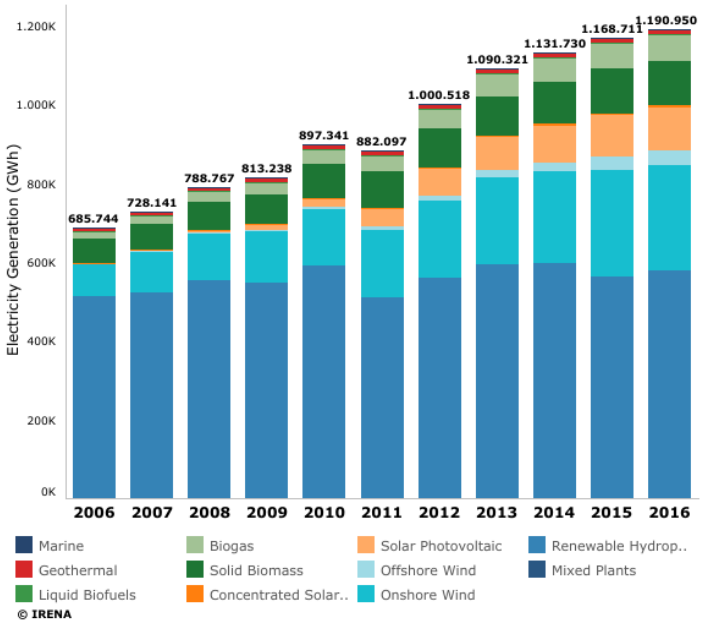



**Figure 2. Trends in Renewable Energy (Electricity Generation) from 2006 to 2016 in the European Union (source data: Irena).**

European Union, in June 2018, agreed to further increase the binding target of at least 32% share of renewable energy by 2030 (the previous was 27%), more in detail:

- first generation biofuel, based on food crops, must be capped at 2020 levels (with an extra 1%) and in no case exceed 7% of final consumption of road and rail transport;
- The share of advanced biofuels and biogas must be at least 1% in 2025 and at least 3,5% in 2030.

Thus, the EU Members should define their contribution to the achievement of this increased target as part of their Integrated National Energy and Climate Plans. Given the global energy picture, and

even more so the closest one, it is, therefore, necessary to continue with intensity in the effort to find, and study, technology advancements that will support an ever-increasing spread and capitalization of energy from renewable sources.

## 1.2. Renewable energy from biomass

Biomass feedstock such as bio-wastes, food waste, animal manure, and algae can play a key role to provide renewable energy, while simultaneously preserving environmental compartments. There is a net reduction in greenhouse gas emissions if transformed into biofuels compared to fossil fuels, and at the same time, the production of other valuable by-products in the concept of bio-refinery is achievable. Generally speaking, a wide spectrum of technologies to convert biomass to renewable-fuels (solid, liquid and gaseous) and valuable chemicals (food, flavours, feeds, pharmaceuticals, cosmeceuticals, nutraceutical) are available. Most conversion pathways can be based on physical, chemical, biological, thermal or a combination of processes. A detailed flow chart of conversion processes and their main products are shown in **Figure 3**.

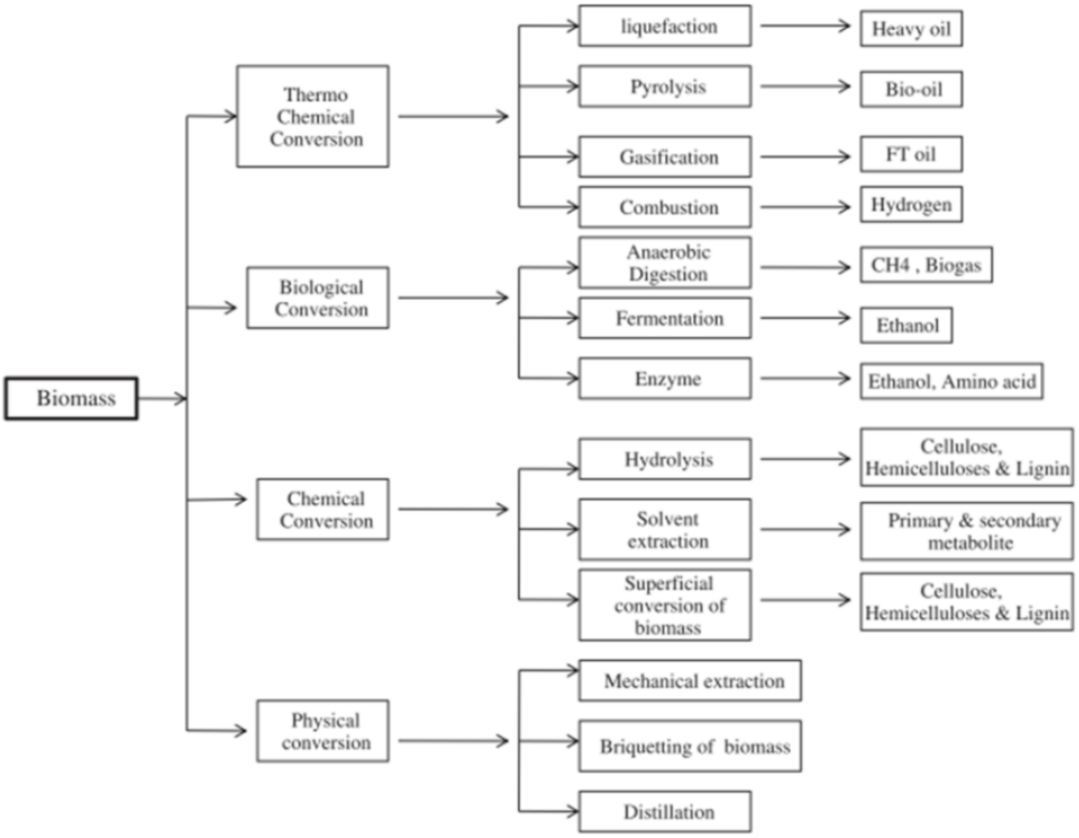



**Figure 3. Biomass conversion processes flow chart.**

Among biological conversions paths, anaerobic digestion (AD) does produce biogas using a variety of wastes and organic sources. Firstly applied for the treatment of sewage sludge, for stabilization purpose, it is currently applied for the treatment of livestock-residues, crop residues, agricultural renewable resources like "energy crops", as well as biological wastes and animal by-products. Besides the production of methane also the effluent, digestate, is usually a valuable by-product that can be spread on land as fertilizer (Guebitz et al., 2015). Compared to thermochemical and thermal conversion techniques, AD is much more flexible in terms of feed materials and it is considered more efficient, both in technical and economic terms (Sarker et al., 2018). Latest statistical numbers released by the European Biogas Association (EBA, 2017) reports that biogas plants among the European Union, in the time period starting from 2009 to 2016, tripled from 6.227 units up to 17.662 units. The strongest growth was recorded between 2009 and 2012 when the number of biogas plants doubled (**Figure 5**). In Italy, the country with the second highest number of AD plants among the European Union, biogas production account for 5% of total renewables production in 2016 (**Figure 4**).

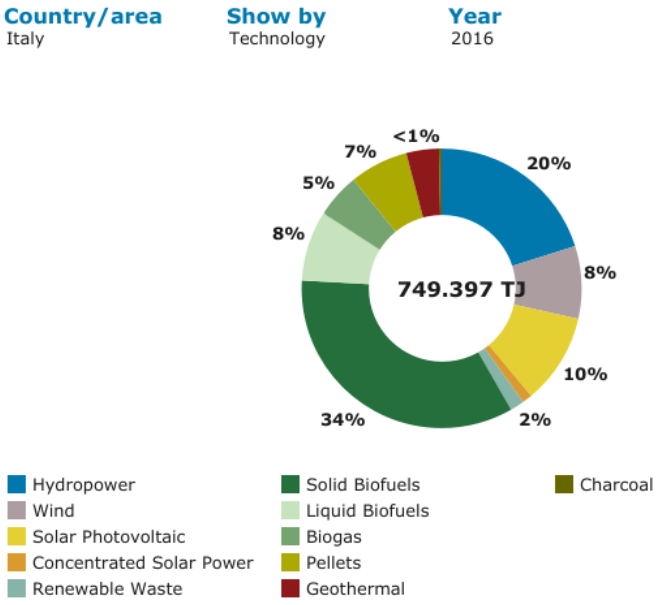



**Figure 4. Italy final renewable energy consumption by country (Irena, 2016).**

In 2016 according to EBA (2017), 12.496 units fed on agricultural substrates, 2.838 plants fed on sewage sludge, 1.604 from landfills and other waste (688 units) (Figure 5). While the World statistic on biogas production reported above 148 TWh in 2017 which nearly 71% is concentrated in the European Union. This global biogas production corresponds to 70% of the IPPC esteem (213 TWh by 2020 AR4, 2011).

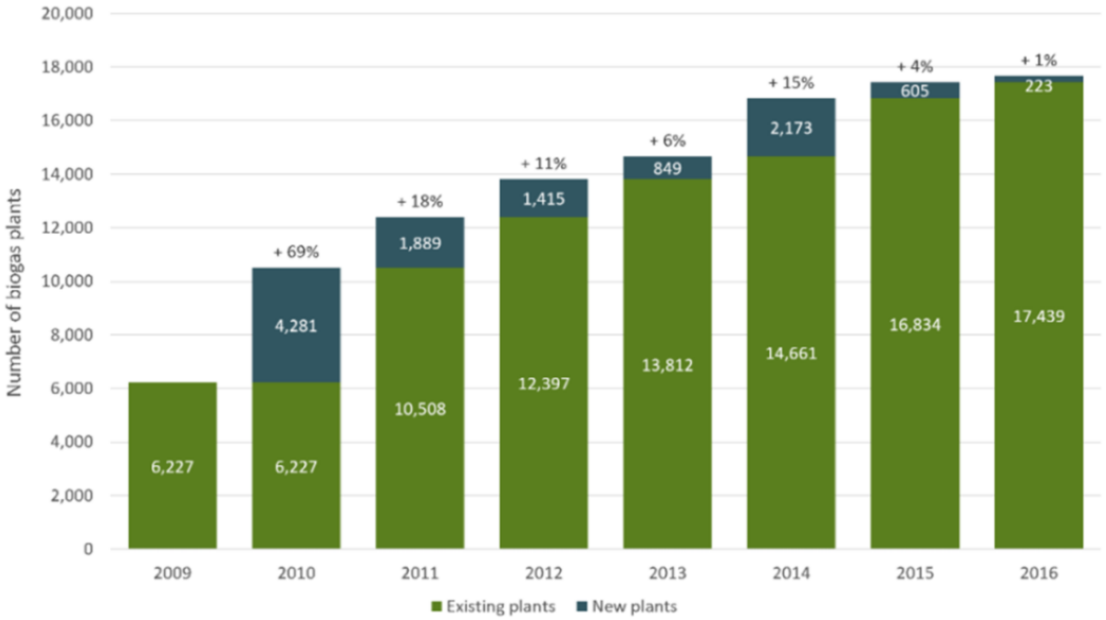



**Figure 5. Evolution of the number of biogas plants in Europe (EBA, 2017).**

One major drawback of biogas from anaerobic digestion of organic substrates is a dilution of $CH_4$ with $CO_2$, and other traces gas ($N_2$, $H_2O$, $O_2$, $H_2S$, $NH_3$ siloxanes) which results in a low biogas calorimetric value (Sun et al., 2015; Awe et al., 2017). The majority of AD installations typically burned biogas in a Combined Heat and Power (CHP) unit providing thermal energy and electricity. Alternatively, it can be purified to a gas comparable to the natural gas to be injected in an existing gas grid or to be used as vehicle fuel. In order to obtain rich-methane biogas, it is necessary to remove $CO_2$, thus obtaining the so-called "biomethane" (Kougias et al., 2017). Specific requirements of biomethane for injection into natural gas grids or for exploitation as a vehicle fuel varies among different countries and in a wide range composition of $CH_4$ 80-96 %, $CO_2$ 2-3 %, $O_2$ 0.2-0.5 % (Patterson et al., 2013; Muñoz et al., 2015) fostering the utilisation of this renewable energy source. Moreover, assisted by the European government policy driver of feed-in tariffs, the biomethane sector is rapidly increasing. In the last decade, biogas purification and upgrading into biomethane have been representing a great opportunity to boost the energy recovery from several types of matrices of wastes and wastewater. From 2011 to 2016, biomethane production increased by +40% (4.971 GWh) (EBA, 2017). Nowadays, in the European countries, there are about 500 biomethane plants, mostly located in Germany (192), the UK (85) and Sweden (63). In Italy 4 plants produce biomethane from the organic fraction of municipal solid waste (OFMSW-FORSU), while three plants are under construction and will come into operation within a few months (Etra - Padua (PD), Acea Pinerolese - Pinerolo (TO); Asja/VUS - Foligno (PG) (**Figure 6**).

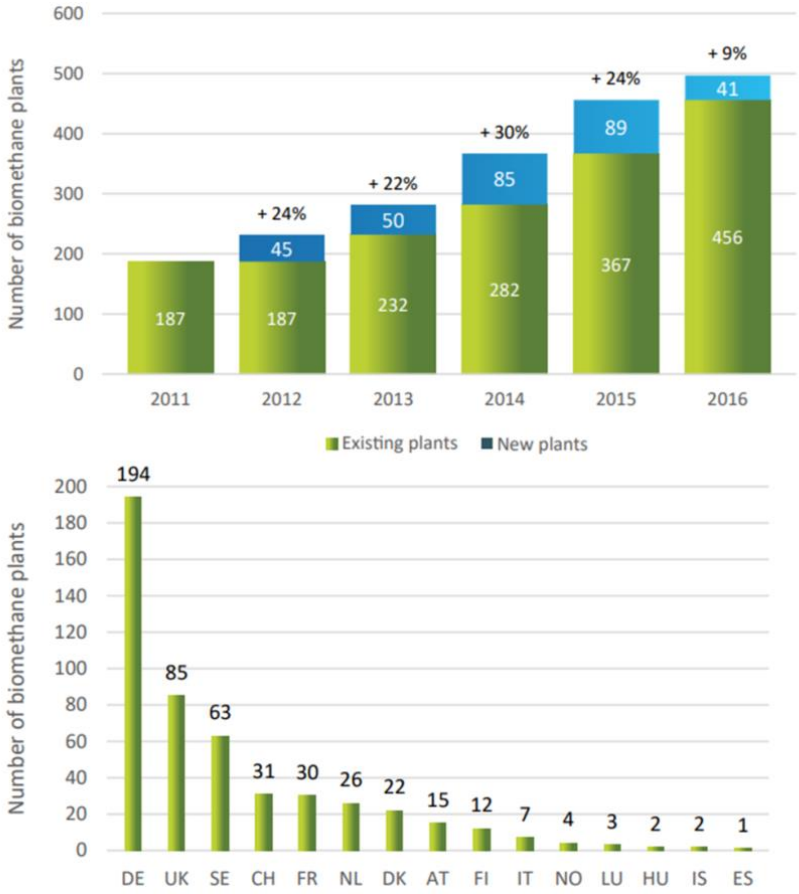



**Figure 6. Number and distribution of biomethane plants in Europe (EBA, 2017).**

Currently, several commercial biogas upgrading technologies are available at full-scale. These systems, simply remove $CO_2$ from biogas, while biological $CO_2$ methanation, discussed in the following paragraphs, is able to capitalize it transforming into extra methane. Moreover, this innovative technology seems to perfectly match the other increasing need to store energy off-peaks from wind and solar power, by transforming it into $H_2$.

## 1.3. Commercial biogas upgrading technologies

Currently available technologies are physical or chemical $CO_2$ absorption (scrubbing with water, $CO_2$-reactive absorbents or organic solvents) pressure (PSA) swing adsorption, membrane, and cryogenic separation. In **Figure 7** the distribution of upgrading technology application in Europe according to IEA (2016) is shown.

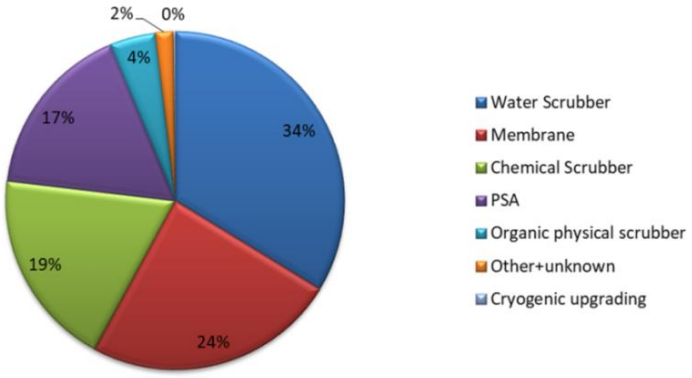



**Figure 7. Upgrading technologies distribution according to IEA 2016.**

Several reviews and studies have comprehensively analysed all commercial technologies taking evaluating technical parameters such as recover efficiency, methane loss, development and commercialisation, energy consumption and economic evaluations. Among these studies: Starr et al., 2012; Scholz et al., 2013; Sun et al., 2015; Khan et al., 2017; Angelidaki et al., 2018. In the following paragraphs, a small hint of the main technologies applied so far at the industrial scale is given.

### 1.3.1. Physical absorption by Water scrubbing

Water scrubbing is the most commonly used biogas upgrading technology in which water is used as a solvent. This process is based on the separation of $CO_2$ and $H_2S$ (only if in low concentrations) due to their greater solubility in water if compared to $CH_4$ (Angelidaki et al., 2018). Since $H_2S$ can cause corrosion problems, a pre-separation of $H_2S$ is normally necessary when it's present in high concentration (Khan et al., 2017). In water scrubbing process the biogas, previously compressed to 6-10 bar at temperatures as low as possible (10-35°C) to increase the relative solubility, is injected into the bottom of the packed absorption column while water is supplied counter-currently as depicted in **Figure 8.**

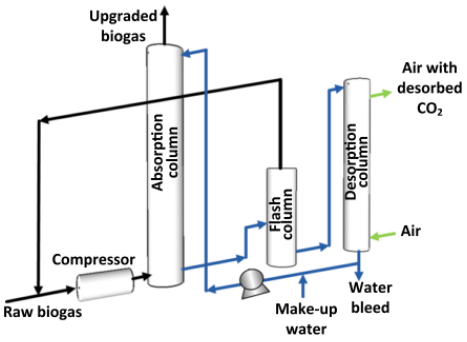



**Figure 8. Water scrubbing scheme; in blue the water stream in black the biogas stream (Muñoz et al., 2015).**

While upgraded biogas is then released from the top of the scrubber, the liquid stream containing $CO_2$, optionally $H_2S$, is sent to the flush column, where the pressure is reduced (2.5–3.5 bar) in order to recover $CH_4$ traces dissolved in the water. Then the water flux is regenerated in the following desorption column, performing the remove $CO_2$ and $H_2S$ usually by means of air stripping.

Regeneration step is crucial for this upgrading technology due to the large quantities of water needs. To upgrade 1000 $Nm^3/h$ of raw biogas 180-200 $m^3/h$ of water is requested, depending on the pressure and water temperature (Bauer et al., 2013). The $CH_4$ content achievable ranges from 80 up to 99%, while, depending on the decompression method, $CO_2$ purity achievable is 80%-90% (Sun et al., 2015).

### 1.3.2. Physical absorption with organic solvents

This method, like water scrubbing, allows $CO_2$ and $H_2S$ to be absorbed using an organic solvent instead of water. Generally, mixtures of methanol and dimethyl ethers of polyethylene glycol are employed. A simplified scheme of the system is depicted in **Figure 9**. The main difference with the water scrubber system is the higher solubility of $CO_2$ in an organic solvent, for instance up to 3 times higher in the case of Selexol®, and as a consequence both smaller dimensions of the upgrading unit and lower product consumption are achievable.

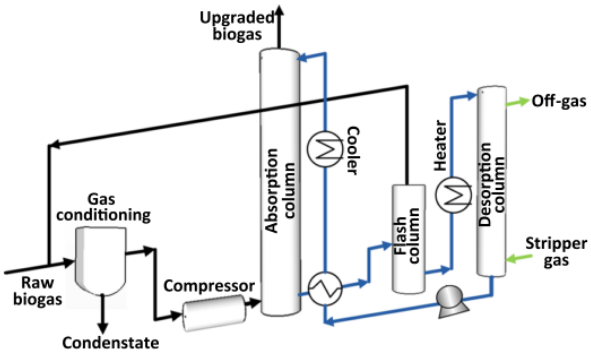



**Figure 9. Organic solvents absorption scheme (Muñoz et al., 2015).**

However, the difficult regenerative capacity of the solvent is a major obstacle. With this process, it is also possible to remove the $H_2S$, but since the temperature to be adopted is proportional to its concentration, it is preferable to provide for a gas pre-treatment for its removal.

In common with other technologies, raw biogas is compressed (7-8 bar) and cooled to about 20 °C before injection from the bottom of the absorption column. Subsequently, the organic solvent is regenerated at high temperature (80 °C) and led to desorption column, where the pressure is reduced to 1 bar (Bauer et al., 2013; Sun et al., 2015). The final $CH_4$ content achievable is up to 98% (Angelidaki et al., 2018).

### 1.3.3. Chemical absorption with amines

In amine-absorption technologies, (**Figure 10)**, solutions such as mono-, di- or tri-ethanolamine are used to chemically bind the $CO_2$ and $H_2S$ contained in the biogas. The raw biogas is slightly pressurised (1-2 bar) and sent to the absorption column from the bottom of the tank, while the amine solution flows counter-currently from above.

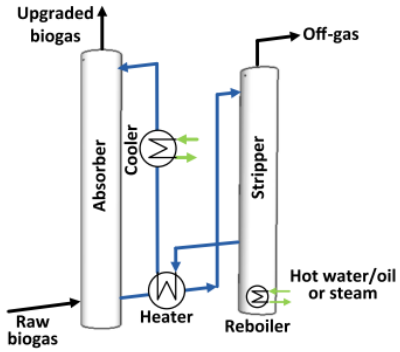



**Figure 10. Chemical absorption simplified scheme (Muñoz et al., 2015).**

Subsequently, the solution used, rich in $CO_2$ and amine, is conveyed to a stripping unit operating at 1.5-3 bar at a temperature of 120-160 °C to recover the amine. The steam-rich in $CO_2$ is cooled in a condenser allowing the condensate to recirculate to the stripper and release the trapped $CO_2$. The main disadvantages of this method include the toxicity of the solvents to humans and to the environment and the high energy requirement for the chemical solutions regeneration. By applying this technology, a final methane purity in the output gas of 99% is achievable. Moreover, due to the fact that that chemical reaction is highly selective, it allows containing methane loss up to 0.1% (Bauer et al., 2013).

### 1.3.4. Pressure swing adsorption

Pressure swing adsorption (PSA) process involves the transfer of solute gases, such as $CO_2$, to the surface of an absorbent material by means of physical or van der Waals forces. As reported in **Figure 11**, a flow diagram for a PSA process consists of a variable number of parallel columns (from 4 up to 9), allowing to continuously feed raw biogas into regenerated columns. Raw biogas needs to be compressed (4-10 bar) before it can be fed. Generally, the most common adsorbents used are activated carbons or zeolites. Recirculation is often used to recover methane still contained in the purified flow. In PSA, a preliminary removal of the $H_2S$ gas is generally adopted due to the irreversible absorption of this gas.

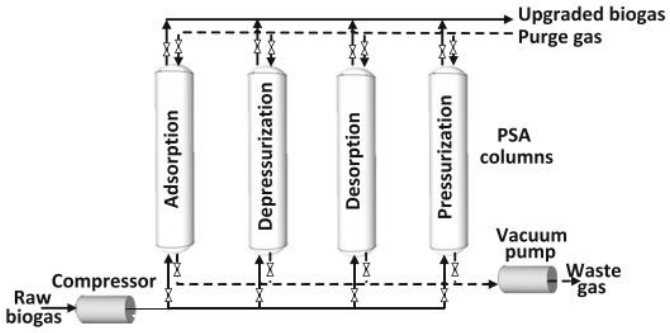



**Figure 11. PSA upgrading process scheme (Muñoz et al., 2015).**

$CH_4$ content achievable in the upgraded biogas varies between 96–98% and up to 4% methane can be lost in the off-gas stream (Angelidaki et al., 2018).

### 1.3.5. Membrane

Membrane upgrading technology exploits the ability of the semi-permeable membrane to select biogas components: $CO_2$ and $H_2S$ pass through as permeates while methane flux is retained. Greater is the difference between the permeability of $CH_4$ and $CO_2$, the more $CH_4$ losses are minimized. In this upgrading process, the raw biogas needs to be pressurised before being treated (5-20 bars). The $CO_2$ capture efficiency varies depending on membranes typology and material: hollow fiber polymeric membranes made of cellulose acetate or polyamide are the most commonly used (Bauer et al., 2013).

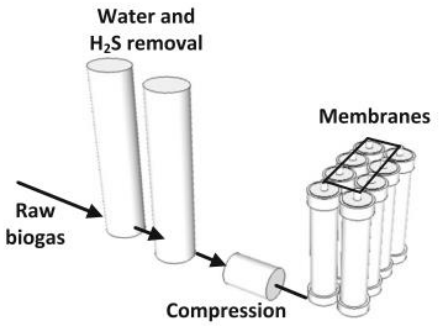



**Figure 12. Membranes technology simplified scheme (Muñoz et al., 2015).**

In order to achieve the highest methane content, an optimal combination of single or multi-stage layouts can be adopted (single stage, two-stage plus a recirculation loop, the two-stage with sweep biogas stream and the three-stage with sweep biogas stream), a simplified scheme can be seen in **Figure 12**. However, the multistage membrane compared to single stage process has lower investment and operating costs and permit to achieve higher $CH_4$ purity: $CH_4$ could rise from 80 to 99.5% using multistage membrane process (Khan et al., 2017). In 2018, a membrane biogas upgrading plant from sewage sludge was completed at the Bresso/Niguarda wastewater treatment plant (220'000 population equivalent PE) in Milan, Italy. The plant which treats civil, industrial and meteoric wastewater is equipped with AD treating mixed primary and secondary sludge in two reactors of (600 $m^3$) at the mesophilic condition. The treatable raw biogas flow is of about 100 $m^3/h$ and methane content in the gas output achievable is > 99.5%. Biomethane is than fed into the Italian Snam gas network and used for transport purposes.

### 1.3.6. Cryogenic

The still little used and very expensive cryogenic technology is based on the different condensation/liquefaction temperature of the biogas components (Zabranska & Pokorna, 2017). By

gradually reducing the temperature is possible to operate a selective separation: $H_2O$, $H_2S$, $CO_2$ are removed while the output stream is already in the form of liquefied methane (free of $O_2$ and $N_2$) at temperatures between -162 and -182 °C. The cryogenic biogas upgrading can be carried out at constant pressure (10 bar) in order to avoid the sudden solidification of $CO_2$ below -78°C. The common procedure involves a preliminary biogas drying step followed by a multi-stage compression (with intermediate cooling) up to 80 bar, then the biogas is gradually cooled to -45 and -55 °C achieving the liquefaction of most part of the $CO_2$, and then expand to 8-10 bar in a flash tank (-110°C) to facilitate the purification of the biomethane through the solidification of the $CO_2$. Cryogenic upgrading can provide biomethane with a purity higher than 97%, with methane losses of less than 2%. Water, $H_2S$, siloxanes, and halogens must be removed before $CO_2$ to avoid operational problems such as pipe clogging or heat exchangers damages (Bauer et al., 2013).

### 1.3.7. Biogas upgrading technology comparison

All commercial biogas upgrading technologies are characterized by specific advantages and disadvantages, and beyond the cost, relevant aspects to be compared are energy, chemicals or water consumption, space availability, tolerance of other gas trace compounds, output gas purity characteristics, modularity, and the methane recovery. The main advantages and disadvantages of the different technologies are summarised in **Table 1**.

**Table 1. Summary of pro and cons of commercial upgrading technology (Ryckebosch et al., 2011).**

| Technology | Pro | Cons |
|---|---|---|
| Water Scrubber | Easy to manage; adaptable by changing pressure or temperature; reduced $CH_4$ losses<br>Tolerate impurities in biogas | High investment and operating costs<br>Clogging for bacterial growth<br>Possible foaming effects<br>Low flexibility on variations of biogas volumes to be treated |
| Ammine Scrubber | Low operating costs<br>Regenerative<br>Higher solubilized $CO_2$ per unit volume (compared to the Water Scrubber) | High investments<br>Heat demand for regeneration<br>Possible corrosion<br>Degradation or undesirable reactions of amines in the presence of $O_2$ or other chemicals<br>Salt precipitation<br>Possible foaming effects |

| | | |
|---|---|---|
| Physical Scrubber | Regenerative<br>Lower energy costs compared to water scrubbers<br>Reduced $CH_4$ losses | High investment and operating costs<br>Reduced capacity if glycol diluted with water<br>Operational difficulties and incomplete regeneration during stripping/vacuuming |
| PSA | Reduced energy consumption: high pressures but regenerative<br>The compact system, also suitable for small volumes Tolerated impurities in the biogas | High investment and operating costs<br>High control required during operation<br>Possible $CH_4$ leakage (in case of valve malfunction) |
| Membrane -Gas/gas | Simple installation<br>Economically flexible in case of reduction of treated volumes | Low membrane selectivity: reduced purity and $CH_4$ volumes achievable<br>Required more steps (in modular systems) to obtain high purity |
| Membrane -Gas/liquid | Low operating and investment costs<br>Obtainable pure $CO_2$ | |
| Cryogenic | $CH_4$ and $CO_2$ of high purity<br>Reduced extra costs to obtain LBM (liquid biomethane) | High investment costs<br>High operating costs |

## 1.4. Power to gas

Many EU countries achieved the target of 25% renewable electricity in the grid (Nastasi & Lo Basso, 2017) as a yearly average. But, the energy production from naturally fluctuating renewables during the day can exceed the permissible grid value (up to 50%). The energy "extra load" (e.g. high wind peak loads) due to their contribution in emphasizing market volatility for a frequent sudden drop in electricity prices, drove the market to new solutions exploitation (Nastasi & Lo Basso 2017). Moreover, the expected increase in energy from renewable sources in recent years is bringing out the need to store energy to match both sides of the energy demand. Energy storage systems include electro-chemical (lithium-batteries, flow-batteries), electro-mechanical (pumped hydropower, compressed air), chemical (hydrogen, synthetic natural gas) and thermal storage (molten-salt storage) (REN21, 2018).

Among all, the chemical solution, characterized as long term-energy storage solution, consist in convert electricity in another energy carrier with higher energy density, using an electrolyser. This energy storage is integrated into the so-called "power to gas" system (P2G) (Petersson & Wellinger, 2009). P2G is achievable mainly via a two-step process: 1) utilisation of excess renewable energy for water electrolysis and subsequent production of hydrogen, 2) conversion of hydrogen by means of chemical or biological reactions with external CO and $CO_2$ sources into $CH_4$ (Collet et al., 2016). The chemical carbon dioxide methanation comprises two main disadvantages if compared to the

biological. The chemical reaction is performed under high temperatures and pressures (around 573 K and 50–200 bar) and a catalyst, typically nickel-based, aiming to reduce the activation energy of the reaction is needed. In addition, gas impurities ($H_2S$) have to be removed prior to the catalytic step (Leonzio, 2017). Whilst the biological process takes place at moderate temperature and pressure (37 to 55 ◦C, ambient pressure) and is more flexible towards gas impurities. From the perspective of an energy smart-grid, P2G has the inherent advantage of exploiting the existing infrastructure of the natural gas grid. In Italy, it has been recently reported that the foreseen technical potential excess energy from wind and solar power, in principle available for electrolysis and P2G, may reach about 51 TWh/y by 2050 (Guandalini et al., 2017).

## 1.5. Biogas and Methane

Taking a step backward, it is noteworthy that it was the Italian Alessandro Volta, who first described ("Lettere sull'aria inflammabile nativa delle paludi", 1777) his phenomenal finding in Angera (Maggiore Lake, Italy) of a "flammable air" naturally generated from, oxygen-free, pouches rich in organic matter of wetlands. Only later in 1868, scientific studies started, settling the microbiological basis for the anaerobic digestion process. It was Béchamp the first researcher who demonstrated that methane was derived from a microbiological process (Kougias & Angelidaki 2018). Methane formation for ages has been responsible for carbon recycling in such diverse environments: marshes, rice paddies, benthic deposits, deep ocean trenches, and animals' intestines. Biogas is indeed a natural gas derived by degradation of complex organic matter to methane and carbon dioxide called anaerobic digestion (Speece, 1983; Schink et al., 1997). This process is widely utilized for treatment and valorisation of organic residues in several countries representing an optimal pathway to turn wastes into valuable products: biogas and fertilizer (Kougias & Angelidaki 2018).

## 1.6. Biomethane from WWTP

Biogas output from traditional anaerobic digestion (AD) reactor contains about 45-70% $CH_4$ and 24-40% $CO_2$ depending on the feedstock (landfills, sewage sludge or wastes). Furthermore, biogas contains traces of other gas in variable %: nitrogen ($N_2$) 0–3%, vapour water ($H_2O$) 5–10%, (higher if AD is operated at thermophilic conditions), oxygen ($O_2$) at concentrations of 0–1%, hydrogen sulfide ($H_2S$) at concentrations of 0–10,000 ppmv, ammonia ($NH_3$), hydrocarbons at concentrations of 0–200 mg/m$^3$ and siloxanes of about 0–41 mg/m$^3$ (Sun et al., 2015, Awe et al., 2017). All compounds in biogas beside $CH_4$ can be considered as pollutants, reducing its calorific value ($CO_2$

and $N_2$) or potentially corrosive and/or toxic ($H_2S$, Si, volatile organic compounds (VOCs), siloxanes, CO, and $NH_3$) (Corbellini et al., 2015). Anaerobic digestion of sewage sludge is a long-established technology to attain both objectives of sludge stabilization, as well as the simultaneous reduction of pathogens. According to the Italian Gestore Servizi Energetici (GSE, 2016), AD is available in only 77 large wastewater treatment plants (WWTPs,) producing only a quite limited fraction 44.2 MW of electricity (3%) of biogas by all sources in Italy. Italian biogas production from sludge accounts for only 4% of total EU production. For instance, in Lombardy region the methane production computed over 14 WWTPs serving from 25000 to 720000 population equivalent (P.E.) was accounted to be 6.1 $LCH_4$ $P.E.^{-1}$ $d^{-1}$ on average (data source: Lombardy Region) which is well below the average European values 10-14 $LCH_4$ $P.E.^{-1}$ $d^{-1}$ for primary sludge only and 18 $LCH_4$ $P.E.^{-1}$ $d^{-1}$ when secondary sludge is also digested (Bodík et al., 2011). These data clearly suggest that in Italy a large room exists in fostering and enlarging the application and integration of AD processes in WWTPs, as well as in extending the exploitation of the many pros that AD offers. So far the biogas produced has been valorised by means of co-generators or micro-turbines with sizes ranging from 50 to 500 kW (Arecco and Ghelardi, 2018). Potential biomethane production, from sewage sludge, is estimated to be approximately 300 million cubic metres per year according to data from Biomethane Platform, (2017). Moreover, for the Italian law (Italian DM 2 March 2018), sewage sludge belongs to the matrices that are allowed to produce so-called “advanced biomethane”, thus accessing an increased double-counting incentive.

# 2. Biological biogas upgrading

As reported above $CO_2$ conversion to $CH_4$, can be biologically catalysed in a process which gained more and more attention for its applicability into the P2G field (Guandalini et al., 2017). The main advantage of biological upgrading technology is the inherent possibility of transforming $CO_2$ into other energy-containing products, $CH_4$, while commercial technologies (physical or chemical) only separate it from the raw biogas, requiring further steps, and additional energy, for its final disposal (Bauer et al., 2013). Moreover, biological treatments, by nature, operate under mild conditions in terms of pressure, and moderate temperature levels, thus contributing to significantly increase their overall benefits (Angelidaki et al., 2018). This condition may reduce investment and operative costs if compared to commercial upgrading technologies (Agneessens, 2018). This reaction is based on the action of chemo-autotrophic microorganisms which utilize $CO_2$ in the metabolism, and that utilise $H_2$ as an electron donor in the energy-yielding reaction described in the following equation (Strevett et al., 1995):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^0 = -135.6\ kJ/mol$$

In the past decades, the methanation of $H_2$ and $CO_2$ by using microbial monocultures has been accomplished, but a new interest on bioprocess development has only recently become a re-emerging focus (Guebitz et al., 2015).

## 2.1. Biogas production

Complex organic matter degradation, in a natural or controlled environment in the absence of oxygen, is attainable only by the fascinating cooperation of microorganisms, including hydrolytic and fermenting bacteria and methanogenic archaea. An uncountable number of parallel or simultaneous reactions either biochemical and physicochemical take places (Pavlostathius & Giraldo-Gomez 1991). Among biochemical reactions, four main have been identified as relevant to describe the whole process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis interconnected in a trophic chain depicted in **Figure 13** (Batstone et al., 2002).

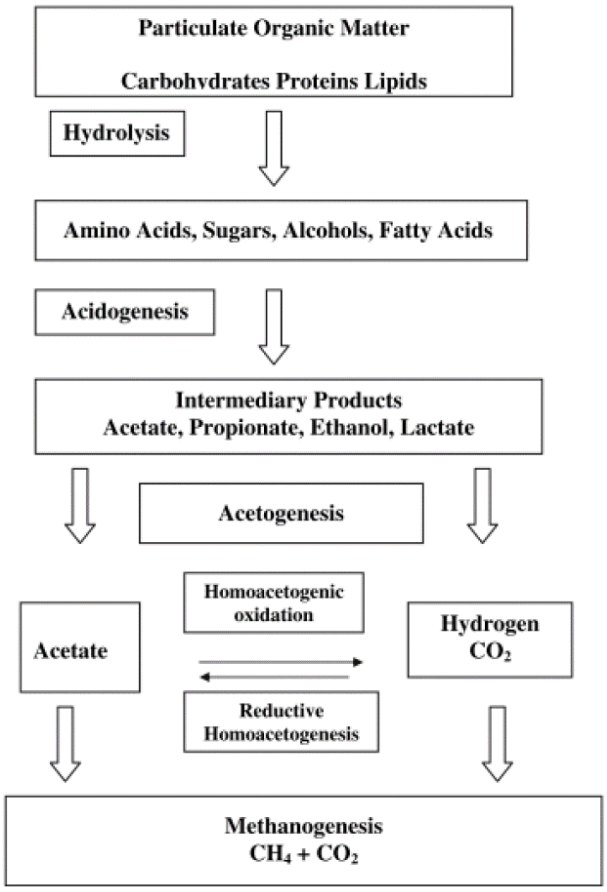



**Figure 13. Schematic representation of anaerobic digestion (Demirel & Scherer 2008).**

In the first phase, fermenting and hydrolytic bacteria excrete enzymes which hydrolyse complex organic matters into simpler molecules down to monosaccharides, amino acids, long chain fatty acids (LCFA) and alcohols (Pavlostathius & Giraldo-Gomez, 1991). This step is carried out by anaerobic bacteria such as *Bacteroides*, *Clostridium*, and *Streptococcus* (Liu & Whitman, 2008). Hydrolytic simpler soluble compounds are then fermented or anaerobically oxidized into short-chain fatty acids, hydrogen, carbon dioxide and ammonia by acidogens. It is important to stress that the major products of carbohydrate fermentation are ethanol, acetate, $H_2$ and $CO_2$ in the absence of methanogenic bacteria. But in anaerobic consortia $H_2$-utilizing bacteria and archaea are present, thus a reduction in ethanol and an increase in acetate production is usually observed (Pavlostathius & Giraldo-Gomez, 1991). In the third phase, syntrophic acetogenic and obligate $H_2$-producing bacteria oxidize to acetate short-chain and long-chain fatty acids. Syntrophic acetogenesis is thermodynamically favourable only at low $H_2$ pressures (<10 Pa) (Dolfing, 1988) and can occur only in syntrophic relation with $H_2$-scavenging microorganisms. The reverse syntrophic reaction, reductive homoacetogenesis is performed mostly by *Clostridium* and *Acetobacterium* genera which grow chemo-litho-autotrophically on $H_2$ plus $CO_2$ as energy and cell carbon source to produce acetate. Homoacetogens utilize the Wood-Ljungdahl pathway to produce acetate (**Figure 14**). These

microorganisms do not compete well with hydrogenotrophic methanogens for hydrogen consumption; they outcompeted only in some environments (Liu & Whitman, 2008) at a high level of $H_2$.

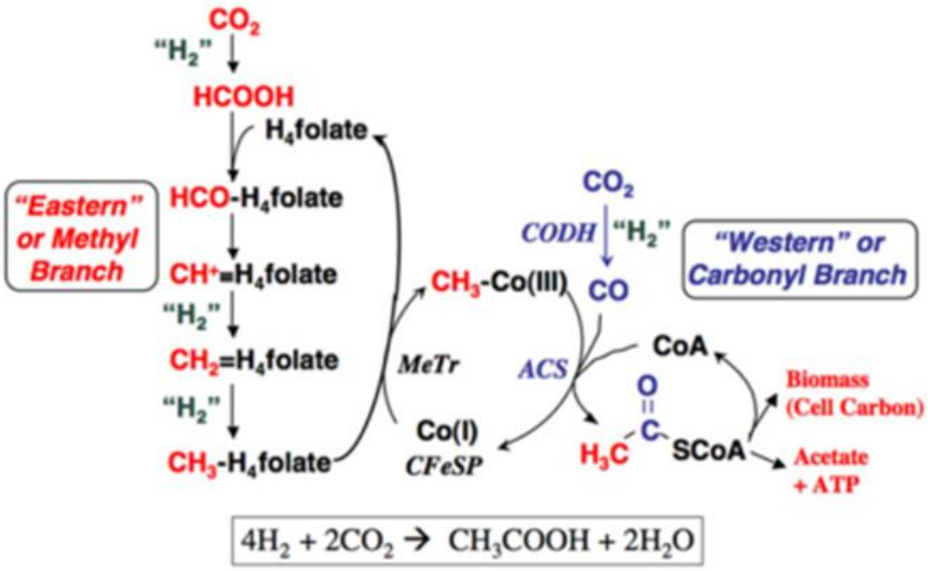



**Figure 14. The Wood-Ljungdahl pathway (Agneessens, 2018).**

The final anaerobic trophic chain is methanogenesis in which acetate and carbon dioxide plus $H_2$ are converted to methane respectively by acetoclastic and hydrogenotrophic methanogenic strains. In this phase, the accumulation of $H_2$ (by hydrogen-utilising methanogens) and of short-chain fatty acids (by acetoclastic methanogens) is therefore avoided by their conversion to $CH_4$ (Pavlostathius & Giraldo-Gomez, 1991). $CO_2$ plus $H_2$ and acetate, as described above, can be converted one in another via syntrophic and its reverse reaction (homoacetogenesis) (**Table 2**).

**Table 2. Equation and free energy changes for a reaction involving anaerobic oxidation of ethanol, propionate, butyrate to acetate by respective catabolizing bacteria, Syntrophic acetate degradation, Homoacetogenesis, acetoclastic and hydrogenotrophic methanogenesis (Dolfing, 1988).**

| Process | Reaction | ΔG0' [kJ/mol] |
|---|---|---|
| Syntrophic Ethanol deg. | $C_2H_5OH + H_2O \rightarrow CH_3COOH + 2\ H_2$ | 9.6 |
| Syntrophic Propionate deg. | $C_3H_6O_2 + 2\ H_2O \rightarrow CH_3COOH + 3\ H_2 + CO_2$ | 76.2 |
| Syntrophic Butyrate deg. | $C_4H_8O_2 + 2\ H_2O \rightarrow 2\ CH_3COOH + 2\ H_2$ | 48.8 |
| Syntrophic Lactate deg. | $C_3H_6O_3 + H_2O \rightarrow CH_3COOH + 2\ H_2 + CO_2$ | -4.2 |
| Syntrophic Acetate deg. | $CH_3COOH + 2\ H_2O \rightarrow 2\ CO_2 + 4\ H_2$ | 104.6 |

| | | |
|---|---|---|
| Homoacetogenesis | $2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O$ | -104.6 |
| Acetoclastic methanogenesis | $CH_3COOH \rightarrow CH_4 + CO_2$ | -31 |
| Hydrogenotrophic methanogenesis | $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$ | -135.6 |

Three are the major methanogenesis pathways depending on the used substrate and the energy source for $CH_4$ production (**Figure 15**): the $CO_2$ reduction (Wood Ljungdahl pathway), the acetotrophic pathway and the methylotrophic pathway (Aryal et al., 2018).

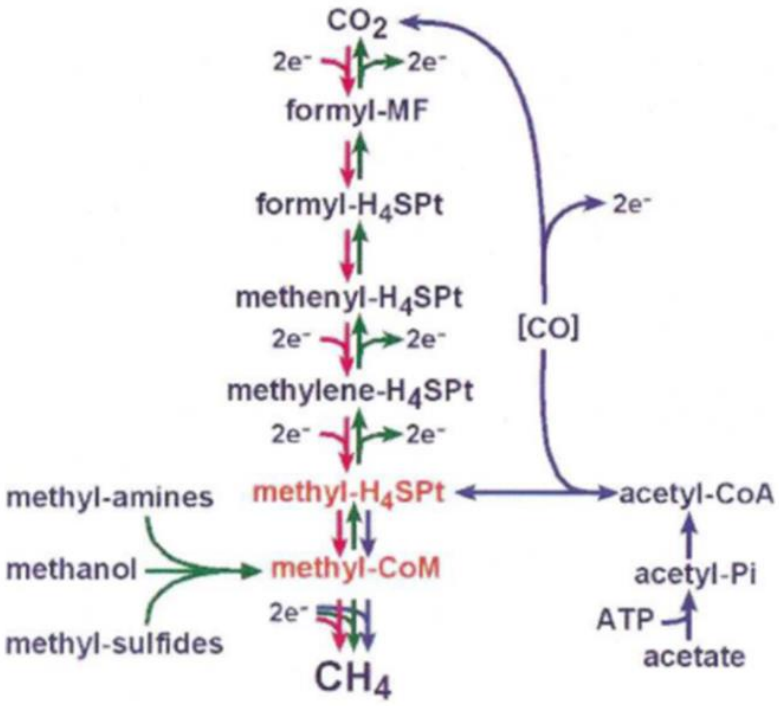



**Figure 15. Reactions in hydrogenotrophic methanogenesis (fuchsia arrows), acetoclastic methanogenesis (blue arrows) and methylotrophic methanogenesis (green arrows). All three pathways share the reduction of methyl-CoM to methane. CoM, coenzyme M; H4SPT, tetra-hydrosarcinapterin; MF, methanofuran (Agneessens, 2018).**

Hydrogenotrophic methanogens, described in the following paragraph, have a key role in maintaining $H_2$ under critical concentrations allowing acetogenesis by syntrophic propionate and butyrate oxidation. The other, acetoclastic methanogenic pathway, generally accounts for two-thirds of methane production (Smith & Mah, 1966). *Methanosaeta* or *Methanosarcina* represent the dominant family in the methanogens members (Liu & Whitman, 2008) but only *Methanosarcinaceae* are capable of both hydrogenotrophic and acetoclastic $CH_4$ production

(Demirel & Scherer, 2008). Typical methanogenesis reaction occurring in during AD is reported in **Table 3**.

**Table 3 Methanogenic reactions (Demirel & Scherer, 2008).**

| | | |
|---|---|---|
| 1. | Hydrogen | $4\ H_2 + CO_2 \rightarrow CH_4 + 2\ H_2O$ |
| 2. | Acetate | $CH_3COOH \rightarrow CH_4 + CO_2$ |
| 3. | Formate | $4\ HCOOH \rightarrow CH_4 + 3\ CO_2 + 2\ H_2O$ |
| 4. | Methanol | $4\ CH_3OH \rightarrow 3\ CH_4 + CO_2 + 2\ H_2O$ |
| 5. | Carbon monoxide | $4\ CO + 2\ H_2O \rightarrow CH_4 + 3\ H_2CO_3$ |
| 6. | Trimethylamine | $4\ (CH_3)_3N + 6\ H_2O \rightarrow 9\ CH_4 + 3\ CO_2 + 4\ NH_3$ |
| 7. | Dimethylamine | $2\ (CH_3)_2NH + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 2\ NH_3$ |
| 8. | Monomethylamine | $4\ (CH_3)NH_2 + 2\ H_2O \rightarrow 3\ CH_4 + CO_2 + 4\ NH_3$ |
| 9. | Methyl mercaptans | $2\ (CH_3)_2S + 3\ H_2O \rightarrow 3\ CH_4 + CO_2 + H_2S$ |
| 10. | Metals | $4\ Me^0 + 8\ H^+ + CO_2 \rightarrow 4Me^{++} + CH_4 + 2H_2O$ |

## 2.2. Hydrogenotrophic methanogens: stoichiometry and kinetics

As mentioned above, methanogens are limited to three major substrates: $CO_2$, methyl-group containing compounds, and acetate. Hydrogenotrophic methanogens play a key role allowing anaerobic trophic chain natural running due to their efficient $H_2$ scavenging, produced during the carbohydrates and proteins fermentation, but also for the anaerobic oxidation of fatty acids (Pavlostathius & Giraldo-Gomez, 1991). $CO_2$ conversion by means of a biological coupling with $H_2$ was at first proposed by Barker and van Niel in 1936 (Zinder & Koch, 1984). This process is carried by hydrogenotrophic methanogens which are present in normal anaerobic digesters with their key role in maintaining a low $H_2$ partial pressure (P<10 Pa) required by acetogenic bacteria for its normal running. Hydrogenotrophic methanogens are able to convert carbon dioxide to methane, according to the following reaction:

$$4H_2 + CO_2 = CH_4 + 2H_2O \quad \Delta G^0 = -135.6\ kJ/mol$$

On a molar base, 5 moles of reagents are transformed in 1 mole of methane. In this reaction, carbon dioxide serves as a carbon source and electron acceptor while hydrogen as an electron donor. An

overall stoichiometry reaction of the hydrogenotrophic methanogens including the bacterial growth is reported in the following equation (Speece, 1983):

$$H_2 + 0.256CO_2 + 0.004HCO_3^- + 0.004NH_4^+ = 0.004C_5H_7O_2N + 0.239CH_4 + 0.517H_2O$$

From this reaction it is therefore expected a yield growth of 0.004 mol ·113 g/mol =0.45 g of the new cell each mole $H_2$ compared to the acetoclastic methanogens according to the following formula:

$$0.125CH_3COO^- + 0.119H_2O + 0.002CO_2 + 0.002NH_4^+ = 0.002C_5H_7O_2N + 0.121CH_4 + 0.123HCO_3^-$$

Result that every 0.125 mol of acetate 0.002·113 g/mol =0.226 g of the new cell is expected thus 1.8 g cell every mole. In **Table 4**, kinetics constant both for acetoclastic and hydrogenotrophic methanogens are presented. Hydrogenotrophic showed both higher affinity and much lower ($k_s$) and maximum specific utilization rate ($k_{max}$) compared to acetoclastic. It is been observed that this microorganism literally starves of $H_2$ in normal operating anaerobic digesters (Aryal et al., 2018).

**Table 4. Kinetic data for acetoclastic and hydrogenotrophic methanogens (Pavlostathius & Giraldo-Gomez 1991; Angelidaki et al., 2018).**

| | **Acetoclastics** | **Hydrogenotrophs** | |
|---|---|---|---|
| $\mu_{max}$ | 0.1-0.4 | 1-4 | $d^{-1}$ |
| $k_{max}$ | 2-7 | 25-35 | gCOD/gCOD/d |
| $k_s$ | 50-600 | 0.01-0.6 | mgCOD/L |
| Y | 0.02-0.05 | 0.045-0.13 | gSSV/gCOD |
| $k_d$ | 0.02-0.04 | 0.04-0.088 | $d^{-1}$ |

Hydrogenotrophic methanogens comprise five orders belonging to the domain of Archaea: *Methanobacteriales*, *Methanococcales*, *Methanomicrobiales*, *Methanosarcinales*, and *Methanopyrales* (Zabranska & Pokorna, 2017). Members of the genus *Methanosarcina* obtain energy by the reduction of $CO_2$ with $H_2$ or CO as the electron donor. In the *Methanosarcinales* family, genera of *Methanosaetaceae* is composed of three species, among these, only the Methanosaeta genus is able to both convert acetate to $CH_4$ and $CO_2$. All the other four orders obtain energy for growth only by the reduction of $CO_2$ to $CH_4$ referred to as obligate $CO_2$-reducing species (Ferry, 2010).

## 2.3. $H_2$ role as an intermediate

As indicated above, $H_2$ represents a key intermediate in the anaerobic trophic chain as an electron donor in the Wood-Ljungdahl pathway (**Figure 14**), both for some acetogenic bacteria and archaea (Campanaro et al., 2016). In a normal AD process, $H_2$ concentration must be extremely low for thermodynamic reasons to allow the conversion of volatile acids and alcohols to acetate (**Figure 16**; **Table 2**) (Aryal et al., 2018). Due to its regulatory role in the anaerobic digestion, the hydrogen content in the biogas is known to be a proxy variable of imminent failure or destabilisation. In the case of overloads, it tends to accumulate at high concentration, whilst its presence at low concentration may indicate an under-loaded process (Rodriguez et al., 2006). The syntrophic interaction occurring between $H_2$-producing organisms (e.g. propionate-oxidizing bacteria) and $H_2$-consuming organisms (e.g. homacetogens and hydrogenotrophic methanogens) is named interspecies "interspecies electron transfer (IET)" and permit to cooperatively transform organic compounds into methane (Kouzuma et al., 2015). Bryant in 1967 was the first who reported the electron transfer in a co-culture system in which *Methanobacterium ruminantium* consumed the $H_2$, that was produced by microbes, to reduce $CO_2$ to $CH_4$ (Aryal et al., 2018). Thus, from a theoretic point of view, the syntrophic acetate oxidation is thermodynamically favourable only when $H_2$ is present at a low partial pressure (2.6-74 Pa) (Sarker at al., 2018).

Thus, the effect of exogenous hydrogen addition may directly stimulate the hydrogenotrophic methanogenesis, and obtaining the willing $CO_2$ conversion into extra methane production. Alternatively, homoacetogens may be partial consumers of the added $H_2$ (Agneessens, 2018). In the following paragraphs the two principal biological biogas upgrading technology, *in-situ* and *ex-situ* so far studied at lab-scale, are presented.

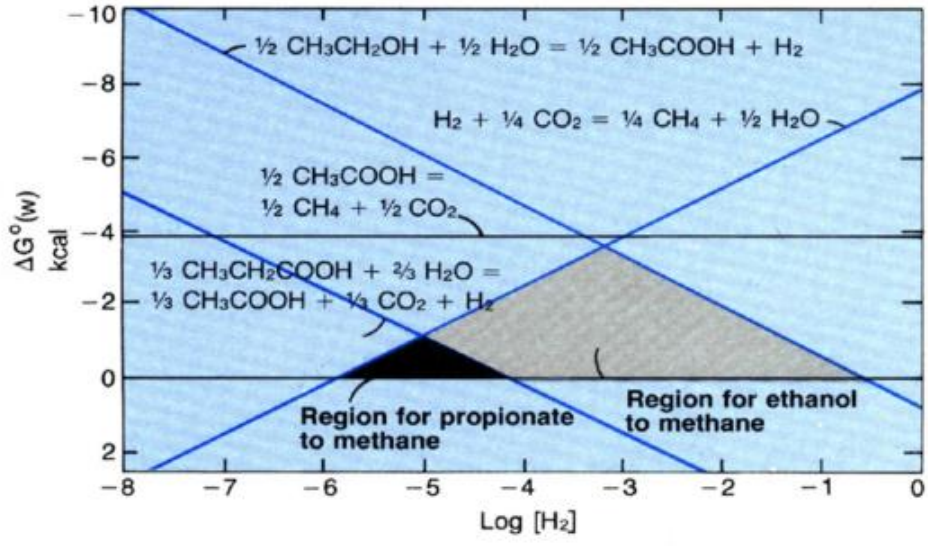



**Figure 16. Change in free energy available over $H_2$ concentration (Speece, 1983).**

## 2.4. Biological upgrading configurations

Two main principle applications of chemo-autotrophic biogas upgrading were mainly studied at lab-scale: *in-situ* in which $H_2$ is injected directly into the main digester where also anaerobic degradation of organic matter takes place and the *ex-situ,* where $H_2$ and $CO_2$ (from biogas or other sources) are provided in a separated tank containing an enriched hydrogenotrophic methanogenic consortia or a pure culture (Luo & Angelidaki 2013; Bassani et al., 2017; Kougias et al., 2017). The *in-situ* biological upgrading exploits the action of autochthonous archaea hydrogenotrophic methanogens to convert the endogenous $CO_2$ produced in the anaerobic digester if $H_2$ from an external source is provided (**Figure 17**). Therefore the biological conversion of carbon dioxide takes place in the same reactor where anaerobic consortia co-operate for the organic matter degradation and may influence the overall anaerobic trophic chain.

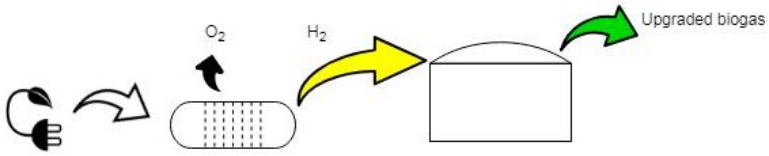



**Figure 17. *In-situ* biological biogas upgrading configuration's scheme.**

In this configuration, the exogenous hydrogen injection, into the main digester, can cause disadvantages the anaerobic digestion process, such as pH increase due to the $CO_2$ removal and process inhibition due to higher $H_2$ partial pressure. Moreover, due to its low solubility, a technical challenge is the limitation of $H_2$ transfer-rate to the liquid fraction.

Regarding the first effect, it's well-known that in the range of normal anaerobic digestion (6-8) pH is regulated by the $H^+$ concentration according to the following equilibrium equation:

$$[H^+] = K_1 \frac{[H_2CO_2]}{[HCO_3^-]}$$

$H_2CO_2$ concentration represents the carbonic acid in equilibrium with $CO_2$ percentage in the headspace of the digester, while $HCO_3^-$ is the bicarbonate alkalinity in the liquid phase, finally K1 is the ionization constant for acid carbon. In **Figure 18**, is reported a graph in which this fundamental relationship between bicarbonate alkalinity and carbon dioxide in the gas phase is shown.

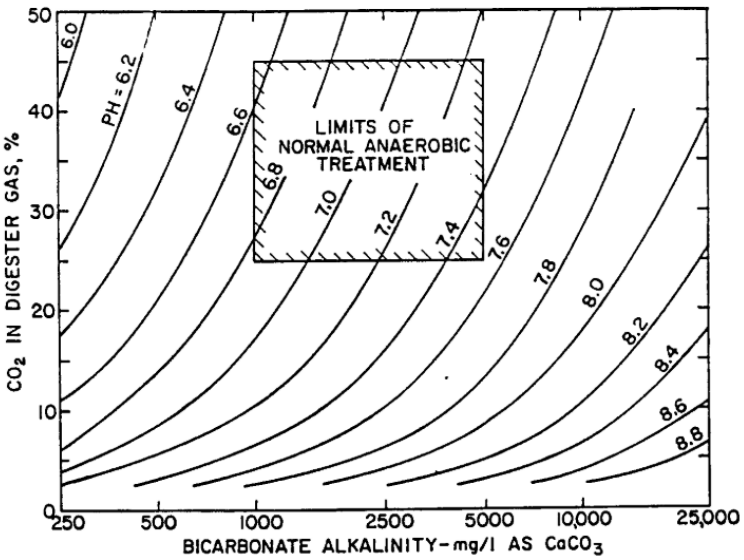



**Figure 18. $CO_2$ in the gas phase and bicarbonate alkalinity relationship at 95°F equal to 35°C (McCarty, 1964).**

At mesophilic conditions (35°C), two are the main effects that can be obtained if lower gas phase $CO_2$ content is the target depending on the pH control. To obtain lower $CO_2$ content in the gas phase means operating also very low alkalinity and neutral pH, or high alkalinity but basic pH. Indeed, the increase in the pH value in the reactor is related to the $HCO_3^-$ direct usage by hydrogenotrophic methanogens that causes the reduction of $H^+$ ions concentration. In literature, this outcome was already proven for *in-situ* upgrading of biogas with pH values above 8.5 which led to a slight methanogenesis inhibition (Luo & Angelidaki, 2012). In order to avoid the rise of pH above the normal anaerobic range, two ways have been tested that could make the *in-situ* technology feasible: co-digestion with an acid waste such as cheese whey (Luo & Angelidaki, 2013a) and parametric control of the pH paired to a minimum alkalinity threshold that avoids failure of anaerobic digestion. In the case of pH control, a significant alkalinity consumption (-73%) to the final value below 1000 mg/L was observed due to HCl addition (Wang et al., 2013). Hydrogen is an important metabolite and as mentioned above, its partial pressure at equilibrium must be lower than 10 Pa to allow proper degradation of propionate (Pauss et al., 1990). At higher concentration, there can be, on one hand, the accumulation of reaction intermediates such as butyrate, lactate, propionate caused by the inhibition of acetogenic bacteria, or its consumption towards acetate via homoacetogenesis according to the following equation:

$$4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G^0 = -104.6\ kJ/mol$$

The significant occurrence of homoacetogenic bacteria activity explains a possible decrease in the rate of methane production (Agneessens, 2018). However, prolonged exposure to hydrogen and the

consequent growth of the hydrogenotrophic population limit the occurrence of such problems (Reeve et al., 1997). Moreover, limitation in $H_2$ solubilisation in the liquid phase is then another essential parameter affecting the *in-situ* treatment. Since the solubility of hydrogen gas in the liquid phase is low (Henry constant $K_H$ =7.40 x $10^{-9}$ mol $L^{-1}$ $Pa^{-1}$), it is of primary importance to use an insufflation system that maximizes its solubilisation: the material and type of instrumentation used to insufflate hydrogen, as well as the application of a gas recirculation and reactor design are key elements for the implementation of an *in-situ* system for upgrading the biogas. Different authors have tested the effectiveness of porous media such as hollow-fiber membranes and ceramic sponges, verifying a better usage of hydrogen whose coupling with carbon dioxide leads to better conversion into methane. Luo and Angelidaki (2013b) achieve methane levels of 96% in the gas output using reactors fed continuously with bovine manure in co-digestion with dairy serum, using hollow-fiber membranes to insufflate hydrogen. The gas recirculation allows optimizing the contact time between the gas phase and the microorganisms, increasing the efficiency of the conversion of hydrogen to methane. The presence of a gas recirculation increases the availability of hydrogen and allows also a better mixing of the reactor increasing gas retention time (Mulat et al., 2017). **Table 5** summarized studies on the *in-situ* set-up.

**Table 5. Summary of literature studies of the *in-situ* technology integration of data from Angelidaki et al., 2018.**

| Organic Substrate | Reactor type | OLR (gVS/L/d) | T (°C) | pH | HRT (d) | $H_2$ (L/Lr/d) | $H_2/CO_2$ | $H_2$ conversion (%) | $CO_2$ removal (%) | $CH_4$ (%) | Ref. |
|---|---|---|---|---|---|---|---|---|---|---|---|
| potato starch | UASB | 2.79 | 55 | 8.38 | 7 | 3.5 | 4:1 | 67 | 76 | 82 | (Bassani et al., 2016) |
| cattle manure | CSTR | 1.85 | 55 | 8.3 | 14 | 1.8 | 4:1 | >90 | | 65 | (Luo et al., 2012) |
| cattle manure and cheese whey | CSTR | 1.66 | 55 | 7.7 - 7.9 | 15 | 1.5-1.7 | 4:1 | | 85 | 75 | (Luo and Angelidaki, 2013a) |
| sewage sludge | CSTR | 0.77 | 38 | 7.89 - 8.43 | 20 | 0.3-1.7 | (2:1-10:1) | 58-99 | 43.3-100 | 76.8-100 | (Agneessens et al., 2017) |
| cattle manure and cheese whey | CSTR | 1.66 | 55 | 7.61 - 8.31 | 15 | 0.93-1.76 | 4:1 | | 53-91 | 78.4-96.1 | (Luo and Angelidaki, 2013b) |
| sewage sludge | CSTR | 2 | 37 | 8.0 | 10 | 0.6-1,32 | 3.27:1-5.4:1 | 96 | 99 | 98.9 | (Wang et al., 2013) |

Important information can be drawn related to $H_2/CO_2$ ratio applied versus the methane and carbon dioxide content achieved and the $H_2$ conversion obtained. Among previous studies, performed at mesophilic conditions and treating sewage sludge in CSTR type reactors, only two reported almost pure methane in the gas output (98.9% and 100%). In the first Wang et al., (2013) utilized as gas input, not pure $H_2$ but synthetic coke oven gas (SCOG) composed by $H_2$ and CO in ratio $H_2$/CO 92/8. Thus, taking into account also the $CO_2$ amount derived from the biogas *in-situ* produced, an overall $H_2/CO_2$ ratio that varied from 3.3:1 to 5.4:1was adopted. The other high rich-methane result was presented by Agneessens et al., (2017). In their study, 100% methane content was obtained operating in semi-batch and adopting a ratio of 6:1. But this is referred to the biogas produced from $H_2$ consumption, before mixing it with the biogas present in the reactor headspace before $H_2$ injection. Thus the extra methane corresponded to the stoichiometric conversion 4:1 of $H_2$ to $CH_4$ until the pH was below 8.18±0.05. Above this pH value, methane from $H_2$ was only 58±9% and the rest was converted into acetate, which increased 10-folds respect to control reactor. Furthermore, $CO_2$ content was indeed 16.8% and lastly, this achievement was obtained after only 5 consecutive days of $H_2$-pulse injections. This outcome may indicate homoacetogenesis acting as temporary storage prior to biomethanation. Propionate levels also increased during $H_2$ injections due to acetate accumulation and not due to direct $H_2$ inhibition of syntrophic propionate degraders. Furthermore, this study suggests, during $H_2$ injections, close monitoring of the $H_2$ consumption rate to increase methane production and control volatile fatty acid concentrations. Hence, even if an indication that working above the stoichiometric value could let to achieve higher methane content, further research on the relevance of the $H_2/CO_2$ is yet to be addressed. Current and previous studies operated under stoichiometric ratio or above, but always considering $CO_2$ in the headspace only. An option might be taking into account to adopt a ratio considering $CO_2$ as the sum of the contributions from the liquid and gas phase. However, regarding $CO_2$ solubilized it should be always kept under control the minimum alkalinity threshold to be guaranteed as buffer capacity of the anaerobic system. So far, previous studies on chemoautotrophic biogas upgrading *in-situ* proved that an addition of external $H_2$ can de facto affect the anaerobic digestion process increasing hydrogenotrophic methanogenic activity and shaping the archaeal community structure and a *Methanothermobacter thermautotrophicus* related band appeared in a denaturing gradient electrophoresis gel from the sample of the reactor with hydrogen addition (Luo et al., 2013). However, the direct $H_2$ injection may also stimulate the production of acetate through the homoacetogenesis route (Agneessens et al., 2018). Furthermore, the addition of hydrogen to anaerobic reactor treating cattle manure led to a pH increase (higher than 8.3) due to the depletion of $CO_2$ (Luo et al., 2012).

The other biological biogas upgrading configuration, the *ex-situ*, consists of the conversion of carbon dioxide and hydrogen, both from an external source, into methane. The treatment takes place into a dedicated tank containing a pure or enriched hydrogenotrophic culture as depicted in **Figure 19**.

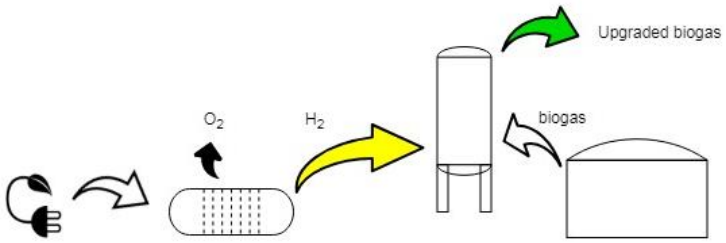



**Figure 19. *Ex-situ* biological biogas upgrading configuration's scheme.**

This method, compared over the *in-situ* concept, shows several advantages:

- do not affect the stability of the anaerobic digestion process of the organic substrate; only methanogenesis step occurred;
- other external sources of residual $CO_2$ or CO can be fed to the upgrading unit, making the process more flexible;
- the process is independent of the type of substrate used.

But in this configuration, the hydrogen solubilisation plays a fundamental role in obtaining a gas with high methane content. Optimization studies in literature have been performed testing diverse liquid/gas dispersion systems, adopting specific reactor configurations, implementing gas recirculation or increasing the liquid phase mixing: Kougias et al., (2017), demonstrated that by using series up-flow reactors or fine bubble columns it is possible to achieve a methane output content of 98% by using conventional hydrogen diffusers instead of advanced membrane systems. Studies focused on reactors types, such as trickle-bed bioreactors, up-flow, bubble column CSTR or fixed-bed lead at achieving 88-98 methane percent in the output, as reported in **Table 6**. Savvas et al., (2017) assessed the ex-situ using a small glass cylinder (1.5 L working volume) and providing the gas mixture directly by introducing it into a centrifugal pump and recirculating the liquid from bottom to top (rate 6 L $min^{-1}$), a methane formation rate per liter reactor volume of 12 $LCH_4/L$ reactor was achieved and a total upgrading to methane was accomplished. Recently a system based on a venturi-type has been proposed, demonstrating that $H_2$ transfer increased with increasing $H_2$ injection rates, but resulting in very low $H_2$ conversion 10–26% of the injection rates (Jensen et al.,

2018). The major outcome, indicate that there still a technical challenge regarding the low $H_2$ gas-liquid mass-transfer rate.

**Table 6 Summary of literature studies of the ex-situ technology integration of data from Angelidaki et al., 2018.**

| **Inocula** | **Reactor type** | **T (°C)** | **pH** | **Gas retention time (h)** | **Gas recirculation (L/Lr/h)** | **$H_2$ conversion (%)** | **$CO_2$ removal (%)** | **$CH_4$ (%)** |
|---|---|---|---|---|---|---|---|---|
| Anerobic digestate | Batch | 55 | 7.7-8.2 | 24 | - | | | 92 |
| Immobilised hydrogenotrophic enriched culture | Trickle-bed | 37 | 7.4-7.7 (7.2-7.4) | 2.5-3.5 (4) | - (-) | - (100) | >96 (100) | >96 (98) |
| Digestate from sewage sludge | Trickle-bed | 37 | - | 2-7 | Yes | 94-100 | - | 92.8-97.9 |
| Digestate from sewage sludge | Fixed-bed | 35 | - | 1.5-6.5 | - | - | 68-100 | - |
| Mixed culture | Fixed-bed | 50 | 6.9 | 4 | | 97.1 | - | >90 |
| Mixed culture | Biofilm plug-flow | 37 | | 0.24 | No | | | >98 |
| Hydrogenotrophic enriched inoculum | Up-flow | 55 | 8.3-8.81 | 4-15 | 2.88-20.14 | 96.8-100 | 85.5-100 | 89.5-96.3 |
| Digestate | Up-flow in series | 55 | 8.5 | 16 | 4 | <100 | 50 | 98 |
| Digestate | CSTR | 55 | 8 | 8 | 4 | >60 | 33 | 79 |
| Digestate | Bubble column | 55 | 8.3 | 8 | 4 | <100 | 83 | 98 |
| Enriched Hydrogenotrophic culture | CSTR | 55 | 7.8 | 1-8 | - | 72-95 | 88-96 | 95 |
| Digestate | CSTR | 37 | 8.17 | - | - | 93 | 71 | 88.1 |
| Digestate | CSTR | 35 | 7.1-7.3 | - | - | <100 | 98 | 92 |
| Pure culture (*M. thermoautotophicus*) | CSTR | 60 | 7.35 | 4 | Yes | 89 | - | - |

Finally, whatever the application is considered (*in-situ* or *ex-situ*) if hydrogen is produced by means of water electrolysis, it could be a valuable product also the $H_2$ co-product, $O_2$. Indeed, when this upgrading technology will be implemented in a wastewater treatment plant also $O_2$ could be recovered for aeration purposes. This aspect, considering that generally aeration represents around 40% of total running costs (Shen et al., 2015) could also lead to significant economic savings, especially when energy surplus is the energy source for the electrolyser in the concept of P2G mentioned above.

# 3. Thesis objectives and outline

In this thesis, the effort was concentrated on knowing and deepening the innovative process of biological biogas upgrading through several experimentations at lab-scale in batch, semi-continuous and continuous operations. These studies were mainly focused on the *in-situ* set-up and anaerobic reactors fed on municipal sludge, except one in which co-digestion feeding (agro-industrial wastewater) of a new-designed third set-up, called Hybrid, was assessed.

The first study, presented in **Chapter 4**, is focused on the fundamentals of the hydrogenotrophic methanogenesis. More in detail, manometric batch tests were performed aiming to propose a simple standard protocol for the measurement of the specific hydrogenotrophic methanogenic activity. The standardization of this measurement can find its interest both for the selection of most suitable sludge as start-up inoculum, but most importantly as a very useful method of measuring the activity during continuous processes at full-scale, both for *in-situ* and *ex-situ* set-ups. A further experiment was focused on how efficiently achieve the enrichment of hydrogenotrophic methanogens of anaerobic consortia. This fundamental theme is the core of **Chapter 5** of this dissertation. A semi-continuous hydrogenotrophic enrichment study has been conducted in two lab-scale reactors at mesophilic conditions fed on municipal sludge. The aim of this work was to study a rapid enrichment strategy capable of limiting the organic degradation unbalance and allowing a fast start-up phase of the *in-situ* biogas upgrading reactors, at pilot or full-scale. The approach was tested with 2+1 control lab-scale CSTRs filled with anaerobic sludge collected from a WWTP. The experimentation lasted 50 days and it was divided into 5 phases: the anaerobic digestion (AD) start-up followed by four $H_2$ injection phases ($H_2/CO_2$ ranging from 1:1 to 4:1 molar basis).

Afterward, during a research period at DTU (by Bioenergy group headed by full professor Irini Angelidaki) presented in **Chapter 6**, a new Hybrid biogas upgrading configuration composed of two-stage thermophilic reactors was designed and evaluated in a 4 months lasted experimental trial. This innovative set-up exploits the combination of the *in-situ* and the *ex-situ* processes in a combined configuration. $H_2$ was directly injected in both reactors and, in particular, the output gas from the first CSTR reactor (*in-situ* biogas upgrade) was subsequently transferred to a second up-flow reactor (*ex-situ* upgrade), in which the inoculation of an enriched hydrogenotrophic culture was responsible for the hydrogenation of $CO_2$ to $CH_4$. Reactors were hydraulically disconnected, but intensive gas recirculation ensured an efficient gas-phase connection. In this experiment, co-digestion of potato starch and cattle manure in the *in-situ* reactor was applied, while the *ex-situ* was fed with degassed digestate only to provide the supplement of nutrient to the biomass. The overall objective of the work was to perform initial methane enrichment in the *in-situ*, avoiding

deterioration of the process due to elevated pH levels and subsequently to complete the biogas upgrading process in the *ex-situ* chamber. Moreover, biogas production and upgrading performances *in-situ* and *ex-situ* conditions were compared. The effect of $H_2$ injection on the microbial community in both reactors was analyzed by 16S rRNA gene amplicon sequencing.

The purpose of the following study, discussed in **Chapter 7** was to evaluate the biological biogas upgrading in- situ in continuous-mode with two parallel CSTR lab-scale reactors (11L each) with pH controlled to 7.4. The aim was to confirm the enrichment strategy proposed in Chapter 4, and its importance, and to further assessing the relationship between biogas upgrading performance to the $H_2/CO_2$ ratio adopted, increasing it up to 6:1 and 7:1. Overall reactors performance and other process parameters such as alkalinity, volatile fatty acids (VFAs) speciation composition and COD balance were measured.

Finally, with the hypothesis that the $H_2$ addition selectively stimulated the hydrogenotrophic related pathway, and in order to deepen the knowledge of the effect of $H_2$ injections the biogas microbial community, 16S rRNA amplicon sequencing was carried out during the experiment. Microbial results, as well as correlations with process parameters, were analysed and presented in **Chapter 8**.

# References


Agneessens, L. M. (2018) The Power of the Small: Microbial Conversion of Hydrogen to Methane during Anaerobic Digestion. PhD thesis (October).

Agneessens, L. M., Ditlev, L., Ottosen, M., Vinther, N., Lund, J., Jonge, N. De, Holst, C., Vedel, M., and Kofoed, W. (2017) Bioresource Technology In-situ biogas upgrading with pulse H 2 additions : The relevance of methanogen adaption and inorganic carbon level. Bioresource Technology, 233, 256–263. http://dx.doi.org/10.1016/j.biortech.2017.02.016.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., and Kougias, P. G. (2018) Biogas upgrading and utilization: Current status and perspectives. Biotechnology advances, (July 2017), 0–1.

Aercco e Ghirlardi (2018), Biometano da biogas Manuale per la progettazione, autorizzazione e gestione degli impianti ISBN: 9788866272366.

Aryal, N., Kvist, T., Ammam, F., Pant, D., and Ottosen, L. D. M. (2018) An overview of microbial biogas enrichment. Bioresource Technology, 264(June), 359–369.

Awe, O. W., Zhao, Y., Nzihou, A., Minh, D. P., and Lyczko, N. (2017) A Review of Biogas Utilisation, Purification and Upgrading Technologies. Waste and Biomass Valorization, 0(0), 0. [online] http://link.springer.com/10.1007/s12649-016-9826-4.

Bassani, I., Kougias, P. G., Treu, L., Portè, H., Campanaro, S., and Angelidaki, I. (2017) Optimization of hydrogen dispersion in thermophilic up-flow reactors for ex situ biogas upgrading. Bioresource Technology, 234(March), 310–319.

Batstone D.J., Keller J., Angelidaki I., Kalyuzhnyi S.V., Pavlostathis S.G., Rozzi A., Sanders W.T.M. HS and VAV (2002). Anaerobic digestion model No 1;1.

Bauer, F., Hulteberg, C., Persson, T., and Tamm, D. (2013) Biogas upgrading – Review of commercial ( Biogasuppgradering – Granskning av kommersiella tekniker ).

Bodík, I., Sedláèek, S., Kubaská, M., and Hutòan, M. (2011) Biogas Production in Municipal Wastewater Treatment Plants – Current Status in EU with a Focus on the Slovak Republic. Chemi. Biochem. Eng., 25(3), 335–340.

British Petroleum BP (2018) 67th edition. Statistical Review of World Energy, 1–56.

Bryant MP (1972) Commentary on the Hungate technique for culture of anaerobic bacteria. Am J Clin Nutr 25 : 1324- t 328.

Campanaro, S., Treu, L., Kougias, P. G., De Francisci, D., Valle, G., and Angelidaki, I. (2016) Metagenomic analysis and functional characterization of the biogas microbiome using high throughput shotgun sequencing and a novel binning strategy. Biotechnology for Biofuels, 9(1), 26.

Collet, P., Flottes, E., Favre, A., Raynal, L., Pierre, H., Capela, S., and Peregrina, C. (2016) Techno-economic and Life Cycle Assessment of methane production via biogas upgrading and power to gas technology. Applied Energy, 192, 282–295.

Corbellini V., Insabato G. Malpei F. (2015) Biometano: tecnologie di upgrade, norme e valutazioni economiche. Politecnico di Milano, ISBN: 8897962424, 9788897962427.

Demirel, B. and Scherer, P. (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Reviews in Environmental Science and Biotechnology, 7(2), 173–190.

Department of Economic and Social Affairs of the United Nations (2017) World populations prospects,

Dolfing J. (1988) Biology of the anaerobic microorganisms. Edit by Alexander J. B. Zehnder. John Wiley & Sons, Inc.

EBA statistical report 2017 (http://european-biogas.eu/2017/12/14/eba-statistical-report-2017)

Eurostat (2016) Renewable energy statistics.

Ferry, J. G. (2010) The chemical biology of methanogenesis. Planetary and Space Science, 58(14–15), 1775–1783. http://dx.doi.org/10.1016/j.pss.2010.08.014.

Guandalini, G., Robinius, M., Grube, T., Campanari, S., and Stolten, D. (2017) Long-term power-to-gas potential from wind and solar power: A country analysis for Italy. International Journal of Hydrogen Energy, 42(19), 13389–13406. http://dx.doi.org/10.1016/j.ijhydene.2017.03.081.

Guebitz, G. M., Bauer, A., Bochmann, G., Gronauer, A., and Weiss, S. (2015) Biogas Science and Technology, Advances in Biochemical Engineering/Biotechnology 151 Series Editor: T. Scheper.

GSE (2016), Rapporto Statistico,

https://www.gse.it/documenti_site/Documenti%20GSE/Rapporti%20statistici/Rapporto%20statistico%20GSE%20-%202016.pdf

IPCC, (2011) Renewable Energy Sources and Climate Change Mitigation Special Report of the Intergovernmental Panel on Climate Change.https://www.ipcc.ch/report/renewable-energy-sources-and-climate-change-mitigation/

Jensen, M. B., Kofoed, M. V. W., Fischer, K., Voigt, N. V., Agneessens, L. M., Batstone, D. J., and Ottosen, L. D. M. (2018) Venturi-type injection system as a potential H 2 mass transfer technology for full-scale in- situ biomethanation. Applied Energy, 222(October 2017), 840–846. https://doi.org/10.1016/j.apenergy.2018.04.034.

Khan, I., Hafiz Dzarfan Othman, M., Hashim, H., Matsuura, T., Ismail, A. F., Rezaei-DashtArzhandi, M., and Wan Azelee, I. (2017) Biogas as a renewable energy fuel – A review of biogas upgrading, utilisation and storage. Energy Conversion and Management, 150(August), 277–294.

Kougias, P. G. and Angelidaki, I. (2018), Biogas and its opportunities — A review. , 12(3). Front. Environ. Sci. Eng. 2018, 12(3): 14

Kougias, P. G., Treu, L., Peñailillo, D., Boe, K., Campanaro, S., and Angelidaki, I. (2017) Bioresource Technology Ex-situ biogas upgrading and enhancement in different reactor systems. Bioresource Technology, 225, 429–437. http://dx.doi.org/10.1016/j.biortech.2016.11.124.

Lema JM, Soto M, Mendez R (1991) Inhibition/toxicity on the anaerobic digestion process caused by the presence of NaCl and other sea salts. In: VI international symposium on anaerobic digestion, Sao Paulo

Leonzio G. Design and feasibility analysis of a Power-to-Gas plant in Germany. J Clean Prod 2017;162:609–23. doi:10.1016/j.jclepro.2017.05.168.

Liu, Y. and Whitman, W. B. (2008) Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Annals of the New York Academy of Sciences, 1125(August 2018), 171–189.

Luo, G. and Angelidaki, I. (2012) Integrated biogas upgrading and hydrogen utilization in an anaerobic reactor containing enriched hydrogenotrophic methanogenic culture. Biotechnology and Bioengineering, 109(11), 2729–2736.

Luo, G. and Angelidaki, I. (2013a) Co-digestion of manure and whey for in situ biogas upgrading by the addition of H2: Process performance and microbial insights. Applied Microbiology and Biotechnology, 97(3), 1373–1381.

Luo, G. and Angelidaki, I. (2013b) Hollow fiber membrane based H2 diffusion for efficient in situ biogas upgrading in an anaerobic reactor. Applied Microbiology and Biotechnology, 97(8), 3739–3744.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., and Angelidaki, I. (2012) Simultaneous hydrogen utilization and in situ biogas upgrading in an anaerobic reactor. Biotechnology and Bioengineering, 109(4), 1088–1094.

McCarty Anaerobic Waste Treatment Fundamentals, PUBLIC WORKS October, 1964.

Mulat, D. G., Mosbæk, F., Ward, A. J., Polag, D., Greule, M., Keppler, F., Nielsen, J. L., and Feilberg, A. (2017) Exogenous addition of H 2 for an in situ biogas upgrading through biological reduction of carbon dioxide into methane. Waste Management, 68, 146–156.

Muñoz, R., Meier, L., Diaz, I., and Jeison, D. (2015) A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Reviews in Environmental Science and Biotechnology, 14(4), 727–759.

Nastasi, B. and Lo Basso, G. (2017) Power-to-Gas integration in the Transition towards Future Urban Energy Systems. International Journal of Hydrogen Energy, 42(38), 23933–23951. http://dx.doi.org/10.1016/j.ijhydene.2017.07.149.

Patterson, T., Esteves, S., Dinsdale, R., Guwy, A., and Maddy, J. (2013) Life cycle assessment of biohydrogen and biomethane production and utilisation as a vehicle fuel. Bioresource Technology, 131, 235–245. http://dx.doi.org/10.1016/j.biortech.2012.12.109.

Pauss, A., Andre, G., Perrier, M., and Guiot, S. R. (1990) Liquid-to-Gas mass transfer in anaerobic processes: Inevitable transfer limitations of methane and hydrogen in the biomethanation process. Applied and Environmental Microbiology, 56(6), 1636–1644.

Pavlostathius SG, Giraldo-Gomez E. Kinetics of anaerobic treatment. Wal Sci Tech Vol 24, No8, Pp 35-59, 1991

Petersson, A. and Wellinger, A. (2009) Biogas upgrading technologies – developments and innovations Task 37 -Energy from biogas and landfill gas IeA Bioenergy aims to accelerate the use of environmental sound and cost-competitive Bioenergy on a sustainable basis, and thereby achieve a substant. IEA Bioenergy, 13.

Reeve, J. N., Morgan, R. M., and Nölling, J. (1997) “Environmental and molecular regulation of methanogenesis” in Water Science and Technology., 1–6.

REN21, (2018) Renewables 2018 global status report. http://www.ren21.net/gsr-2018/chapters/chapter_06/chapter_06/

Ryckebosch, E., Drouillon, M., and Vervaeren, H. (2011) Techniques for transformation of biogas to biomethane. Biomass and Bioenergy, 35(5), 1633–1645.

Rodríguez, J., Ruiz, G., Molina, F., Roca, E., and Lema, J. M. (2006) A hydrogen-based variable-gain controller for anaerobic digestion processses. Water Science and Technology, 54(2), 57–62.

Sarker, S., Lamb, J. J., Hjelme, D. R., and Lien, K. M. (2018) Overview of recent progress towards in-situ biogas upgradation techniques. Fuel, 226(April), 686–697. https://doi.org/10.1016/j.fuel.2018.04.021.

Savvas S, Donnelly J, Patterson T, Dinsdale R, Esteves SR. Closed nutrient recycling via microbial catabolism in an eco-engineered self regenerating mixed anaerobic microbiome for hydrogenotrophic methanogenesis. Bioresour Technol 2017;227:93–101.

Scarlat N, Dallemand JF, Fahl F. Biogas: Developments and perspectives in Europe. Renew Energy 2018;129:457–72. doi:10.1016/j.renene.2018.03.006.

Schink, B. (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and molecular biology reviews : MMBR, 61(2), 262–280.

Scholz, M., Melin, T., and Wessling, M. (2013) Transforming biogas into biomethane using membrane technology. Renewable and Sustainable Energy Reviews, 17, 199–212.

Shen, Y., Linville, J. L., Urgun-Demirtas, M., Mintz, M. M., and Snyder, S. W. (2015) An overview of biogas production and utilization at full-scale wastewater treatment plants (WWTPs) in the United States: Challenges and opportunities towards energy-neutral WWTPs. Renewable and Sustainable Energy Reviews, 50, 346–362.

Smith, P. H. and Mah, R. A. (1966) Kinetics of acetate metabolism during sludge digestion. Applied microbiology, 14(3), 368–71.

Souto, T. F., Aquino, S. F., Silva, S. Q., and Chernicharo, C. A. L. (2010) Influence of incubation conditions on the specific methanogenic activity test. Biodegradation, **21**(3), 411–424.

Speece, R. E. (1983) Anaerobic biotechnology for industrial wastewater treatment. Environmental Science and Technology, 17(9), 416A–427A.

Starr, K., Gabarrell, X., Villalba, G., Talens, L., and Lombardi, L. (2012) Life cycle assessment of biogas upgrading technologies. Waste Management, 32(5), 991–999.

Strevett, K. A., Vieth, R. F., and Grasso, D. (1995) Chemo-autotrophic biogas purification for methane enrichment: Mechanism and kinetics. Chemical Engineering Journal and the Biochemical Engineering Journal, 58(1), 71–79.

Sun, Q., Li, H., Yan, J., Liu, L., Yu, Z., and Yu, X. (2015) Selection of appropriate biogas upgrading technology-a review of biogas cleaning, upgrading and utilisation. Renewable and Sustainable Energy Reviews, 51, 521–532.

Wang, W., Xie, L., Luo, G., Zhou, Q., and Angelidaki, I. (2013) Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and in situ biogas upgrading. Bioresource Technology, 146, 234–239.

Weiland, P. (2010) Biogas production: Current state and perspectives. Applied Microbiology and Biotechnology, 85(4), 849–860.

Zabranska, J. and Pokorna, D. (2017) Bioconversion of carbon dioxide to methane using hydrogen and hydrogenotrophic methanogens. Biotechnology Advances, (November), 0–1. http://linkinghub.elsevier.com/retrieve/pii/S0734975017301556.

Zinder, S. H. and Koch, M. (1984) Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Archives of Microbiology, 138(3), 263–272.

# 4. Towards a standard method to measure the specific hydrogenotrophic methanogenic activity (SHMA)


## ABSTRACT

In the last decade, the innovative biological biogas upgrading by means of hydrogenotrophic methanogenesis gained more and more interest and scientific attention in developing it. As a result, it became significant to study and to develop methods to measure the specific hydrogenotrophic methanogenic activity SHMA, in order to: select the most promising sludge for a rapid process start-up; evaluate the organic load treatable and as a key control parameter during the continuous process. Even if, in the past, some attempts aiming at the optimization of the measurement have been done, so far, there is still the lack of a standard protocol for this specific assessment. In the present study, a simple protocol based on manometric batch tests on three anaerobic sludges taken from municipal full-scale digesters is proposed. Two different apparatus (automatic pressure measurement OxiTop® and manual pressure measurement) were tested. The activity determinations were conducted at substrate concentration well above Ks value to ensure to operate in the zero order kinetics conditions. Both apparatus allowed to measure the SHMA but some constrains were identified especially for the automatic method, thus the non-automatic method is overall perceived as the most reliable.


### 4.1. Introduction

An uncountable number of parallel or simultaneous reactions, both biochemical and physicochemical take place during the anaerobic digestion process (Pavlostathius & Giraldo-Gomez, 1991). Four main biochemical steps have been identified as relevant to describe the whole process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, all interconnected in a trophic chain (Batstone et al., 2002). Methanogens are a diverse group of microorganisms that can utilize only three kinds of substrates: $CO_2$, acetate and methyl-compounds. Indeed, organic compounds such as carbohydrates, fatty acids and alcohols must be pre-processed by anaerobic bacteria or eukaryotes that actually precede them in the aforementioned trophic chain. But it is in this last step that methanogens produce valuable methane as the end-product of their anaerobic respiration (Liu & Whitman, 2008). To assess anaerobic sludge activity characterization the overall anaerobic degradation phases can be considered or a focusing on the activity of different species that participates at the complex organic matter anaerobic degradation (Soto et al., 1993). Usually, and in most of the cases, when a measure of the specific methanogenic activity is evaluated, the

acetoclastic activity is tested, being this group highly vulnerable to both pH and VFAs accumulation. Thus most of the standardization efforts in literature can be found for this specific methanogenic strain (Rozzi & Remigi, 2004). In recent years, the chemoautotrophic biogas upgrading process, which is based on the enhancement of the hydrogenotrophic methanogenesis, has gained more and more attention. Thus, there is a need to investigate the Specific Hydrogenotrophic Methanogenic Activity (SHMA) and to develop a standard protocol to measure it. The SHMA measure will support the implementation of the process at full-scale in evaluating the initial substrate loads that can be treated (Souto at al., 2012) and could allow to carry out toxicity tests with respect to certain substrates (Lema at al., 1991).

There are several possibilities to measure specific bacterial activity. In general terms, methods can be direct or indirect: the former when a parameter directly related to the activity such as the depletion of a substrate or a product over time is involved; the latter when the variation of a parameter related to the chemical-physical reaction is evaluated (Rozzi & Remigi, 2004).

Among direct methods, anaerobic activity methods can be further distinguished in the manometric method where the pressure in headspace generated (or reduced) by gas production (or reduction) is measured and volumetric methods in which the volume of gas produced is quantified (Rozzi & Remigi, 2004). In both cases, the procedure is based on the fact that biological reactions generally involve poorly soluble gas production or consumption, thus from the relationship between the measured pressure variation and the amount of gas produced in the reactor it is possible to derive the specific activity (Scaglione et al., 2009). Manometric tests can be performed both utilizing automatic devices and manual set-up: for instance, automatic methods may employ sensors able to transmit the pressure value measured on a membrane that is contained in the headspace of a sealed glass reactor. In 1984 Shelton and Tiedje refined a method to determine anaerobic biodegradation potential, firstly proposed by Gledhill (1979) where a pressure transducer was used to measure gas pressure along batch experiment, evaluating the variability and reproducibility of this method on more than 100 chemicals substrates. Another example of an automatic apparatus is the Oxitop® device (WTW, Xylem Inc.,) developed for the assessment of the biological oxygen demand BOD. Otherwise, the manual assessment method relies on measuring the reactor internal pressure by means of a manometer connected via a needle directly to the rubber septa. This procedure result to be highly time-consuming due to the fact that activity test often takes more than 8 h (normal working laboratory time) and labour-consuming in case of a large number of parallel replicas (Angelidaki et al., 1998).

For some specific test such as biochemical methane potential (BMP) or acetoclastic methanogenic activity that results in a mixture of gas as products ($CO_2$ and $CH_4$), both in manual and automatic

mode, gas composition measurement (during or at the final point) it would be requested to derive the specific activity. To avoid this, a carbon dioxide traps (i.e. NaOH) prior to the pressure measurement can be used or in case Oxitop® system $CO_2$ absorption can be performed by NaOH tablets placed inside the bottles (Scaglione et al., 2009). Automatic measurement has the main advantage of allowing monitoring remotely the process but generally, costs and maintenance of the equipment such as membranes for gas measurement are higher if compared to manual equipment.
Volumetric method is based on biogas or methane production measurement, here again, both manually and automatically measure can be performed. There are several kind of equipment but are based on a similar procedure in which reactor is connected to a glass tube system where the gas is collected when a pre-set level is reached, a photoelectric sensor drives a motor that resets the volume and counts the number of resets from which the volume of gas produced is obtained. In manual mode, a Mariotte flask or a eudiometer can be used. The first consists of a closed container with an inlet tube inserted deep into the barrier solution and an outlet tube at the same level: this ensures that a constant head is kept under tension for the duration of the test, and therefore a constant discharge per unit of biogas volume is generated. The eudiometer consists of a gas collection tube mounted above the reactor and a tank; the production of biogas pushes the barrier solution towards the tank, thus altering the two levels. After reading, the tank is lowered to restore the levels, i.e. to bring the overpressure back to the atmospheric value (Rozzi & Remigi, 2004). Some attempts in the past have been made to assess the specific hydrogenotrophic activity aiming to optimize the SHMA test, but to date has not been standardized yet. Hydrogenotrophic methanogens are able to convert carbon dioxide to methane, according to the following reaction (Bryant 1979):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^0 = -135.6\ kJ/mol$$

In this reaction, carbon dioxide serves as a carbon source and electron acceptor while hydrogen as an electron donor. On a molar basis, from the 4 $H_2$ moles plus 1 of $CO_2$ of reagents only 1 methane mole gas is produced. Dolfing and Bloemen (1985) assessed SHMA, on granular sludge, basing their methodology on gas chromatographic methane analysis of samples taken from the headspace with a gas pressure lock syringe, into bioreactors initially pressurized with the reagent stoichiometric mixture ($H_2/CO_2$ 80/20; 100–150 kPa). Later, Coates et al, (1996) reported a pressure test system application according to the reaction stoichiometry, measuring SHMA by the under-pressure generated inside the bioreactor, pressurized with a mixture of hydrogen and carbon dioxide at the starting point ($H_2/CO_2$ 80/20; 100–150 kPa). Results obtained were compared with the biogas

composition at the end of the test to verify that from pressure trend it is possible to follow the reaction assuming a stoichiometric factor 4:1 ($H_2/CH_4$). It was reported that low amounts of biomass can prevent the negative effects of $H_2$ mass transfer resistance. Authors set some specifications in order to increase $H_2$ gas-liquid transfers: the adoption of a 1:5 ratio between the liquid and headspace, and providing a high shaking value (180 rpm) placing horizontally the vials to increase exchange area between gas and liquid phase. To derive the activity, they calculate the substrate uptake using a portable pressure transducer device recorded in mV derived $CH_4$ volume production. Also, Soto et al., (1993) measuring anaerobic activity (methanogenic and non-methanogenic) set some fundamental operational parameters regarding the inoculum size and substrate initial concentration starting from the specific expected kinetic constants.

This study aims to provide fundamental elements of the procedure to measure the specific hydrogenotrophic methanogenic activity (SHMA) moving towards a simple standard measurement. Manometric batch tests assessing the specific hydrogenotrophic methanogenic activity of three anaerobic sludges taken from municipal full-scale digesters are presented. Two different experimental set-ups were utilized for comparison: an automatic manometric device and a manual measurement system. Statistical analysis was carried out aimed at assessing the reproducibility of replicates for each sample of sludge performed with the two different apparatus.

## 4.2. Materials and methods

***Batch tests equipment and preparation***

Batch tests were made utilizing two different apparatus: OxiTop® (WTW, Xylem Inc.,) consisting of a glass bottle (V= 0.322 L) with two lateral holes sealed by rubber septa for gas injections and venting (**Figure 1**) and a pressure transducer plus data logger located inside a measuring head. During the batch test, the pressure (P) reduction due to $H_2$ consumption is measured in hPa and automatically registered to allow an automatic pressure profile over time. An OxiTop® transducer, even allowing a semi-continuous and automatic pressure trend, is able to measure a pressure variation within a range of ±0.350 hPa (0.345 atm), out of this range the measurement is interrupted. The second apparatus consisted of glass serum vials (V=0.570 L) equipped with rubber septa and aluminium caps (henceforth Standard Bottle or STB) in which pressure is manually measured by means of a manometer (**Figure 1**). All tests were done in duplicate with a blank (**Table 1**) having the following characteristics: a surface-to-height ratio of the liquid fraction respectively of 5.4 cm and 10 cm for OxiTop ® and STB.

**Figure 1. OxiTop® bottle (on the left) and standard bottle (on the right) used in batch tests.**

**Table 1. Overview of SHMA tests performed.**

| WWTPs | OxiTop® | STB | Blank |
|---|---|---|---|
| **Bresso (BR)** | 2 | 2 | 2 (1 for each apparatus) |
| **Cremona (CR)** | 2 | 2 | 2 (1 for each apparatus) |
| **Sesto S. Giovanni (SSG)** | 2 | 2 | 2 (1 for each apparatus) |

### *Sludge characteristics and trace elements recipe*

Anaerobic sludges were taken from mesophilic digesters of different full-scale municipal waste-water treatment plants: Bresso, Cremona and Sesto S. Giovanni (Northern Italy) which have a capacity respectively of 220000, 180000 and 150000 population equivalent (P.E.). The main characteristics of all the three sludge tested, are reported in **Table 2**, while the recipe of trace elements used to provide useful elements to the biomass in **Table 3**.

**Table 2. Characteristics of the three anaerobic sludges tested.**

| Parameters | Cremona | Bresso | Sesto S. Giovanni |
|---|---|---|---|
| **HRT** | 20 | 25 | 20 |
| **pH** | 7.37 | 7.17 | 7.39 |
| **Alkalinity [mg$CaCO_3$/l]** | 8454 | 5666 | 7676 |
| **CODs [mgCOD/l]** | 212 | 121 | 217 |
| **SST [g/l]** | 37.3 | 25.7 | 12.9 |
| **SSV [g/l]** | 19 | 15.8 | 9.8 |
| **Acetic acid [mg/l]** | 86.76 | 123 | 290 |

**Table 3. Recipe of the trace elements solution employed in the SHMA test.**

| Traces elements in 1 liter of distilled water | G |
|---|---|
| Anhydrous potassium dihydrogen phosphate ($KH_2PO_4$) | 0.27 |
| Disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12H_2O$) | 1.12 |
| Ammonium chloride ($NH_4Cl$) | 0.53 |
| Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$) | 0.075 |
| Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) | 0.1 |
| Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) | 0.02 |
| Sodium sulphide nonahydrate ($Na_2S \cdot 9H_2O$) | 0.1 |

***Tests procedure***

The sludges to be tested were placed overnight in a thermostatic cell at the test temperature of 35°C. Then protocol procedure consists of the following steps:

- Vials were filled by sludge with a ratio between liquid and headspace set 1:1;
- Volatile solids (VS) concentration was set to be no lower than 5 g/L, thus based on the initial sludge concentration; water was eventually added to adjust final VS concentration;
- A trace elements solution was further added with a proportion of 10% to the liquid volume;
- pH was eventually adjusted if its value was out of normal anaerobic digestion range (7-8.5);
- Vials were closed and fluxed 5 minutes with $N_2$, and again for 5 minutes with the reagent gas mixture $H_2/CO_2$ (80/20 %) pressurized at 1.2 atm. Gas flux was performed with a system of 2 needles threaded into the septa, one connected directly to the cylinder containing the gas mixture or $N_2$, the other connected to a bottle that vented under the leaf to avoid $O_2$ inputs as showed in **Figure 2**;
- All vials were incubated in a thermostat shaker (MPM Instruments s.r.l, Italy) at 35°C with a mixing speed of 150 rpm to overcome possible $H_2$ liquid solubilization limits;
- Test duration of 6 h. Gas compositions were measured only as a control measure of reagents injected at the beginning point;
- During the test, the headspace pressure was measured every 30 minutes in the standard bottle set-up, while OxiTop® automatically provided 1 point pressure measured every 12 minutes.

**Figure 2. Gas fluxing systems for the OxiTop® (on the left) and for STBs (on the right).**

***Calculations***

The $H_2/CO_2$ kinetics uptake to produce methane can be considered Monod-type kinetics (Batstone et al., 2002). Thus, in order to obtain the maximum specific activity, the test must be zero order kinetics with respect to the substrate. In this case, hydrogen concentration must be higher than its half-saturation constant (S>>Ks). Ks values within the range 0.01-0.6 mgCOD/L (8gCOD/g$H_2$) were reported in the literature (Pavlostathius & Giraldo-Gomez, 1991). Thus, the aforementioned initial headspace pressure of 1.2 atm $H_2/CO_2$ (80/20) result in a hydrogen partial pressure of:

$$P_{H_2} = P_{tot} \cdot \frac{H_2}{CO_2} = 1.2 \cdot 0.8 = 0.96\ atm$$

Where $P_{H2}$ is the $H_2$ partial pressure, Ptot is the initial test pressure. Then, assuming that the $H_2$ solubility value in aqueous solution (at 35ºC is H = $7.4 \cdot 10^4$ atm) can be valid also for sludge, the $H_2$ molar fraction $x_{H2}$ (ratio of dissolved gas moles to liquid moles) according to Henry's gas low is:

$$P_{H_2} = H \cdot x_{H2} \quad [atm]$$

$$x_{H2} = \frac{P_{H_2}}{H} = \frac{0.96}{7.4 \cdot 10^4} = 1.29 \cdot 10^{-5}\ [-]$$

As the number of total moles ($n_{t,H2O}$) present in 1L water solution (neglecting other solutes) is:

$$n_{t,H2O} = \frac{P_{water}}{PM_{water}} = \frac{1000}{18} = 55.6\ [moles/L]$$

The number of $H_2$ moles ($n_{H2}$) present in water solution:

$$n_{H2} = x_{H2} \cdot n_{t,H2O} = 1.29 \cdot 10^{-5} \cdot 55.6 = 7.21 \cdot 10^{-4} \left[\frac{mol\ H_2}{L}\right]$$

Finally, $H_2$ concentration in water solution in equilibrium with the previous calculated $P_{H2}$ is:

$$C_{H2} = n_{H2} \cdot PM_{H2} = 7.21 \cdot 10^{-4} \cdot 2 \cdot 10^3 \left(\frac{mg}{g}\right) = 1.44 \left[\frac{mgH_2}{L}\right] \cdot 8 \left[\frac{gCOD}{gH_2}\right] = 11.52 \left[\frac{mgCOD}{L}\right]$$

Thus the zero order condition (S>>Ks) considering the more restrictive Ks value of 0.6 mgCOD/L the equivalent in terms of $H_2$ is 0.075 $mgH_2/L$ is respected until a pressure value of 0.05 atm is reached. This pressure value can be calculated simply turning aforementioned formulas.

The *SHMA* value was calculated expressed as $NmLCH_4 \cdot gVS^{-1} \cdot d^{-1}$ as follows:

$$\mathbf{SHMA} = \frac{dVCH_4}{dt}\frac{1}{X} \cdot 24h$$

Where: *X* (gVS) is the VS content of the biomass tested; $dV(CH_4)/dt$ $NmLCH_4 \cdot h^{-1}$ the slope of cumulative methane production. The first calculation procedure step is to remove gas production, even if very low, of the blank test. At each time-step, the blank pressure value is subtracted to the test pressure value as reported in equation (1). $CH_4$ production, at each step, is then derived from gas headspace pressure measurement according to gas low (2) and assuming that pressure reduction is due to the sole reaction of $H_2$ and $CO_2$ to give $CH_4$. $CH_4$ gas moles produced at step i+1 are equal to the difference between gas moles at step i and gas moles at step i+1 divided by the stoichiometric value of 4 (3). Then, methane volume for each step ($mlCH_4$) is calculated by multiplying $CH_4$ moles for the molar volume at 35°C (25.28 L/mol) (4):

$$P_{net} = P_i - P_{i,blank} \qquad (1)$$

$$n_{tot(i)} = \frac{(P_{net}) * V_{headspace}}{R * T} \qquad (2)$$

$$n_{CH4\ (i+1)=}\frac{n_{tot(i)}-n_{tot(i+1)}}{4} \quad (3)$$

$$(NmlCH_4)_{i+1} = n_{CH4\ i+1} \cdot (\frac{22.414}{25.28}) \quad (4)$$

The zero order kinetic previously assumed to last until 0.05 atm, could not be reached with automatic apparatus due to its own pressure detection limits nor for the manual method due to time and substrate removal rate. Noteworthy, starting from the 8th hour, Sesto SG and Bresso (a) and b) (in **Figure 3**), changed slope indicating substrate depletion trend more approximating to first-order kinetics, due to a possible substrate limitation. Lastly, Cremona samples showed always the same slope for all test duration. This aspect may be related not exactly because the limiting substrate concentration was reached, but because of its transfer into the liquid was limited.

In the kinetic constant calculation $K_0$, the lag phases that affected tests were taken into account. In particular, only the straight line section after the lag phase (circa 2 h) was considered, as shown in the results paragraph. For replicates, statistically similar, $K_0$ was reported for an average cumulative regression line, whilst for replicates considered being statistically different, $K_0$ was reported on a single regression line.

***Analytical Methods***

Total (TS) and volatile solids (VS) were measured according to APHA standard methods for the examination of water and wastewater. Total COD was determined according to Method 5130 APAT/IRSA-CNR (2003), Analytical Methods for Water by digestion with $K_2Cr_2O_7$ and $H_2SO_4$ 95-96% (Velp Scientifica, Heating Digester ECO6) and titration with FAS (ferrous ammonium sulfate). Manual pressure measures were performed using a digital manometer (Keller LEO 2) by puncturing the rubber septum. Gas composition ($CO_2$, $CH_4$, $H_2$, $O_2$, $N_2$) was analyzed by using gas chromatography (DANI Master GC Analyser equipped with two columns HayeSep Q and Molesieve 5A). The pH was directly measured in samples by means portable multi-probe meter (Hach-Lange, HQ40D).

***Statistical analysis***

Statistical analysis was carried out using the software SPSS v.25 aimed at statistically assess the reproducibility of replicates for each sample of the sludge of the two apparatus used. Since variables were not normally distributed, the nonparametric Mann-Whitney U test (significance level = 0.05)

was used. The null hypothesis ($H_0$) is that replicates are statistically reproducible, and it is accepted if the p-value is >0.05 otherwise it is rejected.

### 4.3. Results

In **Figure 3** pressure trends in OxiTop® reactors and $CH_4$ cumulative production over time are reported, for all three samples (Sesto San Giovanni, Bresso and Cremona + relative blanks). Although, as explained above, for the kinetic constants calculation only the 6-hour time frame was considered allowing a direct comparison between the two apparatus, in case of the Oxitop® measurement, all the registered curves are reported until automatic devices went out of its measurability range.

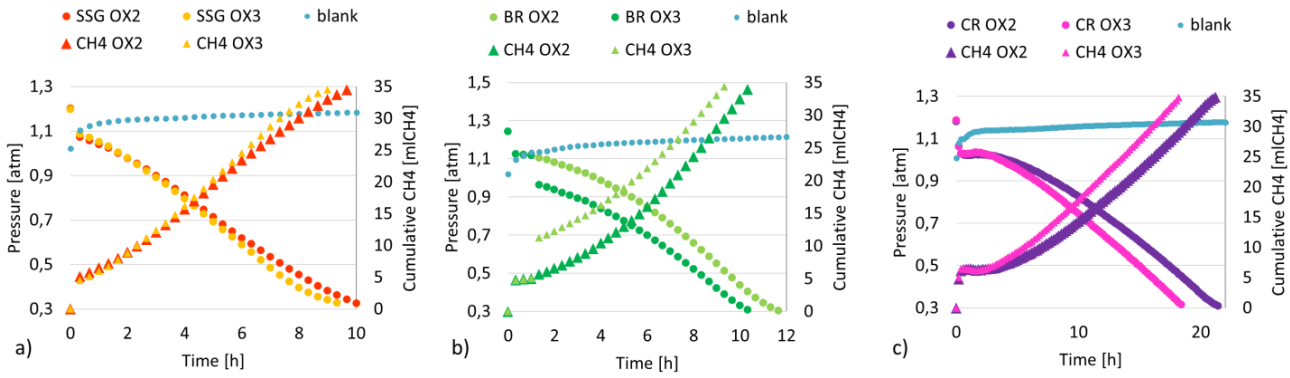



**Figure 3. Pressure trends [atm] and cumulative methane [mlCH$_4$] during SHMA tests performed with OxiTop® apparatus; a) Sesto S. Giovanni sludge; b) Bresso and c) Cremona.**

Despite all samples were taken from full-scale municipal plant digesters operating under mesophilic conditions and similar HRTs, they demonstrated different rates: SSG went out of measurable range in 10 hours, Bresso in 12 hours, while the slowest (Cremona) went out of this range in approximately 20 hours. At the beginning of the batch test, samples showed a variable lag phase (<1 h) due to the adaptation for perturbations generated by reagents fluxing phase in the headspace, and a possible little decrease in temperature to allow fluxing operations (**Figure 3**).

Qualitatively, good reproducibility of replicates is observed in the case of sludge a) and lowers for b) and c). However, the statistical analysis performed with the Mann-Whitney U test, showed that the reproducibility between replicates was found to be statistically significant for sludge from Sesto San Giovanni and Cremona but not for Bresso sludge (SSG_ox; CR_ox; BR_ox; p-values respectively 0.762, 0.406 and 0.019), thus the $H_0$ hypothesis for Bresso sludge replicates, for automatic device, was rejected as reported in **Figure 4**, indicating they cannot be considered similar replicates.

| | Null Hypothesis | Test | Sig. | Decision |
|---|---|---|---|---|
| 1 | The distribution of SSG_ox is the same across categories of Replicate. | Independent-Samples Mann-Whitney U Test | ,762 | Retain the null hypothesis. |
| 2 | The distribution of SSG_st is the same across categories of Replicate. | Independent-Samples Mann-Whitney U Test | ,762[1] | Retain the null hypothesis. |
| 3 | The distribution of BR_ox is the same across categories of Replicate. | Independent-Samples Mann-Whitney U Test | ,019 | Reject the null hypothesis. |
| 4 | The distribution of BR_st is the same across categories of Replicate. | Independent-Samples Mann-Whitney U Test | ,887[1] | Retain the null hypothesis. |
| 5 | The distribution of CR_ox is the same across categories of Replicate. | Independent-Samples Mann-Whitney U Test | ,406 | Retain the null hypothesis. |
| 6 | The distribution of CR_st is the same across categories of Replicate. | Independent-Samples Mann-Whitney U Test | ,291[1] | Retain the null hypothesis. |

Asymptotic significances are displayed. The significance level is ,05.

[1]Exact significance is displayed for this test.

**Figure 4. Summary of the hypothesis test of Mann-Whitney U assessing the reproducibility between each duplicates couples; SSG_ox is the test among OxiTop replicates for SSG sludge; SSG_st is the test among STB replicates for Sesto San Giovanni sludge; BR_ox is the test among OxiTop replicates for Bresso sludge; BR_st is the test among STB replicates for Bresso sludge; CR_ox is the test among OxiTop replicates for Cremona sludge; CR_st is the test among STB replicates for Cremona sludge.**

In **Figure 5** pressure trend performed with the Standard bottle system and the $CH_4$ cumulative production over time are presented, for all three samples, (Sesto San Giovanni, Bresso and Cremona + the relative blanks).

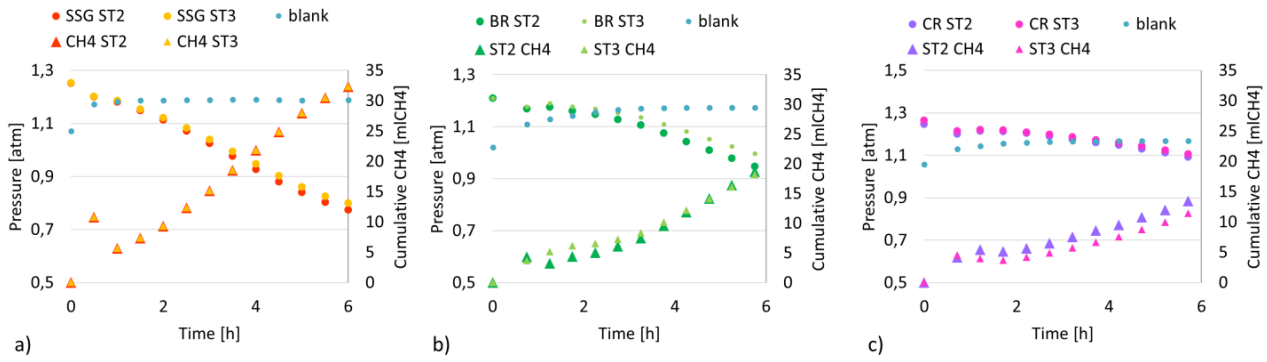



**Figure 5. Pressure trends [atm] and cumulative methane [ml$CH_4$] during SHMA test performed with Standard bottles apparatus; a) Sesto S. Giovanni sludge; b) Bresso and c) Cremona.**

At the beginning of this set of batch tests, a shorter lag phase was observed compared to the automatic apparatus, probably due to the higher sludge volume and a consequent lower effect of the slight temperature variation. This set-up cannot provide continuous measures, but at the same time allows measuring the pressure trend in a wider range of values.

A statistical test was carried out to assess the reproducibility of duplicates, qualitatively more similar if compared to those from the automatic. The Mann-Whitney U test found replicates to be statistically significant for all samples (p-values equal to 0.762; 0.887 and 0.291 for a) b) and c)), the $H_0$ hypothesis was always accepted as reported in **Figure 4**.

**Figure 6** shows for each sludge samples (a) (b) and (c) $CH_4$ cumulative linear regression curves of normalized by the mass of VS of both apparatus in order to be directly compared within the test duration time (6 hours). For each duplicate verified being statistically reproducible one curve each couple of replicates, in case of Bresso sludge (b) for each replica a single linear regression curve was presented. From the directly comparable curves in **Figure 6**, both devices have provided similar slopes for Sesto San Giovanni sludge and Bresso, and no failures have been observed in either of the two devices. Conversely, for Cremona sludge in which automatic device showed a very lower kinetic constant compared to the manual.

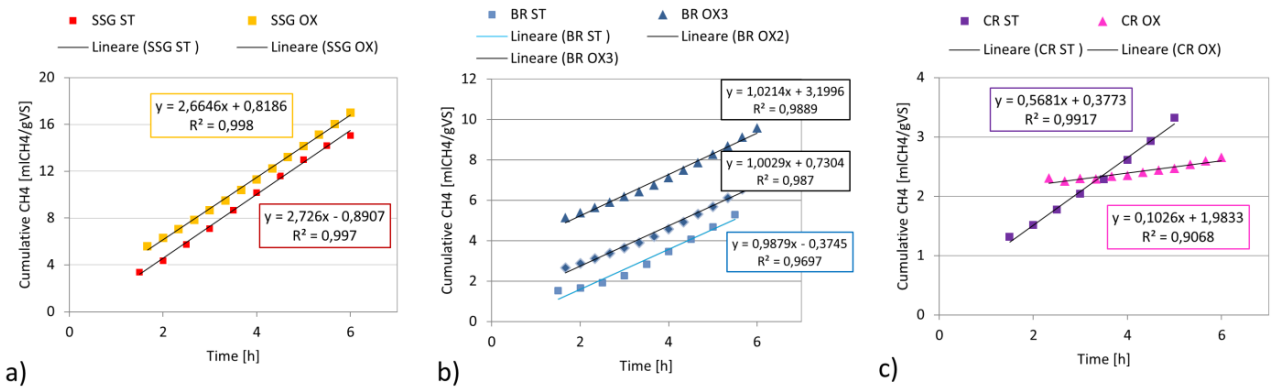



**Figure 6. Specific cumulative methane trends expressed in [mlCH$_4$ /gVS] for each sludge samples for the two apparatus: a) Sesto San Giovanni; b) Bresso; c) Cremona.**

In **Table 4** kinetic constant $K_0$ for each replicate are reported for all sludge tested. The kinetics constant is greater for Sesto S Giovanni sludge then Bresso and lastly Cremona. Moreover, sludge a) and b) both devices have recorded similar kinetic value. For sludge c) while the manual method gave similar values, values calculated for the automatic devices both seem to be outliers, possibly due to a pressure leak of bottles (intrusion of air).

**Table 4. Kinetic constant $K_0$ for each replicates for all sludge tested.**

| $K_0$ [$mlCH_4/VS\ h$] | | | | | | |
|---|---|---|---|---|---|---|
| | OxiTop | | | STB | | |
| | Rep. 1 | Rep. 2 | st.dv | Rep. 1 | Rep. 2 | st.dv |
| Sesto SG | 2.56 | 2.76 | 0.14 | 2.78 | 2.66 | 0.08 |
| Bresso | 1.0 | 0.98 | 0.01 | 0.97 | 0.96 | 0.01 |
| Cremona | 0.05 | 0.14 | 0.06 | 0.60 | 0.52 | 0.06 |

The coefficient of the linear regression represents the value of the SHMA (expressed in $mlC_{H4}/gVS{\cdot}h$), which were transformed into daily values and reported in **Table 5**. Noteworthy, the standard deviation between the devices revealed a good reproducibility among automatic and manual methods, except that for Cremona sludge.

**Table 5. SHMA results for all batch tests of the three municipal inocula expressed [mlCH4/gVS·d].**

| SHMA [$mlCH_4/gVS{\cdot}d$] | OxiTop® | STB | St dev (OxiTop®-STB) |
|---|---|---|---|
| Sesto SG | 63.9±0.1* | 65.3±0.1 | 1.0 |
| Bresso | 23.8±0 | 23.2±0 | 0.42 |
| Cremona | 2.3±0.1 | 13.4±0.1 | 7.9 |

*±Standard deviation.

SHMA obtained in this work measured on dispersed anaerobic sludge are significantly lower than those reported by Coates et al., (1996) (266.4-307.2 $mlCH_4/gVSS/d$) and by Dolfing et al (1985) (291.2 $mlCH_4/gVSS/d$). Both authors tested the specific activity on granular type sludge, thus more active than a dispersed one. Results of this study seemed to be not unusual and do not suggest any indication of test failures. In terms of comparison between the two methods in the case of sludge a) both apparatus resulted in very similar SHMA values, probably due to the fast kinetic, for Bresso sludge (b) even if statistics reported replicates from automatic method not reproducible, SHMA values were similar (st.dev 0.42) for both measurement methods. In the case of sludge (c), only with the manual method, it was possible to the esteem the SHMA. The automatic method showed problems possibly related to the tightness of the bottle. To conclude for all samples, the manual method showed both greater pressure tightness ensuring replicates reproducibility statistically

proved. Regarding the test conditions 1) the duration time of the test can be highly relevant depending on the specific sludge characteristic but the 6-hour period considered in this study allowed reproducible results for both methods and for different sludge. However, unquestionable is the manageability of an automatic system which, despite having a limit pressure range (not affecting the test), guarantees to obtain a semi-continuous curve for a longer period of time. 2) With regard to the ratio between headspace and liquid volume 1:1, no obvious issue was found; further tests can be carried out to evaluate the influence of its variation on SHMA value. 3) An initial gas pressure of 1.2 atm ($H_2/CO_2$ 80/20 %) and 4) a shaking value of 150 rpm were found to be an optimal condition as well as 5) the inoculum concentration of 5 gVS/L.

### 4.4. Conclusions

This work presented manometric batch tests to assess an easy protocol to perform SHMA tests in multiple samples. The manometric test helps to avoid errors of $CH_4$ estimation due to the extraction (and measure) of gas, especially during first reaction steps. Comparison between the apparatus standard deviation on SHMA value of approximately 1 except for one sludge (st.dev = 8), indicated that the process can be easily described only from P measurement trends. Moreover, in this study, automatic tests showed worse performance in terms of both pressure tightness and reproducibility of the replicas if compared to the manual set-up. Further investigations are necessary to test the reproducibility of other automatic systems coupled with bottles with greater pressure-tightness. A noteworthy result is that the statistical non-reproducibility may not, however, lead to an error in the estimation of the kinetics, as seen for the Bresso sludge. Vice versa, statistically reproducible replicas can lead to errors in the estimation of the kinetic parameter. Test conditions of the proposed protocol, time (6 h), the ratio between headspace and liquid volume (1:1), inoculum concentration (5gVS/L), initial pressure P ($H_2/CO_2$ 1.2 atm), and rpm (150) were found to be optimal conditions for the measurement of the SHMA. A further assessment should be done to deepen the shaking speed effect and also the starting overpressure of vials on dispersed sludge compared to granular sludge. Moreover, anaerobic sludge taken from digesters treating another kind of waste should be tested in order to extend the validity of this protocol towards standardization.

## *Acknowledgments*

The authors wish to thank Master student Tommaso Faravelli for his valuable support in the laboratory activities, Dott. Nadia Margariti for her support for the analysis, and WWTPs managers for providing the sludge.

# References


Angelidaki, I., Schmidt, J. E., Ellegaard, L., and Ahring, B. K., 1998. An automatic system for simultaneous monitoring of gas evolution in multiple closed vessels. Journal of Microbiological Methods, 33, 93–100 Journal.

Batstone D.J., Keller J., Angelidaki I., Kalyuzhnyi S.V., Pavlostathis S.G., Rozzi A., Sanders W.T.M. HS and VAV. Anaerobic digestion model No 1 2002;1.

Bryant, M.P., 1979. Microbial methane production—theoretical aspects. J. Anim. Sci. 48, 193–201.

Coates JD, Coughlan MF, Colleran E. Simple method for the measurement of the hydrogenotrophic methanogenic activity of anaerobic sludges. J Microbiol Methods 1996;26:237–46. doi:10.1016/0167-7012(96)00915-3.

Dolfing J, Bloeman WGBM. Acitivity measurements as a tool to characterize the microbial composition of methanogenic environments. J Microbiol Methods 1985;4:1–12. doi:10.1016/0167-7012(85)90002-8.

Gledhill, W. E. 1979. Proposed standard practice for the determination of the anaerobic biodegradability of organic chemicals. Working document. Draft 2, no. 35.24. American Society for Testing Materials, Philadelphia.

Lema JM, Soto M, Mendez R (1991) Inhibition/toxicity on the anaerobic digestion process caused by the presence of NaCl and other sea salts. In: VI international symposium on anaerobic digestion, Sao Paulo

Liu Y, Whitman WB. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann N Y Acad Sci 2008;1125:171–89. doi:10.1196/annals.1419.019.

Pavlostathius, S. G. and Giraldo-Gomez, E. (1991) Kinetics of anaerobic treatment. Wal. Sci. Tech. Vol. 24, No.8, pp. 35-59, 1991., 24(8), 35–59.

Rodríguez J, Ruiz G, Molina F, Roca E, Lema JM. A hydrogen-based variable-gain controller for anaerobic digestion processses. Water Sci Technol 2006;54:57–62. doi:10.2166/wst.2006.486.

Rozzi A, Remigi E. Methods of assessing microbial activity and inhibition under anaerobic conditions: A literature review. Rev Environ Sci Biotechnol 2004;3:93–115. doi:10.1007/s11157-004-5762-z.

Scaglione, D., Buttiglieri, G., Ficara, E., Caffaz, S., and Lubello, C. (2009) Microcalorimetric and manometric tests to assess anammox activity. Water Science and Technology, 60(10), 2705–2711.

Shelton, D. R. and Tiedje, J. M. (1984) General Method for Determining Anaerobic Biodegradation Potential. Applied and Environmental Microbiology, 47(P-7017), 850–857.

Soto M, Mendez R, Lema JM. Methanogenic and non-methanogenic activity tests. Theoretical basis and experimental set up. Water Res 1993;27:1361–76. doi:10.1016/0043-1354(93)90224-6.

Souto TF, Aquino SF, Silva SQ, Chernicharo CAL. Influence of incubation conditions on the specific methanogenic activity test. Biodegradation 2010;21:411–24. doi:10.1007/s10532-009-9311-x.

# 5. Hydrogenotrophic biogas upgrading integrated into WWTPs: enrichment strategy[1]

***V. Corbellini*, A. Catenacci* & F. Malpei****

*Department of Civil and Environmental Engineering – DICA, Politecnico di Milano, Environmental Section, Piazza L. da Vinci 32, 20133, Milano IT

## Abstract

Within the European circular economy roadmap, it's important for wastewater treatment plant's (WWTPs) to recover energy and become energy neutral or positive. In the last few years, it has become increasingly interesting to boost energy recovery through biogas upgrading. The aim of this work is to study a rapid hydrogenotrophic methanogenic culture enrichment strategy capable of limiting the organic degradation unbalance and allowing a fast start-up phase of the in-situ biogas upgrading reactors, at pilot or full-scale. The approach was tested with 2+1 control lab-scale CSTRs filled with anaerobic sludge collected from a full-scale WWTP. The experimentation lasted 50 days and was divided into 5 phases: the anaerobic digestion start-up followed by four $H_2$ injection phases ($H_2/CO_2$ ranging from 1:1 to 4:1 on a molar basis). Despite a temporary slight increase in the total concentration of volatile fatty acids during phase II (2.56 $gHac \cdot L^{-1}$), and in phase III a mild pH increment indicating the expected $CO_2$ depletion (anyway below 7.4), the strategy proposed was effective. In the last phase, in the biogas, methane content of about 80% was achieved, thus suggesting that the use of $H_2/CO_2$ above the stoichiometric value could further improve the biological biogas upgrading.

### 5.1. Introduction

Within the circular economy context, it is important for wastewater treatment plant's (WWTPs) to reduce the energy demand towards energy self-efficiency. According to Silvestre et al., (2015), an energy amount of about 3.2 $kJ \cdot gTS^{-1}$ in raw wastewater is contained, and an average amount of energy of 0.35 kJ·gTS-1 is required for sewage treatment: if captured and managed efficiently, sludge generated in WWTPs could yield substantial energy in the form of biogas, potentially turning WWTP into a net energy producer rather than a consumer. However, nowadays this is still far from being viable (Shen et al., 2015).

[1]V. Corbellini, A. Catenacci, F. Malpei; Hydrogenotrophic biogas upgrading integrated into WWTPs: enrichment strategy. Water Sci Technol wst2019096. doi: https://doi.org/10.2166/wst.2019.096

Anaerobic digestion (AD) is the technology commonly used to recover energy from organic streams. Biogas produced from anaerobic digestion of primary and secondary sludge from WWTPs consists mainly of methane (55-70%) and carbon dioxide (30-45%). Besides $CH_4$ and $CO_2$, raw biogas also contains small amounts of nitrogen (0-15%), oxygen (0-3%), water (1-5%), hydrocarbons (0-200 $mg \cdot m^{-3}$), hydrogen sulphide (0-10,000 $ppm_v$), ammonia (0-100 $ppm_v$), and siloxanes (0-41 $mgSi \cdot m^{-3}$) (Sun et al., 2015, Awe et al., 2017).

The lower heating value of biogas is usually found to be roughly around 23'400 $kJ \cdot Nm^{-3}$ depending on methane percentage (Silvestre et al., 2015). Biogas upgrading relies on the contaminant's removal or transformation from the raw biogas, in order to produce a final output gas consisting of higher methane concentration. Typically, the removal of moisture, $H_2S$, and $CO_2$ represent the most important upgrading steps (Miltner et al., 2017). If the upgraded biogas is purified to natural gas standards, then the final gas product is called biomethane (Kougias et al., 2017).

Different technologies are currently used for biogas cleaning and upgrading. Physical (condensation) and chemical (adsorption or absorption) drying methods are used to remove water. Two procedures commonly used to remove $H_2S$ during digestion are air/oxygen dosing to the biogas, and the addition of $Fe_2^+$ or $Fe_3^+$ in the form of $FeCl_2$, $FeCl_3$ and $FeSO_4$ into the digester or to the organic feed. Techniques such as adsorption on iron oxide or hydroxide or activated carbon, absorption with gas-liquid contactors (spray or packed bed towers using water, organic solvents or aqueous chemical solutions with $H_2S$ conversion to elemental sulfur or metal sulfide) and membrane separation are commonly adopted to remove $H_2S$ after digestion (Moñoz et al., 2015). Subsequently, trace components like siloxanes, hydrocarbons, ammonia, oxygen, carbon monoxide, and nitrogen can require extra removal steps, if not sufficiently removed by other treatment steps. Finally, the bulk $CO_2$ content must be separated from $CH_4$. Several commercial technologies are currently available: pressure (PSA) or vacuum (VSA) swing adsorption, membrane separation, physical or chemical $CO_2$ absorption (scrubbing with water, $CO_2$-reactive absorbents or organic solvents) and cryogenic separation (Ryckebosch et al., 2011, Awe et al., 2017).

Nowadays, those commercial $CO_2$ separation techniques are facing significant challenges in terms of energy/chemicals consumption and operating costs, making the upgraded gas expensive and not always affordable from an economic point of view. An alternative solution currently attracting many researchers is the biological biogas upgrading via the enhancement of hydrogenotrophic methanogenesis (Kougias et al., 2017).

The methane formation, known as methanogenesis, is the final AD process step and it is exclusively carried out by methanogenic members belonging to the Archaea domain. Organic substrates are converted to methane by distinct but concomitant methanogenic pathways operative in

phylogenetically diverse methanogens: the acetoclastic methanogens, which convert acetate into $CH_4$ according to Eq.1, and the hydrogenotrophic methanogens, which convert $H_2$ and $CO_2$ into $CH_4$ without other organic carbon sources, according to Eq.2 (Kern et al., 2016).

Eq. 1 $CH_3COOH \leftrightarrow CO_2 + CH_4$ $\Delta G^0 = -31\ kJ \cdot mol^{-1}$

Eq. 2 $4H_2 + CO_2 \leftrightarrow CH_4 + 2H_2O$ $\Delta G^0 = -135.6\ kJ \cdot mol^{-1}$

Although acetoclastic methanogens have a major role in $CH_4$ production (approximately 70 %) (Rozzi et al, 2002), methane can be also produced from hydrogen plus carbon dioxide or formate (Smith & Mah 1966). At high $H_2$ concentrations (e.g. >500 Pa), acetogenesis or methanogenesis from $H_2 + CO_2$ are favored, and at low concentrations (e.g. <40 Pa), oxidation of the acetate occurs (Demirel & Scherer 2008). Thus, at low $H_2$ partial pressure (i.e. the normal anaerobic process), hydrogenotrophic methanogens maintain low $H_2$ partial pressure necessary for the growth of intermediate syntrophic bacteria (Zinder 1994).

In the last few years, three applications which rely the enhancement of hydrogenotrophic methanogens for biogas upgrading were mainly studied: *in-situ*, *ex-situ* (Luo & Angelidaki, 2013; Kougias et al., 2017) and more recently a *hybrid* system, which couples the *in-situ* and the *ex-situ* in one operational unit, to benefits from both system advantages (Corbellini et al., 2018).

Hydrogenotrophic biogas upgrading process has several advantages mainly related to the higher $CH_4$ final volume and to $CO_2$ removal from biogas, which would decrease the costs for the upgrading of biogas to natural gas quality. However, in order to convert the major part of $CO_2$, $H_2$ has to be generated by an external source. Recent literature reports about bio-electrochemical systems (BESs), already applied for nutrient, metal and energy recovery as well as for wastewater treatment, coupled with anaerobic digestion in order to enhance $CH_4$ production while removing $CO_2$ in biogas. Specifically, bio-electrochemically assisted AD (AD-BEC) consists in applying , a relatively low, external potential to a conventional anaerobic digester, then making possible the simultaneous biogas production and upgrading: hydrogenotrophic methanogenesis and electro-methanogenesis are the two main processes through which $CO_2$ and electrons from the cathode electrode are directly used for $CH_4$ production AD-BEC systems (Dou et al., 2018). The other sustainable technology to produce $H_2$ is from the water electrolysis utilizing excess energy from windmills or solar power stations (Ullah Khan et al., 2017); in this respect, biological upgrading represents a highly promising approach to connect electricity net to the natural gas grid via water electrolysis (Lecker et al., 2017).

Moreover, as for the *in- situ* pathway, the utilization of the existing infrastructure of biogas plants and the need for lower technical requirements, resulting in reduced operational and investment cost

and energy compared to available technologies. Finally, possible unconverted hydrogen mixed with methane would improve the combustion properties of biogas as fuel (5-30% hydrogen by volume) (Luo et al., 2012).

Focusing on the *in-situ* application, three main issues have been identified: 1) the low solubility of $H_2$; 2) the addition of hydrogen to a biogas reactor might cause problems to even a breakdown of the process: the increase of hydrogen partial pressure ($pH_2$) can lead to a subsequent inhibition of Volatile Fatty Acids (VFA) degradation (propionate and butyrate); 3) $H_2$ injection exceeding the 4:1 stoichiometric ratio between $CO_2$ and $H_2$ could result in $CO_2$ depletion, and thus lead to an increase of pH: too alkaline pH values may limit the methanogenic activity, while a depletion of $CO_2$ could entail a substrate inhibition for autotrophic hydrogenotrophic methanogens, which rely on $CO_2$ as a C-source (Luo et al., 2012, Rachbauer et al., 2016). In 2013, Luo & Angelidaki tried to overcome the pH increment by means of co-digestion of cattle manure with an acidic substrate such as cheese whey. However, since co-digestion can't always be adopted especially within the field of municipal WW treatment, other optimization modes need to be identified. Few studies have focused on the development of efficient hydrogenotrophic methanogens enrichment in-situ to overcome the risk of Total Volatile Fatty Acids (TVFA) shock caused by the un-adapted consortia at high $H_2$ concentration. Recently, Agneessens et al., (2017) tested $H_2$ pulse injections in order to induce modulation of the microbial community resulting in an increased $H_2$ uptake; In 2015, Xu et al., performed a continuous cultivation in an up-flow anaerobic sludge blanket (UASB) reactor with $H_2/CO_2$ (4:1) as the sole substrate.

The aim of this work was to develop a fast hydrogenotrophic methanogenic culture enrichment strategy capable of limiting organic biodegradation unbalance and to allow a fast start-up of in-situ biogas upgrading reactors, at the pilot or full-scale, at mesophilic conditions. The effectiveness of the enrichment procedure was evaluated in terms of methane content and specific methane production in the output gas, $H_2$ conversion efficiency, pH trends, Volatile Fatty Acids (VFAs) concentrations, and speciation dynamics, COD mass-balance and by means Specific Hydrogenotrophic Methanogens Activity (SHMA) measurement.

## 5.2. Material & Methods

***Semi-continuous experiment: reactor's set up and operation***

The hydrogenotrophic methanogenic culture enrichment was performed using 3 CSTR reactors (total volume $V_{tot} = 2.4$ L; working volume $V_w = 1$ L), namely R1 as control (no hydrogen injection), and R2 and R3 as two replicates: the experimental set up is shown in **Figure 1**. All reactors were

incubated at 35°C and continuously mixed at 150 rpm, by means of a magnetic stirrer, in order to maximize $H_2$ dissolution into the liquid phase.

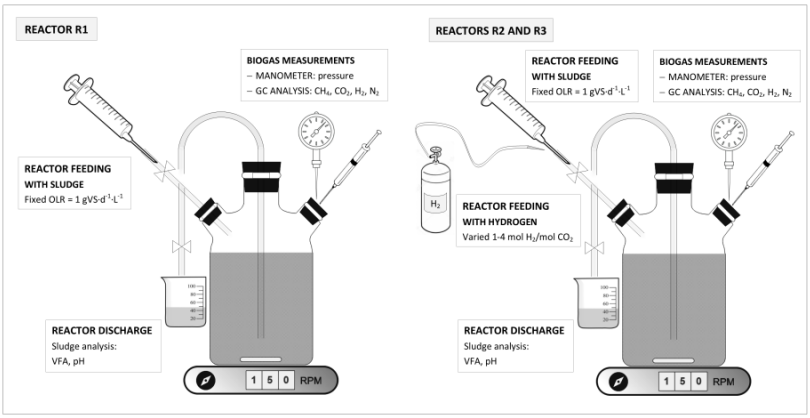



**Figure 1. Graphical representation of the experimental set-up. Reactor R1, on the left, was run as a control reactor; reactors R2 and R3, on the right, were run as replicates of the hydrogenotrophic methanogenic enrichment in-situ test.**

A mixture of primary and biological sludge, collected from a full-scale municipal WWTP (Bresso - Seveso Sud, Milan, Italy), was manually fed in semi-continuous mode (5 days per week). A dose of 70 mL of fresh sludge mixture was adopted, corresponding to an Organic Loading Rate (OLR) of 1 gVS·L-1·d-1, and to a Hydraulic Retention Time (HRT) of 15 days. Main characteristics of substrate and inoculum used in the tests are summarized in **Table 1.**

.

**Table 1. Average characteristics of inoculum and feeding substrate used. The sludge mixture was composed of primary and biological sludge.**

| Parameters | Unit | Sludge mixture | Inoculum |
|---|---|---|---|
| Total Solids (TS) | $g_{ST}$/kg | 22.4±3[(1)] | 24.9±3 |
| Volatile Solids (VS) | $g_{SV}$/kg | 14.7±2 | 14.7±3 |
| VS/TS | % | 66±0 | 59±1 |
| TKN | $mg_N$/kg | 749 | 1350 |
| COD | g/kg | 10 | 5.5 |
| TVFA | mgHac/L | 824 | 255 |

Notes: [(1)] ± Standard Deviation

The three reactors were inoculated with digestates taken from the full-scale digester in the same WWTP (Bresso - Seveso Sud, Milan, Italy) where the sludge mixture used as feeding was collected. Bottles, stored at mesophilic conditions (35°C), were then flushed with nitrogen gas ($N_2$) in order to ensure anaerobic conditions. A mineral medium solution, containing macro and micro-nutrients, was added to the three anaerobic reactors in the ratio 1:10 with respect to the working volume, in

order to avoid lack of trace elements during culture enrichment. The mineral medium was prepared according to Angelidaki et al., (2009).

The enrichment test on reactors R2 and R3 was divided into five phases; the operating conditions of each of them are shown in Table 2. The start-up phase (I), aimed at the acclimation of the biomass, lasted 19 days; during phase I, no hydrogen was added but only the sludge mixture; then, four enrichment phases (from II to V), lasting a total of 29 days, were implemented: during phases II to V, besides the feeding of the sludge mixture, $H_2$ was dosed at different and increasing $H_2/CO_2$ ratios. Specifically, the $H_2/CO_2$ ratio was raised from 1:1 mol $H_2$/mol $CO_2$ to the stoichiometric value of 4:1 mol $H_2$/mol $CO_2$. Before the daily addition of the sludge mixture by means of a syringe, gas volume and composition were measured and reactors were vented to the atmospheric pressure. The volume of hydrogen to be dosed was then calculated based on the average daily flow rate of $CO_2$ produced during the previous phase; thus, $H_2$ was injected using a gas-tight syringe. Table 2 summarizes the operative conditions that were adopted for the enrichment reactors (R2 and R3); the organic loading rate is reported with reference to the contribution given by the sludge mixture only ($OLR_{SM}$), and to the total COD fed including both the hydrogen (8 g COD/g $H_2$) and the sludge mixture ($OLR_{tot}$).

**Table 2. Operational parameters adopted during the five experimental phases of the enrichment trial.**

| Experimental Phase | | Duration [days] | $OLR_{SM}$ [$g_{COD} \cdot L^{-1} \cdot d^{-1}$] | $OLR_{tot}$ [$g_{COD} \cdot L^{-1} \cdot d^{-1}$] | $H_2/CO_2$ ratio [mol $H_2$/mol $CO_2$] |
|---|---|---|---|---|---|
| I | Start-up | 19 | 1 | 1 | - |
| II | Enrichment | 7 | 1 | 1.05 | 1:1 |
| III | Enrichment | 7 | 1 | 1.07 | 2:1 |
| IV | Enrichment | 7 | 1 | 1.09 | 3:1 |
| V | Enrichment | 8 | 1 | 1.12 | 4:1 |

***Monitoring of the process and analytical methods***

Reactors were fed five times per week: total solids (TS), volatile solids (VS) and COD were measured in the feeding sludge mixture according to Standard Methods 2540 for solids and 5220 for COD (APHA, 2005). Total Kjeldahl Nitrogen (TKN) was also measured according to the ISO 5663-1984.

Corresponding to each feeding, digestate was discharged and analysed for TS, VS, pH, and VFA. The pH, which was not controlled during the experiment in order to follow its variation over time then simulating real conditions, was directly measured in samples by means of a portable multi-probe meter (Hach-Lange, HQ40D). The VFA (acetic, propionic, isobutyric, butyric, isovaleric and

valeric) concentrations were determined according to Standard Methods 5560 (APHA, 2005), using a gas chromatograph (DANI Master GC) coupled with a flame ionization detector (FID Nukol fused silica).

The manometric method was used for monitoring biogas production. The pressure was daily measured using a digital manometer (Keller LEO 2) by puncturing the rubber septum; the volume of gas produced during the anaerobic degradation was computed from pressure data, according to the ideal gas law. Biogas composition ($CO_2$, $CH_4$, $H_2$, $O_2$, $N_2$) was analysed three times per week by using a gas chromatograph (DANI Master GC Analyser equipped with two columns HayeSep Q and Molesieve 5A).

The amount of hydrogen ($Dose(H_2)_{phase\_i}$) to be daily dosed in reactors R2 and R3 during the *i-th* enrichment phase (from II to V) was expressed as $mLH_2 \cdot d^{-1}$ and calculated according to Eq. 3:

$$Dose(H_2)_{phase\_i} = (H_2/CO_2)_{phase\_i} \cdot Q(CO_2)_{phase_{i-1}} \quad (3)$$

Where: $(H_2/CO_2)_{phase\_i}$ is the hydrogen to carbon dioxide molar ratio to be used in phase "*i"*, while $Q(CO_2)_{phase\_i}$ is the rate of carbon dioxide daily produced and released in biogas during the previous phase "*i-1*" and expressed in $mLCO_2 \cdot d^{-1}$.

Two coefficients, both expressed as percentages, the $CO_2$ conversion efficiency, and the $H_2$ utilization efficiency, were also evaluated in order to monitor the enrichment evolution. $CO_2$ conversion efficiency (%) of the *i-th* enrichment phase was derived according to Eq. 4:

$$CO_2 conversion\ efficiency = \frac{(CO_2)_{phase\_I} - (CO_2)_{phase\_i}}{(CO_2)_{phase\_I}} \quad (4)$$

where: $(CO_2)_{phase\_I}$ is the rate of $CO_2$ daily produced at the stable point of phase I ($mLCO_2 \cdot d^{-1}$), while $(CO_2)_{phase\_i}$ is the average rate of carbon dioxide produced during phase *"i"*. The $H_2$ utilization efficiency was calculated according to Eq. 5:

$$H_2 utilization\ efficiency = \frac{Dose(H_2)_{phase\_i} - \left(\frac{mlH_2}{d}\right)_{gas\ output\ phase\ i}}{Dose(H_2)_{phase\_i}} \quad (5)$$

where $(H_2)_{phase\_i}$ is the daily hydrogen amount measured in the gas phase ($mLH_2 \cdot d^{-1}$).

Furthermore, the COD mass-balance was evaluated for both reactors. Influent COD was calculated

considering both the sludge mixture and the $H_2$ injected, while the effluent COD was the sum of the COD discharged in both the liquid and the gas phases, then including: the non-degradable volatile solids (VS), multiplied by a conversion factor of 0.7 $gCOD \cdot gVS^{-1}$ based on the substrate characteristics; for methane was used 0.35 $NmLCH_4/gCOD$; the un-converted $H_2$ multiplied by a factor of 0.7 $mgCOD/NmLH_2$.

***SHMA procedure***

The Specific Hydrogenotrophic Methanogenic Activity of the biomass was assessed both at the beginning of the experimentation and at the end of each enrichment phase. SHMA was assessed at mesophilic conditions (35±0.5 °C) adopting an internal protocol based on the manometric method. In detail, the digestate taken from the two reactors was tested in serum vials of 40 mL, fluxed with nitrogen gas and the with a gas mixture composed of $H_2$ and $CO_2$ in the stoichiometric ratio of 4:1 (molar base). Tests were run for 7 hours, at mesophilic conditions and adopting a ratio between the headspace volume and the liquid volume of 2. $H_2$ diffusion in the liquid phase was ensured by means of a magnetic stirrer set at 150 rpm. Headspace pressure was measured every 20 minutes, and methane production was derived assuming that one mole of $H_2$ produces 0.25 moles of $CH_4$; the *SHMA* expressed as $NmLCH_4 \cdot gVS^{-1} \cdot h^{-1}$ was calculated as follows (Eq.6):

$$SHMA = dVCH_4/dt \cdot 1/X \quad (6)$$

Where $X$ $[gVS \cdot L^{-1}]$ is the amount of volatile solids of the digestate dosed in each bottle, and the term $dV(CH_4)/dt$ $[NmLCH_4 \cdot h^{-1}]$ refers to the maximum slope of the cumulative methane production trend over time.

***Statistical analysis***

Statistical analysis was carried out using the software SPSS v.25 aimed at statistically assess: (i) the significance of the observed differences of reactors R2 and R3 with the control reactor R1; (ii) the reproducibility of the two replicates, reactors R2 and R3. Since variables were not normally distributed, the nonparametric Mann-Whitney U test (significance level = 0.05) was used to compare the dependent variables (methane content in the biogas and biogas rate) for two independent groups (R1 and R2, R1 and R3, R2 and R3).

## 5.3. Results and Discussion

**Performance of the semi-continuous reactors**

**Table 3** summarizes the results obtained during the five experimental phases in the three reactors: analyses on biogas and on discharged effluents are shown, as well as performance parameters.

In **Figure 2**, trends over time of the daily biogas volume produced and the daily methane, carbon dioxide, and hydrogen percentages measured, are represented for the three reactors. Process stability was monitored by measuring pH and VFA's total amount and distribution in order to detect possible accumulation and consequent methanogens inhibitory effect. **Figure 3** shows the distribution of VFA measured in the three reactors during all five experimental phases.

In general terms, reproducible results, in terms of biogas composition and methane production, were observed for reactors R2 and R3 until phase IV, thus suggesting that the equipment used was appropriate for the enrichment tests. More significant differences were found in VFAs composition, indicating that intermediate compounds in the anaerobic degradation chain may be more affected by slight differences in environmental conditions, between parallel reactors. The Mann-Whitney U test, considering all methane content data collected during all five phases, showed that the distribution between R2 and R3 (U = 170, Exact sig. = 0.204) were the same (mean ranks: R2 = 23.9; R3 = 19.1). Moreover, since biogas rate data in R3 during phase V were not available, only data collected from phases I to IV were used to test the distribution of biogas rate. This distribution resulted R2 and R3 being comparable (U = 113, Exact sig. = 0.195), with mean ranks equal to 20.2 for R2 and 15.7 for R3. Furthermore, the result was the same, displaying only an exact significance value (0.095) closer to the significance level of 0.05, if all biogas rate data available (phases from I to V for R2, and from I to IV for R3) were considered.

During phase I, the acclimation of the biomass taken from the full-scale digester to the new operative conditions was ensured: at the end of this phase, indeed, methane production achieved a steady state of about 157 $NmLCH_4 \cdot gVS^{-1}$, resulting in a biogas composed for the 73% of methane and for the 27% of carbon dioxide.

**Table 3. Summary of reactors performance parameters for all phases; reactors R1 (control), R2 and R3 (enrichment reactors).**

| **Experimental phase** | **I (pre-$H_2$)** | | | **II ($H_2$/$CO_2$ 1:1)** | | | **III ($H_2$/$CO_2$ 2:1)** | | | **IV ($H_2$/$CO_2$ 3:1)** | | | **V ($H_2$/$CO_2$ 4:1)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Reactor | **R1** | **R2** | **R3** | **R1** | **R2** | **R3** | **R1** | **R2** | **R3** | **R1** | **R2** | **R3** | **R1** | **R2** | **R3** |
| $H_2$ flow rate [NmL·d$^{-1}$] | NA* | NA * | NA | NA * | 62 | 62 | NA * | 102 | 98 | NA * | 133 | 124 | NA * | 222 | 204 |
| Biogas rate [NmL·d$^{-1}$] | 217±20 | 205±9 | 213±18 | 215±20 | 218±26 | 197±7 | 226±31 | 262±4 | 209±17 | 234±5 | 253±16 | 253±30 | 233±30 | 250±17 | Nd** |
| Biogas composition | | | | | | | | | | | | | | | |
| - $CH_4$[%] | 72.6±1.1 | 73.0±1.0 | 72.4±0.5 | 72.2±0.4 | 73.5±0.2 | 74.5±0.3 | 72.5±0.3 | 75.2±0.3 | 76.3±0.3 | 71.3±0.9 | 77.2±0.3 | 77±0.5 | 71.3±0.5 | 80.2±0.9 | 75.31±1.1 |
| - $CO_2$[%] | 27.5±0.7 | 27.0±1.0 | 27.6±0.6 | 26.3±0.5 | 26.5±0.2 | 25.4±0.2 | 27.5±0.7 | 24.8±0.6 | 23.6±0.6 | 28.6±0.1 | 22.8±0.1 | 22.3±0.1 | 26.3±0.2 | 20.3±0.2 | 24.7±0.2 |
| - $H_2$ [%] | Na* | Na* | Na* | Na* | 2.6±1.4 | 0.1 | Na* | 0 | 0 | Na* | 0 | 0 | Na* | 0 | 3.6±1.5 |
| Methane yield [NmL$CH_4$·g$_{VS}$$^{-1}$) | 157 | 149 | 153 | 156 | 160 | 146 | 164 | 169 | 160 | 162 | 194 | 194 | 161 | 195 | Nd** |
| $H_2$ utilization efficiency [%] | Na* | Na* | Na* | Na* | 96.0% | 99.8% | Na* | 100% | 100% | Na* | 100% | 100% | Na* | 100% | 100% |
| $CO_2$ conversion efficiency [%] | Na* | Na* | Na* | Na* | 41.5% | 38.6% | Na* | 49.1% | 26.4% | Na* | 25.1% | 13.4% | Na* | 45.9% | Nd** |
| pH [-] | 7.3 | 7.4 | 7.4 | 7.2 | 7.2 | 7.2 | 7.2 | 7.3 | 7.3 | 7.0 | 7.3 | 7.2 | 7.1 | 7.2 | 7.2 |
| TVFA [mg$CH_3COOH$·L$^{-1}$] | 834 | 314 | 943 | 632 | 348 | 2560 | 313 | 275 | 1090 | 269 | 412 | 274 | 209 | 184 | 1012 |

Notes: *NA: not applicable to this period/reactor; **Nd: not determined due to a technical issue

Averagely, at the end of phase I, pH was slightly lower in the control reactor (7.3) compared to values measured in both R2 and R3 (7.4). During the start-up phase, TVFA resulted lower for R2

(0.3 $g \cdot L^{-1}$) compared to both R1 and R3 (approximately 0.7 $g \cdot L^{-1}$). With reference to TVFA speciation, acetic acid prevailed in R2 (85%), while in reactors R1 and R3 the percentage of acetic acid accounted for about 65%, with a content of propionic acid of 15% indicating that new operative conditions could have led a slight unbalance on propionate degradation.

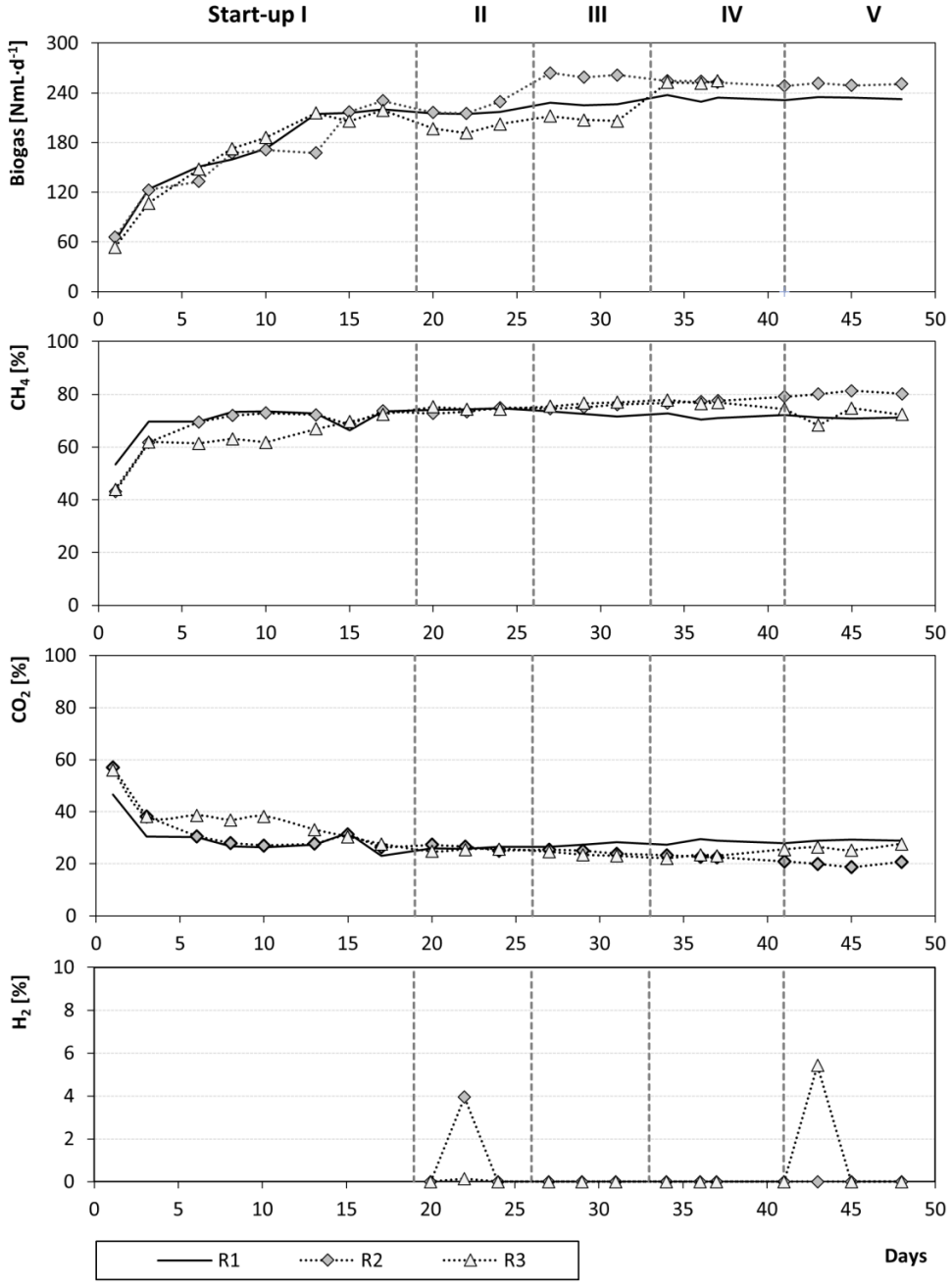



**Figure 2. Biogas rate and composition measured for the three reactors during all experimental phases; R1 is the control reactor; R2 and R3 are the two replicates of the enrichment trial.**

During the first period of the enrichment phases (II), a small $H_2$ dose (approximately 62 $NmLH_2 \cdot L^{-1} \cdot d^{-1}$) determined different effects on R2 and R3, both considering VFA total composition and

speciation and methane yield. Compared to period I, in the control reactor R1, the specific methane yield remained stable at 156 $NmlCH_4 \cdot gVS^{-1}$, while in R2 and R3 it respectively increased and decreased by 5% (160 $NmlCH_4 \cdot gVS^{-1}$ and 146 $NmlCH_4 \cdot gVS^{-1}$, respectively).

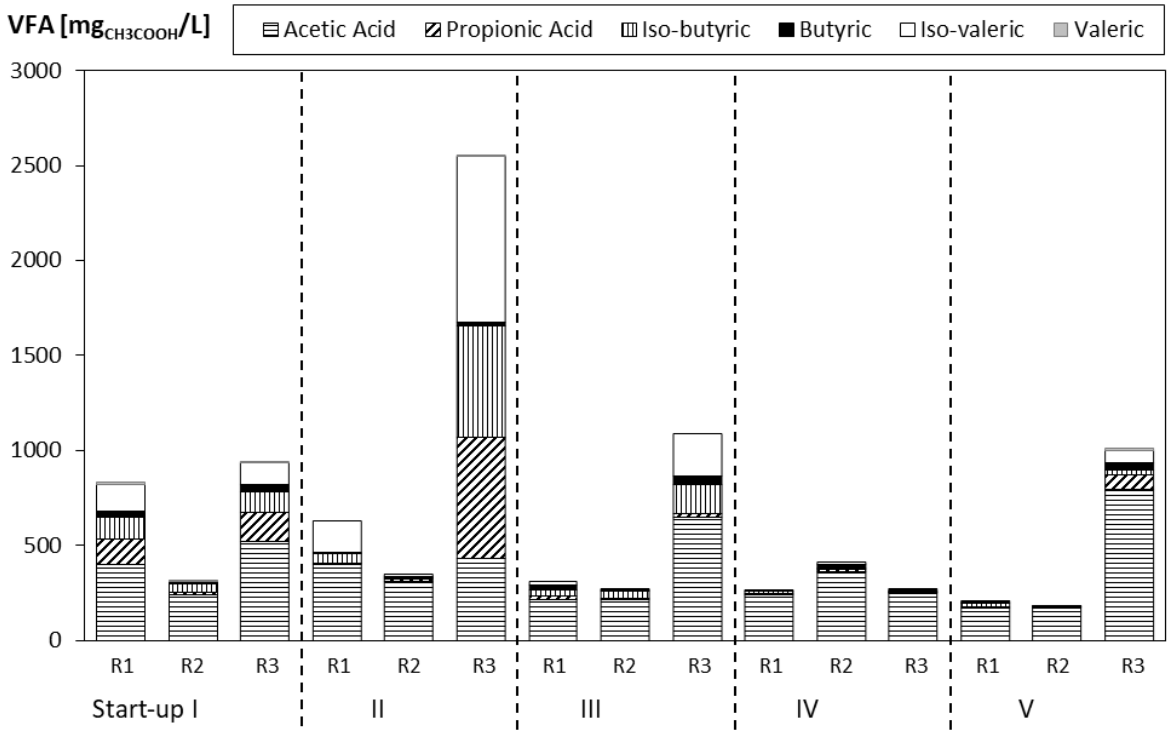



**Figure 3. Volatile fatty acids composition during five experimentation phases; R1 is the control reactor; R2 and R3 are the two replicates of the enrichment trial.**

As expected, TVFA concentration in R1 decreased, indicating a complete adaptation of the biomass to the actual operative condition; on the contrary, R2 and R3 showed different behaviour. More in detail, in R2 a 30% increase of total volatile fatty acids, mainly acetate (94%) was observed, thus indicating the simultaneous utilization of hydrogen by the homoacetogens, in accordance with other studies (Kougias et al., 2017). A significant TVFA change in R3 was observed, as both for concentration (2.56 $gHac \cdot L^{-1}$) and composition: acetate, propionate, iso-butyric and iso-valeric, each accounted for 25% of the total amount, suggesting that a mild inhibition occurred. Moreover, butyrate and valerate isoforms are well-known to be specific indicators of process imbalance as reported in the study of Ahring et al., (1995). TVFA increment and accumulation in both reactors determined a slight pH decrease from 7.4 to 7.2. As for R2, specific methane production was almost the theoretically expected (+7%), thus matching the exogenous $H_2$ conversion by hydrogenotrophic methanogenesis.

The extra methane expected amount was calculated by applying the stoichiometric conversion of 4

$H_2$ moles to 1 mole of $CH_4$, meaning that all the $H_2$ injected in R2 was consumed by the hydrogenotrophic biomass already present, and only partially by homoacetogens. In fact, Kern et al., (2016), assessed that hydrogenotrophic methanogenic strain operates below its physiological capacity. By providing different $H_2$ quantities to three different sludge samples, not acclimatized to exogenous $H_2$, they observed, after only 24h, a positive linear correlation between $H_2$ dose and methane formation rates.

In addition, higher percentages of $CH_4$ in the produced biogas, as well as reduced $CO_2$ contents, were measured in both R2 (73.5% $CH_4$ and 26.5% $CO_2$) and R3 (74.5% $CH_4$ and 25.4% $CO_2$).

Afterwards, during phase III, the $H_2/CO_2$ ratio was further raised up to 2:1, corresponding to a dosage of approximately 100 $NmLH_2 \cdot L^{-1} \cdot d^{-1}$. As a result, an increase of 13% in R2 and 4% in R3 in the specific methane production, and a decrease in $CO_2$ content were observed. Moreover, the resulting $H_2$ utilization efficiency was evaluated as 98% (Table 3).

Despite the same initial conditions, the two enrichment reactors behaved differently: enrichment dynamic was faster in R2 compared to R3. Moreover, during this phase, VFA composition and concentrations measured in R2 (78% acetate, below 300 $mgHac \cdot L^{-1}$) were in the range usually found in well-operating anaerobic reactors (Figure 3). As for reactor R3, TVFA accumulated in the previous phase almost halved (1090 $mgHac \cdot L^{-1}$) and resulted mainly composed of acetate (60%), thus indicating a progressive adaptation to the new increasing $H_2$ partial pressure conditions. Furthermore a slight pH increase (about 7.4) in both reactors was observed, confirming that $CO_2$ in the liquid phase was reduced, corresponding to that observed in the headspace gas composition.

During phase IV, an $H_2$ dose of 129 $NmLH_2 \cdot L^{-1} \cdot d^{-1}$ was adopted, corresponding to an $H_2/CO_2$ ratio of 3:1. The specific methane yield increase of about 30% in both reactors was registered, reflecting a methane content of 77% (Figure 2). Moreover, an effective and stable hydrogenotrophic methanogenic culture enrichment was also confirmed by carbon dioxide content, which was further reduced by 3% in both reactors (22% $CO_2$); also, acetate concentrations (the prevailing component of TVA for about 90%) were well below the suggested threshold of 781 $mgHac \cdot L^{-1}$ (13 mM of acetate) indicated by Ahring et al., (1995).

In the last phase, the stoichiometric value of 4:1 was finally achieved, but no further increments in the specific methane production and in the methane content were observed. However, SHMA values were found higher, compared to the previous phases, as it will be better explained in the next paragraph. High concentrations of TVFA were again registered in R3, even if this time the accumulation was found lower compared to that observed in the II phase. Concluding, the acclimation procedure worked better for R2 than for R3, the last one being less stable and efficient. Further investigations are certainly needed to confirm these results and improve the procedure

proposed.

Moreover, the observed differences between reactors R2 and R3 with the control reactor R1 during the enrichment procedure (phases from II to V) were statistically tested in order to strengthen the main conclusion of the study. The Mann-Whitney U test showed that there was a significant difference between R1 and R2, as to both the biogas rate (U = 140, Exact sig. = 0.003, mean ranks: 9.2 for R1 and 17.8 for R2) and the methane content (U = 160, Exact sig. = $1.9 \cdot 10^{-5}$, mean ranks: 7.7 for R1 and 19.3 for R2). As for R3, phases from II to IV were tested: the methane content measured in R3 resulted being significantly different from that measured in R1 (U = 96, Exact sig. = $1.3 \cdot 10^{-4}$, mean ranks: 5.9 for R1 and 15.1 for R3). Conversely, with reference to the biogas rate, the differences between R1 and R3 were not found to be statistically significant (U = 30, Exact sig. = 0.243); this is likely due to lower biogas rate measured in R3 since the beginning of the test. However, it can be stated that the increment of the methane content in biogas due to the enrichment procedure is statistically significant for both R2 and R3 tested against R1.

A COD mass-balance on the three reactors is shown in Figure 4: the balance closed with errors below 10%, allowing validating data results of the enrichment trial.

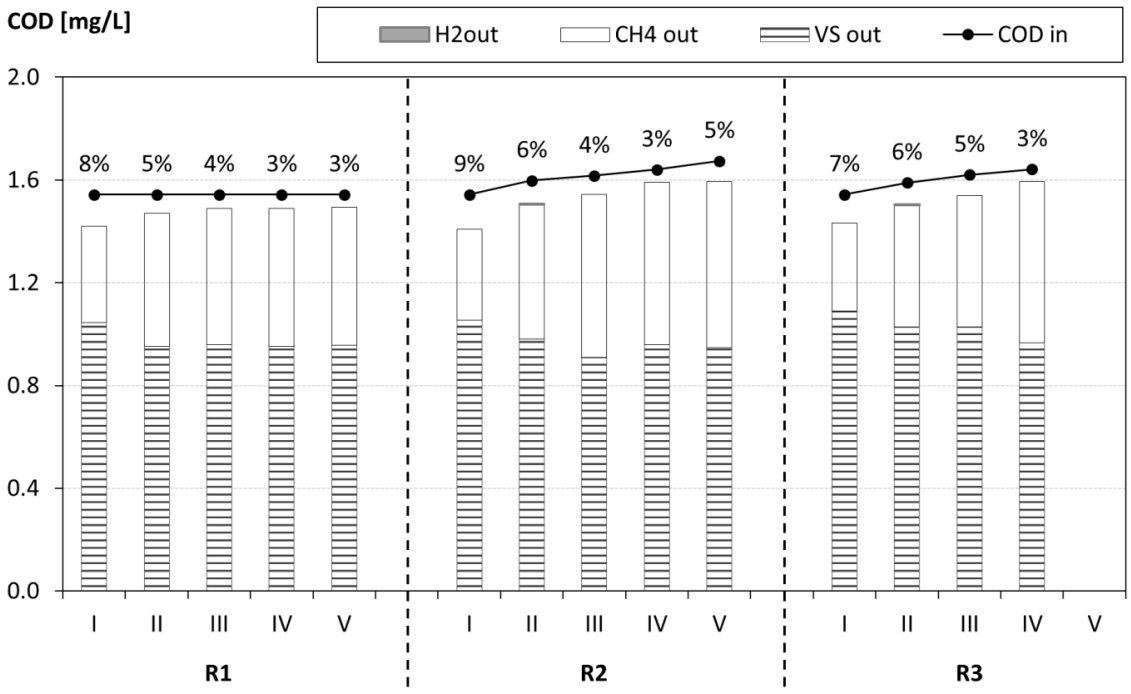



**Figure 4. COD mass-balance during the five enrichment phases for the three reactors; R1 is the control reactor; R2 and R3 are the two replicates for the enrichment trial. Percentages indicate the closing errors evaluated for each phase.**

## Specific hydrogenotrophic methanogenic activity (SHMA) tests

SHMA was measured at the beginning of the experiment and at the end of each experimental phase. As reported in Table 4, the incremental hydrogen dosage resulted in an increased SHMA from values of approximatively 80-90 $mLCH_4 \cdot gVS^{-1} \cdot d^{-1}$ to values in the range 290-360 $mLCH_4 \cdot gVS^{-1} \cdot d^{-1}$. These results are in accordance with Xu et al., (2015) who found, on anaerobic granules from a UASB, an increasing SHMA from 0.2 to 0.6 $gCOD \cdot gVSS^{-1} \cdot d^{-1}$, corresponding to 70 and 210 $mlCH_4 \cdot gVS^{-1} \cdot d^{-1}$. If compared to R2, the lower value measured in R3 at the end of phase II confirms the slight inhibition occurred in that period.

Similar conclusions can be drawn by comparing the SHMA to the methane production of the two reactors during phase V when TVFA accumulation in reactor R3 can be observed. In general, results are shown in Table 4 clearly indicate that the biomass present in the reactors R2 and R3 was effectively enriched in the content of hydrogenotrophic methanogens.

**Table 4. Specific hydrogenotrophic methanogenic activity (SHMA) measured on the effluent digestate at the end of each experimental phase for the three reactors.**

| | | SHMA [$mLCH_4 \cdot g_{VS}^{-1} \cdot d^{-1}$] | | |
|---|---|---|---|---|
| **Experimental phase** | | **R1** | **R2** | **R3** |
| I | Start-up | 81±36 | 87±37 | 91±38 |
| II | $H_2/CO_2$ 1:1 | 90±28 | 105±29 | 98±27 |
| III | $H_2/CO_2$ 2:1 | 85±31 | 108±34 | 115±35 |
| IV | $H_2/CO_2$ 3:1 | 87±29 | 234±36 | 198±29 |
| V | $H_2/CO_2$ 4:1 | 92±33 | 359±29 | 289±38 |

## 5.4. Conclusions

In this work, the effectiveness of an *in-situ* enrichment procedure was evaluated in terms of process stability, biogas composition, and methane production. At the end of first enrichment phase ($H_2/CO_2$ molar ratio 1:1), a significant TVFA accumulation not over 1.8 $g \cdot L^{-1}$ occurred in one of the two replicates; despite this, the system was able to gain stability, TVFA were consumed and $H_2$ injections were never interrupted. During all the four enrichment phases $H_2$ was converted up to 98%. At the end of the last phase, carried out at the stoichiometric $H_2/CO_2$ ratio of 4:1, one of the two reactors, was affected by a second stage of slight process instability, causing TVFA accumulation, probably due to the high $H_2$ partial pressure. It's likely to be that a longer acclimation time is needed when achieving or exceeding the stoichiometric $H_2/CO_2$ ratio. However, during the final phase, a methane content percentage of 81% was achieved in one of the two reactors. thus

making attractive in a biological biogas upgrading process operation the use of an $H_2/CO_2$ ratio above the stoichiometric to boost $CO_2$ conversion resulting in increasing methane yield and methane content in the biogas produced. Further investigations are needed in adopting or exceeding the $H_2/CO_2$ ratio of 4:1 in a longer trial. Furthermore, current results suggest that up to an $H_2$ dosage below the stoichiometric, time duration of one week for each increasing step it seemed to be appropriate for the effective adaptation of the hydrogenotrophic methanogenic culture. A close TVFA dynamics monitoring confirmed to be a proper tool to follow the running of anaerobic degradation chain but also anaerobic consortia acclimation to an increasing hydrogen dosage. Furthermore, SHMA tests demonstrated the effectiveness of hydrogenotrophic enrichment. Concluding, the enrichment procedure here proposed could be an effective tool for the start-up of a pilot and full-scale reactors to be used for *in-situ* biological biogas upgrading applications.


### *Acknowledgments*

The authors wish to thank Dott. Nadia Margariti for her valuable support in the biochemical analysis and Lucia Gambardella for the working activities in the laboratory.


## References


Agneessens, M. L., Ditlev, L., Ottosen, M., Vinther, N., Lund, J., Jonge, N. De, Holst, C., Vedel, M., & Kofoed, W. (2017) In-situ biogas upgrading with pulse H2 additions: the relevance of methanogen adaption and inorganic carbon level. Bioresource Technology, 233, 256-263.

Ahring, B. K., Sandberg, M. & Angelidaki, I. (1995) Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Applied Microbiology and Biotechnology, 43 (3), 559-565.

Angelidaki, I., Alves, M., Bolzonella, D., Borzacconi, L., Campos, J. L., Guwy, A. J., Kalyuzhnyi, S., Jenicek, P., & Van Lier, J. B. (2009) Defining the biomethane potential (BMP) of solid organic wastes and energy crops: a proposed protocol for batch assays. Water Science and Technology, 59(5), 927-934.

APHA 2005 Standard Methods for the Examination of Water and Wastewater 2005 21st edn, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Awe, O. W., Zhao, Y., Nzihou, A., Minh, D. P., & Lyczko, N. (2017) A review of biogas utilisation, purification and upgrading technologies. Waste and Biomass Valorization, 8(2), 267-283.

Corbellini, V., Kougias, P. G., Bassani, I., & Treu, L. (2018) Hybrid biogas upgrading in a two-stage thermophilic reactor. Energy Conversion and Management, 168, 1-12.

Demirel, B. & Scherer, P. (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: a review. Reviews in Environmental Science and Biotechnology, 7(2), 173-190.

Dou Z, Dykstra CM, Pavlostathis SG. (2018) Bioelectrochemically assisted anaerobic digestion system for biogas upgrading and enhanced methane production. Science of the Total Environment, 633, 1012-1021.

Kern, T., Theiss, J., Röske, K., & Rother, M. (2016) Assessment of hydrogen metabolism in commercial anaerobic digesters. Applied Microbiology and Biotechnology, 100(10), 4699-4710.

Kougias, P. G., Treu, L., Peñailillo, D., Boe, K., Campanaro, S., & Angelidaki, I. (2017) Ex-situ biogas upgrading and enhancement in different reactor systems. Bioresource Technology, 225, 429-437.

Lecker, B., Illi, L., Lemmer, A., & Oechsner, H. (2017) Biological hydrogen methanation - A review. Bioresource Technology, 245, 1220-1228.

Luo, G. & Angelidaki, I. (2013) Co-digestion of manure and whey for in situ biogas upgrading by the addition of H2: Process performance and microbial insights. Applied Microbiology and Biotechnology, 97(3), 1373-1381.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., & Angelidaki, I. (2012) Simultaneous hydrogen utilization and in situ biogas upgrading in an anaerobic reactor. Biotechnology and Bioengineering, 109(4), 1088-1094.

Miltner, M., Makaruk, A., & Harasek, M. (2017) Review on available biogas upgrading technologies and innovations towards advanced solutions. Journal of Cleaner Production, 161, 1329-1337.

Muñoz, R., Meier, L., Diaz, I., & Jeison D. (2015) A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Reviews in Environmental Science and Biotechnology, 14(4), 727-759.

Rachbauer, L., Voitl, G., Bochmann, G., & Fuchs, W. (2016) Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Applied Energy, 180, 483-490.

Ryckebosch, E., Drouillon, M., & Vervaeren, H. (2011) Techniques for transformation of biogas to biomethane. Biomass and Bioenergy, 35(5), 1633-1645.

Rozzi, A., Castellazzi, L., and Speece, R. E. (2002) Acetoclastic methanogenic activity measurement by a titration bioassay. Biotechnology and bioengineering, **77**(1).

Shen, Y., Linville, J. L., Urgun-Demirtas, M., Mintz, M. M., & Snyder, S. W. (2015) An overview of biogas production and utilization at full-scale wastewater treatment plants (WWTPs) in the

United States: challenges and opportunities towards energy-neutral WWTPs. Renewable and Sustainable Energy Reviews, 50, 346-362.

Silvestre, G., Fernández, B., & Bonmatí, A. (2015) Significance of anaerobic digestion as a source of clean energy in wastewater treatment plants. Energy Conversion and Management, 101, 255-262.

Smith, P. H. & Mah, R. A. (1966) Kinetics of acetate metabolism during sludge digestion. Applied microbiology, 14(3), 368-371.

Sun, Q., Li, H., Yan, J., Liu, L., Yu, Z., & Yu, X. (2015). Selection of appropriate biogas upgrading technology-a review of biogas cleaning, upgrading and utilization. Renewable and Sustainable Energy Reviews, 51, 521-532.

Ullah Khan, I., Hafiz Dzarfan Othman, M., Hashim, H., Matsuura, T., Ismail, A. F., Rezaei-DashtArzhandi, M., & Wan Azelee, I. (2017) Biogas as a renewable energy fuel – A review of biogas upgrading, utilisation and storage. Energy Conversion and Management, 150, 277–294.

Xu, H., Gong, S., Sun, Y., Ma, H., Zheng, M., and Wang, K. (2015) High-rate hydrogenotrophic methanogenesis for biogas upgrading: the role of anaerobic granules. Environmental technology, 36(1–4), 529–37.

Zinder S.H. (1994) Syntrophic Acetate Oxidation and “Reversible Acetogenesis”. In: Drake H.L. (eds) Acetogenesis. Chapman & Hall Microbiology Series (Physiology / Ecology / Molecular Biology / Biotechnology). Springer, Boston, MA.

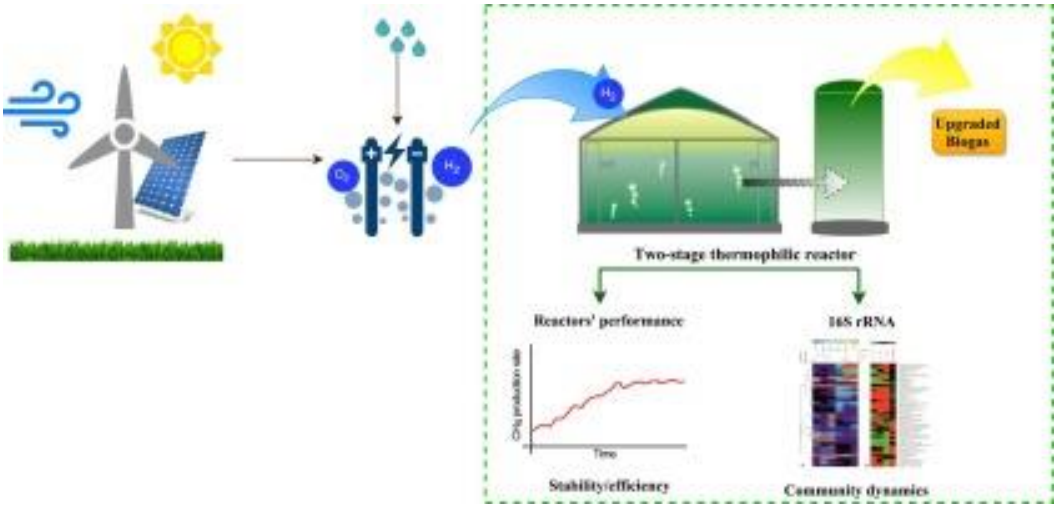
# 6. Hybrid biogas upgrading in a two-stage thermophilic reactor[2]

***Viola Corbellini[1], Panagiotis G. Kougias[2*], Laura Treu[2], Ilaria Bassani[2], Francesca Malpei[1], Irini Angelidaki[2]***

[1]Department of Civil and Environmental Engineering - DICA, Politecnico di Milano, Environmental Section, Piazza L. da Vinci 32, 20133 Milano, Italy

[2]Department of Environmental Engineering, Technical University of Denmark, Kgs. Lyngby, DK-2800, Denmark

## Graphical abstract




## Abstract

This study proposes a hybrid biogas upgrading configuration composed of two-stage thermophilic reactors. This innovative design exploits the combination of the *in-situ* and the *ex-situ* processes in a combined configuration. $H_2$ is directly injected in both reactors and, in particular, the output gas from the first reactor (*in-situ* biogas upgrade) is subsequently transferred to a second up-flow reactor (*ex-situ* upgrade), in which enriched hydrogenotrophic culture is responsible for the hydrogenation of $CO_2$ to $CH_4$. The overall objective of the work was to perform initial methane

[2] Corbellini V, Kougias PG, Bassani I, Treu L. Hybrid biogas upgrading in a two-stage thermophilic reactor. Energy Convers Manag 2018;168:1–12. doi:10.1016/j.enconman.2018.04.074.

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 $CH_4$ 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 (VFA) was observed in the first reactor (*in-situ*) after 8 days of continuous $H_2$ injection reaching a concentration of 5.6 gTVFA/L. Nevertheless, after an adaptation period of one hydraulic retention time (HRT), the system started to recover from the stress and the VFA decreased to 2.5 g/L. No pH drop was recorded during the period characterised by increased VFA concentration mainly due to the consumption of the endogenous $CO_2$ by hydrogenotrophic methanogenesis. Indeed, the bicarbonate contained in the liquid phase of the biogas reactor was coupled with the injected $H_2$, and thus the pH was maintained within the range for optimal methanogenesis (i.e. slightly increased from 8.3 to 8.5) despite the high VFA accumulation. The effect of $H_2$ injection on the microbial community in both reactors was analysed by 16S rRNA gene amplicon sequencing. The results demonstrated an increment in the relative abundance of hydrogenotrophic methanogens and homoacetogens in the *in-situ* reactor, while the microbial community in the *ex-situ* chamber was more simple dominated by hydrogenotrophic methanogens.

## 6.1. 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 increase market volatility and frequency of sudden drop in electricity prices. Unfortunately, the direct storage of the surplus energy produced from RES is yet economically unfavourable. Therefore, several alternative options have been demonstrated in the concept of “Power-to-X”; transforming excess RES into power, heat, and 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 integrating two renewable sources such as biogas and wind/eolic or photovoltaic power generation [1]. 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 production of hydrogen, 2) conversion of hydrogen by means of biological reactions with external CO and $CO_2$ sources into $CH_4$ [2,3].

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. In order to obtain biogas with a 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" [1]. The specific requirements of biomethane for injection into natural gas grids or for exploitation as a vehicle fuel varies among different countries and are in the range of $CH_4$ 80-96 %, $CO_2$ 2-3 %, $O_2$ 0.2-0.5 % [4,5]. Nowadays, more than 280 biogas upgrading plants are in operation worldwide [5]. The commercial technologies implemented are mainly physically or chemically based. In particular, 38% of the upgrading plants utilise water scrubbing, 25%, and 23% organic or chemical scrubber respectively, 9% and 5% physical adsorption and membrane, and lastly, cryogenic technology is used at only 0.4% of the facilities. 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: *in-situ* option, in which $H_2$ is delivered directly inside the biogas digester and there biologically coupled with the endogenous $CO_2$ produced by means of the autochthonous hydrogenotrophic methanogens; the *ex-situ* option, in which $CO_2$ from external sources (e.g. biogas, $CO_2$ storage, syngas) and $H_2$ are injected together inside a reactor containing selected hydrogenotrophic cultures, resulting in their conversion to $CH_4$, and lastly the hybrid biogas upgrade technology, in which *in-situ* and *ex-situ* biogas upgrading are implemented together 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 [6-8]. 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 [9]. Moreover, increased $H_2$ partial pressure, as a result of $H_2$ addition, could affect the interplay of specific bacteria and methanogens. Thus, direct injection of $H_2$ into the anaerobic reactor might inhibit the activity of syntrophic bacteria reducing the anaerobic substrate degradation [10-12]. 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 methanation process, common in both *in-situ* and *ex-situ* concepts, is the poor gas-liquid $H_2$ mass transfer that can be alleviated by using more efficient gas dispersion systems or reactor configurations [7,12,13].

Thus, the aim of the present work was to evaluate the performance of a hybrid biological biogas upgrading system (i.e. *in-situ* and *ex-situ* processes in an integrated system) in respect to the conversion efficiency and final methane content in the output gas. Moreover, 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, VFA and methane yield was performed during the whole experimental work. In addition, in order to better 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**.**

## 6.2. Materials and methods

### 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. Both reactors were operating at thermophilic condition (53 ±1$^{o}$C). The selection of an upflow column reactor was based on previous studies, which demonstrated that such 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 [6]. 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 divided into three periods. During Period I, (days 0-36) the configuration operated as a conventional anaerobic digester. During Period II (days 37-80), the hybrid process was initiated by injecting $H_2$ gas to R1 (i.e. *in-situ* upgrading process) and the output gas was subsequently recirculated to the upflow chamber (i.e. *ex-situ* upgrading process). 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 ($Q_{RC}$) was for 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 [7]. 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 [5].

**Substrate characteristics and feedstock preparation**

A mixture of cattle manure and potato starch was used as influent feedstock for reactor R1. The cattle manure was taken from Snertinge biogas plant, (Denmark), 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. The feedstock mixture was composed by 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 (i.e. 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 1M HCl (i.e. the specific ratio of digestate, HCl and distilled water was 1:0.1:0.3) to reduce the pH from average 8.61 ±0.18 to 6.71 ±0.04. The chemical composition of the used substrates is reported in **Table 1**.

**Table 1. Characteristics of the used substrates; the 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 |
| NH4+-N (g/L) | 0.89±0.04 | 0.49±0.01 | 0.73±0.03 | 4.27±0.05 |
| TVFA (g/L) | 10.21±0.33 | 0.045±0.0 | 2.72±0.12 | 0.94.±0.02 |

**Analytical Methods and calculations**

Total solids (TS), volatile solids (VS) and pH were measured according to APHA standard methods for the examination of water and wastewater [7]. The methane content in the batch assay was

determined using a gas-chromatograph (Shimadzu GC-8A, Tokyo-Japan) as previously described [8]. 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) as previously described [8]. The concentration of volatile fatty acids (VFA) was analyzed using a gas chromatograph (Shimadzu GC-2010, Kyoto, Japan) as previously described [8]. All analyses were done in triplicate samples.

**DNA extraction and 16S rRNA gene 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 (10000 rpm, at 4°C for 10 min) was conducted to obtain around 2 g of cell pellet. The total microbial DNA extraction (DNA isolation and purification) was performed using the PowerSoil® DNA Isolation Kit protocol (MO BIO Laboratories, Carlsbad, CA) with an additional initial cleaning step by Phenol:Chloroform:Isoamyl Alcohol 25:24:1 pH 8 (Sigma-Aldrich, DK). The quality of the purified DNA was examined, and the DNA concentration was analysed with NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA). 16S rRNA gene V4 hypervariable region was amplified with universal primers and sequenced using Illumina MiSeq sequencing technology. The obtained reads were submitted to the NCBI sequence read archive database (SRA) with accession number SRP126498, (http://www.ncbi.nlm.nih.-gov/sra) as BioProject PRJNA421924 with the follow IDs: SAMN08160168 (R1 period I), SAMN08160169 (R1 period II1), SAMN08160170 (R1 period II2), SAMN08160171 (R1 period III), SAMN08160172 (R2 period II2), and SAMN08160173 (R2 period III). The 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 was previously described [15]. 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. Comparison of the microbial abundance between the samples and reactors was performed calculated as a percentage of the total community for each sample. The classification used was carried out considering as highly abundant (>0.5% relative abundance) and lowly abundant (between 0.01% and 0.5% of relative abundance) OTUs, discarded from analysis when lower than

0.01%. Results discussion is focused on the most abundant microbes in the community (>0.5% of relative abundance), some information on the less abundant when statistically significant were reported.

## 6.3. Results and Discussion

### Overview of reactor performance

During Period I, the system operated as a conventional anaerobic reactor co-digesting cattle manure and potato starch. The methane yield of the feedstock at steady-state conditions (approximately after 3 HRTs) reached 211 mL $CH_4$/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. In agreement with previous studies, a difference between the methane and carbon dioxide content was noted among the two reactors [2, 5]. The higher $CH_4$ content in the secondary reactor can be associated with the enhanced dilution of $CO_2$ into the liquid reactor phase due to the gas recirculation system.

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

| | | Period I | | Period II | | Period III | |
|---|---|---|---|---|---|---|---|
| Reactor | | R1 | R2 | R1 | R2 | R1 | R2 |
| Organic Loading rate | gVS/(L*d) | 1.68 | | 1.68±0.2 | | 1.80 | |
| Gas feed ($H_2$ 100%) | mL/(L*d) | | | 550 | | | |
| Gas recirculation | L/(L*hr) | 0.79 | 0.8 | 0.67 | 0.67 | 0.92 | 0.92 |
| Biogas rate | mL/(L*d) | | 472.72±47 | | 400.4±67 | | 552.4±144.5 |
| $CH_4$ production rate* | mL/(L*d) | | 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±0.04 | 8.1±0 | 8.09±0.1 | 7.66±0.17 |
| Total VFA | g/L | 0.2 | 0.03 | 2.7 | 0.1 | 0.6 | 0.1 |

* Methane production rate is calculated considering CH4% from the *ex-situ* reactor

The methane production rate, represented in **Figure 1**, increased upon $H_2$ addition achieving a maximum $CO_2$ removal of 91% (on average 80%) thus as a consequence reaching a maximum methane rate of 434 mL$CH_4$ $Lr^{-1}d^{-1}$ . The methane content in the output gas was on average 85% in the *in-situ* reactor and 88% in the *ex-situ*, while the concentrations of $CO_2$ were 13% and 10%,

respectively. A small content $H_2$ (i.e. approximately 2%) remained unconverted so that 98% of the injected $H_2$ was consumed.

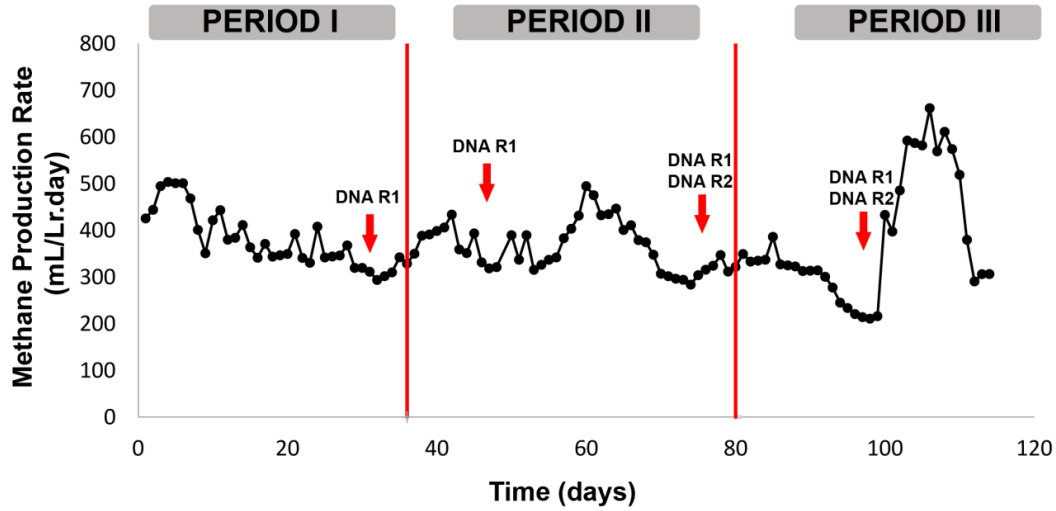



**Figure 1. Methane production rate during the different experimental periods, red arrows indicate DNA extraction for R1 and R2 respectively. CH4 production rate is referred only to R1 reactor volume.**

However, as it can be noticed in **Figure 1**, the increment in methane production rate due to hydrogenation of $CO_2$ did not last long or did not stabilize because was not in balance as indicated by the VFA accumulation (**Figure 2**). 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, respectively in R1. Similarly, a slight VFA accumulation was also recorded in R2 (**Figure 2**). The remarkable increase in VFA concentration reveals a strong inhibition of acetoclastic methanogenesis. As a consequence of the increased $H_2$ partial pressure, the system shifted the metabolic pathway towards homoacetogenesis. This argument was additionally supported by the decrease in the methane production rate. The outcome of the present study is in agreement with previous studies which reported increased acetate concentration upon $H_2$ addition [10; 13]. As it can be noticed from **Figure 2** 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/propionate ratio was inversed in R1, with higher propionate concentration compared to acetate (**Figure 2**). It is has been previously suggested that acetate to propionate ratio can serve as an indicator for process performance [16]; in this context, when propionate exceeds acetate concentration above a certain threshold, an impending digester failure is indicated [17, 18]. The system started to recover as soon as the concentration of propionate was decreased and was found to be lowered compared to acetate.

As it 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 able to metabolize intermediate compounds of AD 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 significantly lower concentration (2.2 and 0.95 gTVFA/L respectively of acetate and propionate) 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 percentage of 95% was indeed achieved (86% in R1). 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 (**Figure 2**). More specifically, the pH values were on average 8.35 and 8.1 for R1 and R2, respectively (**Table 3**). 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.

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

| **Upgrading system** | **Reactor type** | **T °C** | **Substrate (*in-situ*) inoculum (*ex-situ*)** | **OLR g SV/(L*d)** | **$H_2$ flow rate L/(L*d)** | **$CH_4$%** | **$CO_2$ %** | **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 | [8] |
| *In-situ* | CSTR | 55 | Cattle manure | 0.17 | 0.68 | 65 | 15 | 8.3 | [9] |
| *In-situ* | CSTR | 55 | Cattle manure and whey | 1.66 | 0.93–1.76 | 78.4–96.1 | 47-9 | 7.61–8.31 | [10] |
| *Ex-situ* | Up-flow | 55 | Enriched Hydrogentrophic culture | | | 91 | 7 | 8.1 | Current study |
| *Ex-situ* | Up-flow | 55 | Enriched Hydrogentrophic inoculum | | 1-3.6* | 89.5-96.3 | 14.5-0 | 8.03-8.81 | [7] |
| *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.*

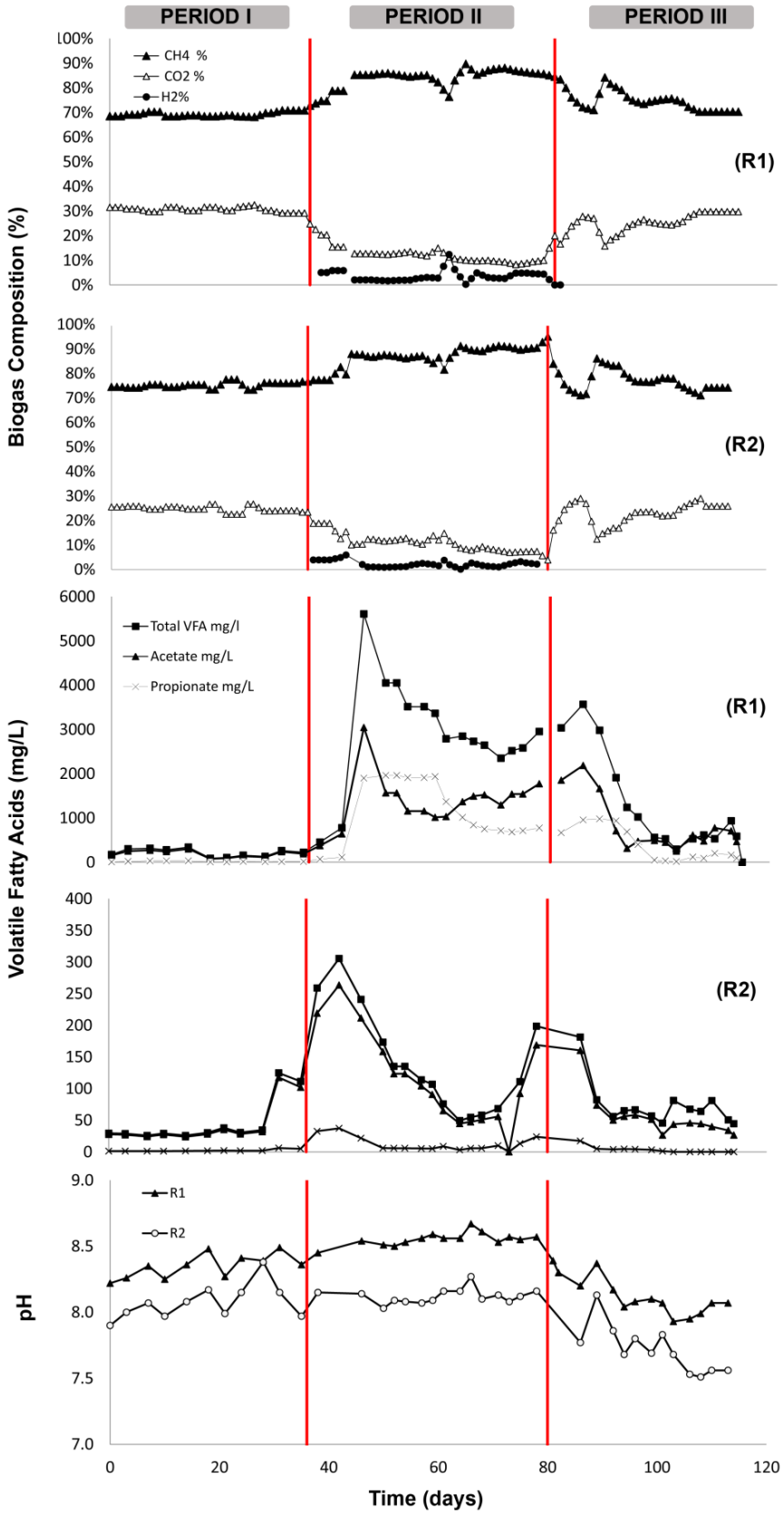



**Figure 2. Gas compositions, VFA and pH values for the in-situ (R1) and ex-situ (R2) biogas upgrading reactors during the whole experiment.**

**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 in R1 an increment in relative abundance of hydrogenotrophic methanogens would occur with a concomitant decrease of other members of the microbial community due 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 [6]) and due to the fact that only gas fermentation was occurring.

Illumina sequencing generated more than 4.76 million of raw reads with an 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.

**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 | 222597 | 56% | 322 | 20 | 79% |
| R1-2 | 1 | II | yes | high | 434264 | 68% | 392 | 16 | 75% |
| R1-3 | 3 | II | yes | high | 315084 | 57% | 460 | 31 | 69% |
| R1-4 | 1 | III | no | standard | 408442 | 72% | 357 | 27 | 73% |
| R2-3 | 3 | II | yes | high | 359785 | 66% | 321 | 20 | 80% |
| R2-4 | 3 | III | no | standard | 411433 | 63% | 391 | 25 | 75% |

Rarefaction curves (Figure 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 Figure 3a.

Samples of 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. **Figure 3b** presents the beta diversity displayed in Principal Component Analysis (PCA) plot.

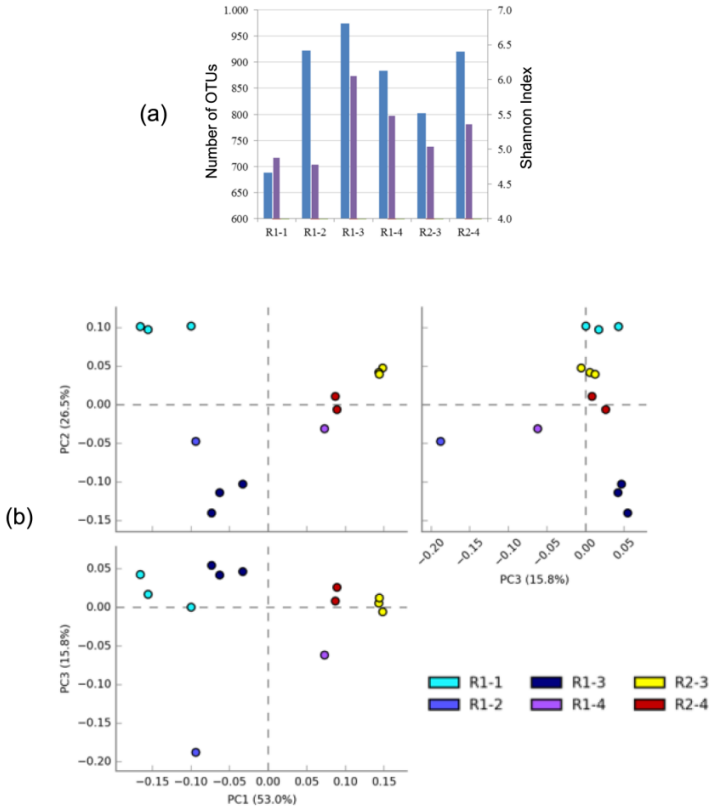



**Figure 3. a) Alpha diversity histograms of OTUs and Shannon Index for all samples of both reactors, b) beta diversity.**

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 from one (purple dot), which clearly showed higher similarity to R2 samples (red and yellow dots) (**Figure 3b**). One replicate of R2-4 was inconsistent with others 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 (**Figure S2**). Table 3 summarised 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%) (**Figure S3**). Notably, only 35% of the OTUs were assigned at the genus level (**Figure S4**) indicating that a high percentage of the microbial

community is composed of uncharacterized species. All further discussions on microbial analysis results will be focused only on the most abundant OTUs, having a relative abundance >0.5%. In particular, 40 OTUs represent the most abundant members and can be considered representative of all the samples. In **Figure 4**, the relative abundance and fold change of the identified OTUs are represented for all collection points from the two reactors. In **Figure 4a**, it can be noted that all samples related to R1 showed a greater diversity 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 (**Figure 3**). This outcome indicates that the microbial community changes 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. 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 the Period I, or microbes whose relative abundance was high in R2 and remained unchanged during the experimental periods.

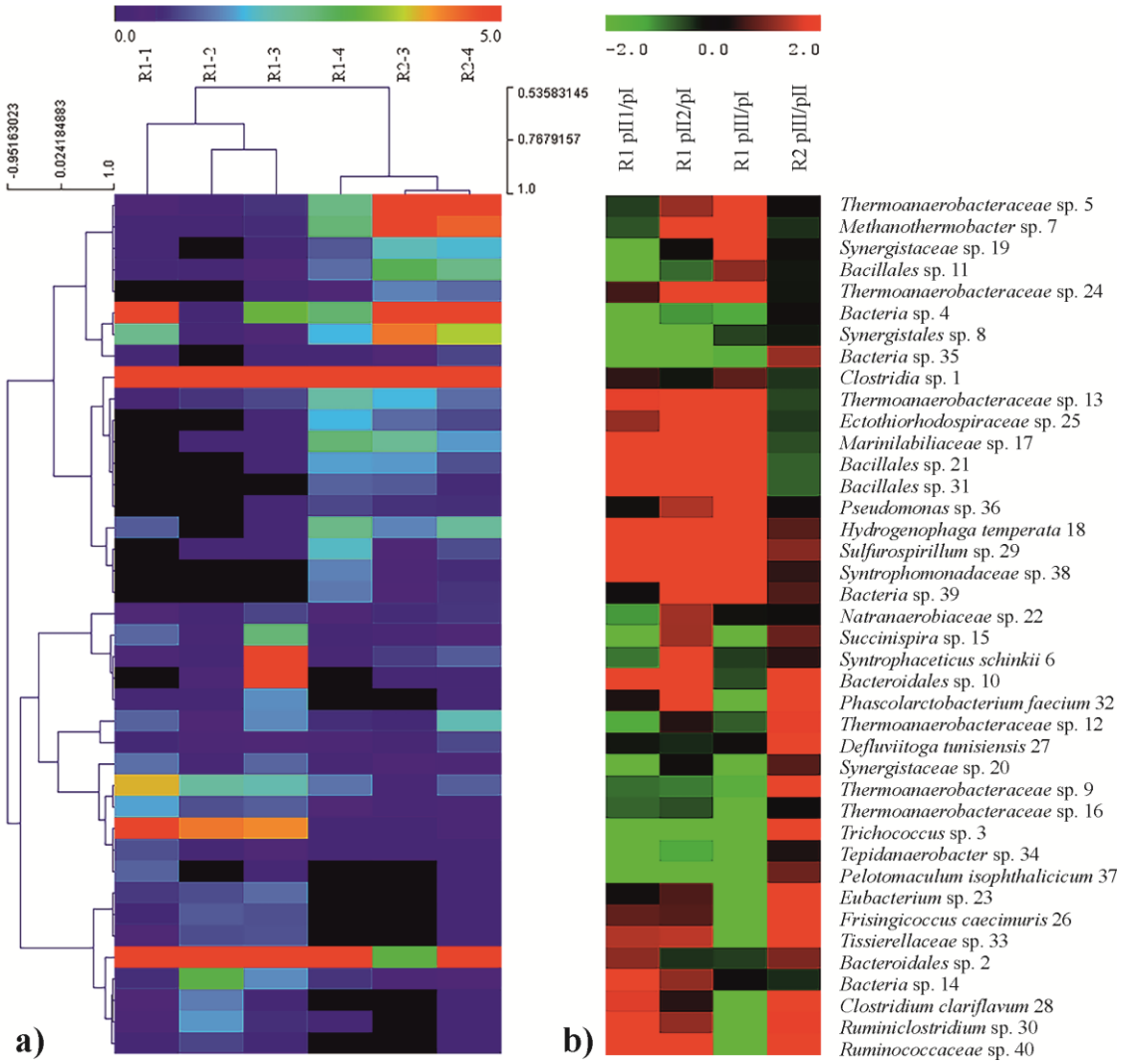



**Figure 4. Heat maps of relative abundance (%) (a), and fold change (log2) (b) of the most abundant OTUs. Colour scales are shown on top of each panel. On the left panel, the most abundant microorganisms are shown in red colour and the less abundant in blue and black. On the right panel, the relative abundance increment in fold change is coloured by red, while the decrease in fold change is coloured in green.**

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 from 0.06% in the Period I and 2.6% period III in R1, stable 5% in R2) belonged to this cluster and was taxonomically assigned to Methanothermobacter genus. The dominance of hydrogenotrophic methanogens in biological biogas upgrading processes is in accordance with previous studies [7,8]. Based on the results from BLASTn search against 16S ribosomal RNA sequences (Bacteria and Archaea) database, *Methanothermobacter* sp. 7 was found 100% similar to *Methanothermobacter thermautotrophicus* and its abundance was enhanced by 45-fold from the Period I to Period III in the R1 reactor. The significant increment of this specific hydrogenotroph is in agreement with previous studies on biogas upgrading systems [6,19].

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 known to be able to grow on autotrophic and/or heterotrophic substrates and metabolize $H_2$ and $CO_2$ producing acetate [20]. 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 $CO_2$ [21]. 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 without or with $H_2$ addition) that were shifting the thermodynamic equilibrium. Thus, the microbial analysis revealed the high abundance of species belonging to *Thermoanaerobacteraceae* family; members of *Thermoanaerobacteraceae* have been previously recognised as homoaceetogens [22]. According to the results of the BLASTn search, the identified OTUs were possibly assigned to *Moorella* genus, which includes several species capable of performing homoacetogenic fermentation [23]. More specifically, *Thermoanaerobacteraceae sp. 5* had 91% similarity to *Moorella humiferrea or Moorella stamsii* and *Thermoanaerobacteraceae sp. 24* was found 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 probably belonging to an unknown microbial species [24]. Both OTUs presented a statistically significant increase in their abundance of more than 8-fold and 459-fold, respectively (**Figure 5**). Similarly, *Syntrophaceticus schinkii* 6 showed a statistically significant increase of 30-fold in Period III (sample R1-3) compared to the Period I (Sample R1-1) (**Figure 5a**). *Syntrophaceticus schinkii* is a well-known syntrophic acetate-oxidizing bacteria previously isolated from sludge digesters [25].

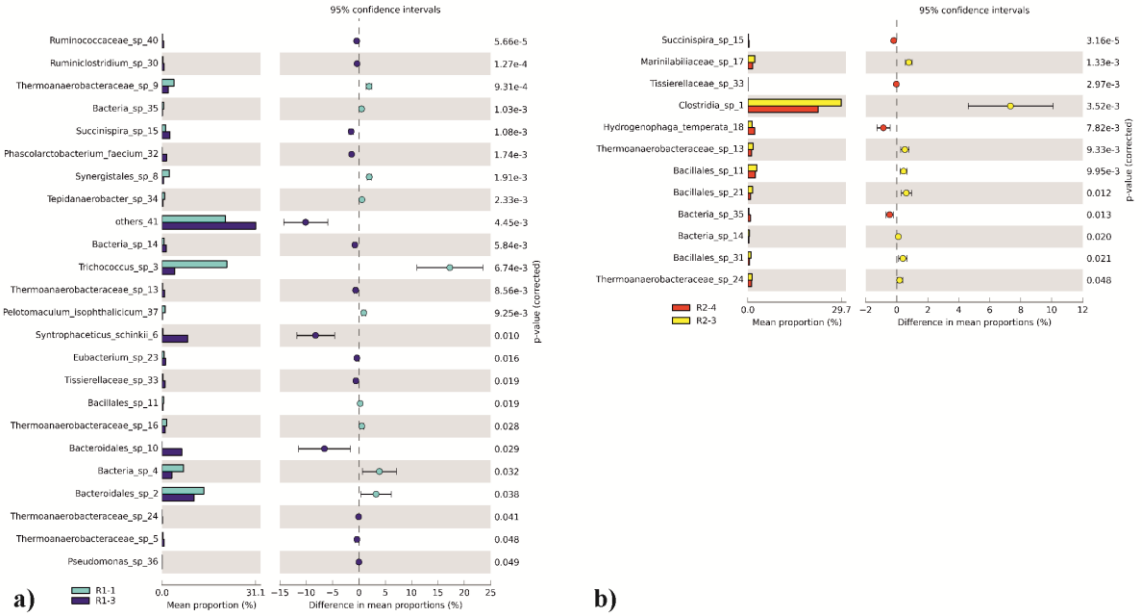



**Figure 5. Statistical comparisons between different extraction point samples; a) comparison between Period III (R1-3) and Period I (R1-1); b) between Periods II (R2-3) and III (R2-4) of R2.**

One of the most dominant OTUs in both reactors was *Clostridia sp. 1, whose relative abundance was significantly increased* upon long-term addition of $H_2$ in R2 (**Figure 5b**). 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 [1,9]. However, the difficulty of assigning this OTU in lower taxonomic classification levels based on the existing public genomic databases highlights its importance as novel microbe residing engineered anaerobic digestion ecosystems.

Finally, it was 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 (**Figure 5b**). Members of this genus are well known to be present in anaerobic reactors processing sludge and dairy manure. Moreover, they are characterized by a homofermentative metabolism with the production of lactic and acetic acids from several carbohydrate substrates [26]. Another bacterium who presented high decrease among this cluster was *Pelotomaculum isophthalicicum* 37 showing 98% identity to *Pelotomaculum isophthalicicum*; this OTU was found to decrease its relative abundance by 84-fold upon $H_2$ addition.

### 6.4. 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 optimisation. An adaptation period has to be taken into consideration as the immediate addition of $H_2$ can lead to a remarkable accumulation of VFA. 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. The close monitoring of the reactors' performance strongly indicates that the addition of a supplemental amount of $H_2$ gas will contribute to further $CO_2$ transformation and thus higher biomethanisation efficiency. Finally, since the upgrading process was achieved in two stages, the removal and subsequent conversion of $CO_2$ to $CH_4$ slightly increased the pH, maintaining it within the range of optimal methanogenesis. Microbial analysis showed that the most abundant microbes belong to uncharacterized taxa. This argument clearly demonstrates that the AD microbiome during hydrogen assisted methanogenesis is strongly stimulated to increase in diversity towards the hydrogenotrophic community contained in *ex-situ* reactor.


### *Acknowledgments*

This work was supported by the Danish Innovation Fund under the project ‘‘SYMBIO – Integration of biomass and wind power for biogas enhancement and upgrading via hydrogen assisted anaerobic digestion”, contract 12-132654 and by Energinet under the ForskEL project “2016-1-12465-BioUpgrade- Ex-situ biogas upgrading through biologically mediated CO2 reduction”.


## References


[1] Kougias PG, Treu L, Peñailillo D, Boe K, Campanaro S, Angelidaki I. Bioresource Technology Ex-situ biogas upgrading and enhancement in different reactor systems. Bioresour Technol 2017;225:429–37.

[2] Nastasi B, Lo Basso G. Power-to-Gas integration in the Transition towards Future Urban Energy Systems. Int J Hydrogen Energy 2017;42:23933–51.

[3] Collet P, Flottes E, Favre A, Raynal L, Pierre H, Capela S, et al. Techno-economic and Life Cycle Assessment of methane production via biogas upgrading and power to gas technology. Appl Energy 2016;192:282–95.

[4] Patterson T, Esteves S, Dinsdale R, Guwy A, Maddy J. Life cycle assessment of biohydrogen and biomethane production and utilisation as a vehicle fuel. Bioresour Technol 2013;131:235–45.

[5] Muñoz R, Meier L, Diaz I, Jeison D. A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Rev Environ Sci Biotechnol 2015;14:727–59.

[6] Bassani I, Kougias PG, Treu L, Angelidaki I. Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environ Sci Technol 2015;49:12585–93.

[7] Bassani I, Kougias PG, Treu L, Port H, Campanaro S, Angelidaki I. Optimization of hydrogen dispersion in thermophilic up-flow reactors for ex-situ biogas upgrading. Bioresour Technol 2017;234:310–9.

[8] Luo G, Angelidaki I. Co-digestion of manure and whey for in-situ biogas upgrading by the addition of H2: Process performance and microbial insights. Appl Microbiol Biotechnol 2013;97:1373–81.

[9] Angelidaki I, Luo G, Kougias P. Simultaneous hydrogen utilization and biogas upgrading by anaerobic microorganisms 2006:2–5.

[10] Luo G, Johansson S, Boe K, Xie L, Zhou Q, Angelidaki I. Simultaneous hydrogen utilization and in situ biogas upgrading in an anaerobic reactor. Biotechnol Bioeng 2012;109:1088–94.

[11] Awe OW, Zhao Y, Nzihou A, Minh DP, Lyczko N. A Review of Biogas Utilisation, Purification and Upgrading Technologies. Waste and Biomass Valorization 2017;0:0.

[12] Rachbauer L, Voitl G, Bochmann G, Fuchs W. Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Appl Energy 2016;180:483–90.

[13] Luo G, Angelidaki I. Hollow fiber membrane based H2 diffusion for efficient in situ biogas upgrading in an anaerobic reactor. Appl Microbiol Biotechnol 2013;97:3739–44.

[14] Guiot SR, Cimpoia R. Potential of Wastewater-Treating Anaerobic Granules for *Biomethanation* of Synthesis Gas 2012:2006–12.

[15] Zhu X, Kougias PG, Treu L, Campanaro S, Angelidaki I. Microbial community changes in methanogenic granules during the transition from mesophilic to thermophilic conditions. Appl Microbiol Biotechnol 2017;101:1313–22.

[16] Wagner AO, Reitschuler C, Illmer P. Effect of different acetate: Propionate ratios on the methanogenic community during thermophilic anaerobic digestion in batch experiments. Biochem Eng J 2014;90:154–61.

[17] D. T. Hill, S. A. Cobb, J. P. Bolte. Using Volatile Fatty Acid Relationships to Predict Anaerobic Digester Failure. Trans ASAE 1987;30:0496–501.

[18] Li L, He Q, Wei Y, He Q, Peng X. Early warning indicators for monitoring the process failure of anaerobic digestion system of food waste. Bioresour Technol 2014;171:491–4.

[19] Treu L, Kougias PG, Campanaro S, Bassani I, Angelidaki I. Deeper insight into the structure of the anaerobic digestion microbial community; the biogas microbiome database is expanded with 157 new genomes. Bioresour Technol 2016;216:260–6.

[20] Gao J, Liu G, Li H, Xu L, Du L, Yang B. Predictive functional profiling using marker gene sequences and community diversity analyses of microbes in full-scale anaerobic sludge digesters. Bioprocess Biosyst Eng 2016;39:1115–27.

[21] Westerholm M, Moestedt J, Schnürer A. Biogas production through syntrophic acetate oxidation and deliberate operating strategies for improved digester performance. Appl Energy 2016;179:124–35.

[22] Win, T. T., Kim, H., Cho, K., Song, K. G., & Park, J. (2016). Monitoring the microbial community shift throughout the shock changes of hydraulic retention time in an anaerobic moving bed membrane bioreactor. Bioresource Technology, 202, 125–132.

[23] Alves, J. I., van Gelder, A. H., Alves, M. M., Sousa, D. Z., & Plugge, C. M. (2013). Moorella stamsii sp. nov., a new anaerobic thermophilic hydrogenogenic carboxydotroph isolated from digester sludge. International Journal of Systematic and Evolutionary Microbiology, 63(PART 11), 4072–4076.

[24] Yarza P, Yilmaz P, Pruesse E, Glöckner FO, Ludwig W, Schleifer K-H, et al. Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat Rev Microbiol 2014;12:635–45.

[25] Westerholm M, Roos S, Schnürer A. Sybtrophaceticus schinkii gen.nov., sp, nov., an anaerobic, syntrophic acetate-oxidizing bacterium isolated from a mesophilic anaerobic filter. FEMS Microbiol Lett 2010;309:100-4.

[26] Rojas-Sossa JP, Murillo-Roos M, Uribe L, Uribe-Lorio L, Marsh T, Larsen N, et al. Effects of coffee processing residues on anaerobic microorganisms and corresponding digestion performance. Bioresour Technol 2017;245:714–23.

# 7. Biological biogas upgrading via *in-situ* hydrogenotrophic methanogenesis from sewage sludge: continuous operation and microbial investigations[3]


## Abstract

Biogas upgrading can be operated by a biologically mediated process via the introduction of exogenous $H_2$ that is coupled with $CO_2$ to form biomethane. Nowadays, the intent of optimizing the process towards scaling-up is gaining more and more attention. However, $H_2$ injection into a biogas reactor may affect the anaerobic degradation process. Furthermore, $H_2$ can be transformed to methane directly via hydrogenotrophic methanogenesis or indirectly via homoacetogenesis plus acetoclastic methanogenesis. Thus, unwanted VFA accumulation or other intermediates, as well as pH increment due to $CO_2$ depletion from the liquid phase can occur. This study investigated biological *in-situ* biogas upgrading from sewage sludge and in continuous mode. 2 parallel CSTRs (11L) were fed on a mixture of sewage sludge at mesophilic conditions at fixed organic loading rate (OLR) of (1.5 gCOD $L^{-1}d^{-1}$), $H_2$ gas injections progressively increased from 0:5:1 to 7:1 ($H_2/CO_2$ ratio) with pH controlled to 7.4. Maximum methane content of 83% and a minimum of 5% of $CO_2$ and 91% of $H_2$ utilization were achieved at 7:1 $H_2/CO_2$ ratio. A noteworthy ethanol accumulation, during the very first $H_2$ Phase ($H_2/CO_2$ of 0.5:1) occurred (up to 2.5-3 gCOD $L^{-1}$). Nonetheless, maintaining the $H_2$ feeding, ethanol was rapidly depleted, thus indicating the system was able to withstand the new operative conditions. A significant alkalinity reduction due to $CO_2$ depletion in the liquid phase of 50% and 17% in R1 and R2 was registered.


## 7.1. Introduction

European policy driver of feed-in tariffs made its effects: the biomethane sector is rapidly increasing. From 2011 to 2016, biomethane production showed growth of +40% which corresponds to 4.971 GWh (EBA, 2017). In the last decade, biogas upgrading into biomethane has been representing a great opportunity to boost the energy recovery from several kinds of matrices of wastes and wastewater. Raw biogas derived from wastewater treatment plant (WWTP) is generally

[3] Paper in preparation

composed by 55-70% $CH_4$, 30-45% of $CO_2$ and other traces gas (nitrogen oxygen, water, hydrocarbons ammonia, and siloxanes) (Awe et al., 2017). Commonly, it is utilised in combined heat and power (CHP) engines or upgraded to biomethane. In the market, different carbon dioxide separation technologies are available, all derived from oil and gas field for natural gas purification (Kohl and Nielsen, 1997). However, despite their long application, these techniques, after separating $CO_2$ from the gas flux it is released back to the environment. Actually, only membrane-based upgrading technology produces a high purity $CO_2$ stream that can be used as a secondary-product for food or industrial purposes (Starr et al., 2012). Furthermore, still considerable challenges in terms of energy/chemicals consumption are driving researchers on less expensive and simpler $CO_2$ separation solutions (Ryckebosch et al., 2011; Muñoz et al., 2015). Among them, the innovative treatment that exploits the biological conversion of $CO_2$ to methane allows capitalizing it in the most profitable way and represents a cheaper and more environmental friendly solution towards sustainable energy production (Alfaro et al., 2018). $CO_2$ conversion by means of a biological coupling with $H_2$ was at first proposed by Barker and van Niel in 1936 (Zinder & Koch 1984). This process is carried by hydrogenotrophic methanogens which are present in normal anaerobic digesters, with their key role in maintaining a low $H_2$ partial pressure (p <10 Pa), as required by acetogenic bacteria. Hydrogenotrophic methanogens are able to convert carbon dioxide to methane, according to the following reaction (Eq. 1):

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^0 = -135.6 \frac{kJ}{mol} \quad (1)$$

In this reaction, carbon dioxide serves as a carbon source and electron acceptor while hydrogen as an electron donor. Hydrogenotrophic methanogens belong to the archaeal domain in the orders of *Methanobacteriales*, *Methanomicrobiales* and *Methanococcales* (Gomec et al., 2008). In the last decade, the biological upgrading pathway has gained more and more attention (Strevett et al., 1995; Angelidaki et al., 2006; Rachbauer et al., 2016; Mulat et al., 2017; Agneessens et al., 2017). Not just as a more sustainable alternative to conventional biogas upgrade technology, but also for its applicability into the power to gas (P2G) field. In P2G, electrical-energy peaks from natural fluctuant renewables are transformed into $H_2$ through water electrolysis that is further converted to methane by a biological or chemical methanation (Guandalini et al., 2017). In very recent years, three main applications of the biological biogas upgrading were mainly studied at lab-scale: *in-situ*, *ex-situ* (Luo & Angelidaki 2013; Bassani et al., 2017; Kougias et al., 2017) and the very recent *hybrid*, that couples the *in-situ* and the *ex-situ* in one operational unit obtaining benefits from both systems advantages (Corbellini et al., 2018).

In the *in-situ* application, $H_2$ is provided into a biogas reactor where it coupled with the endogenous $CO_2$ derived from the organic substrate degradation and converted into $CH_4$ (Angelidaki et al., 2018). For this upgrading application, some constrains were underlined in the literature (Rachbauer et al., 2016; Luo et al., 2012):

- the $H_2$ partial pressure may ($pH_2$) affect the overall anaerobic degradation trophic chain;
- pH may rise due to endogenous $CO_2$ depletion in the liquid fraction
- Scarce $H_2$ solubility may limit an efficient $H_2$ distribution.

To overcome the pH increasing tendency due to $CO_2$ depletion, in the field of agro-residues and zoo-technical wastewater, optimization has been identified in maintaining pH in an optimal range, by co-digestion of cattle manure with an acidic substrate, such as cheese whey (Luo & Angelidaki, 2013a). A pH was not controlled in most of the published researches, pH variations in the range 7-8.5 were observed (Angelidaki et al., 2018; Corbellini et al., 2018). The usage of a hollow-fiber membrane to facilitate the dissolution of $H_2$ has been proposed (Luo & Angelidaki, 2013b) and excellent results were obtained with percentages up to 100 % of $CH_4$ in the gas output (Savvas et al., 2017; Kougias et al., 2017).

In the field of wastewater treatment, an additional advantage in the application of biological biogas upgrading (both *in-situ* or *ex-situ*) can be found. Being the requested $H_2$ produced on site by electrolysis, is it possible to use also the co-product $O_2$ to partly cover the oxygenation needs in the traditional treatment, obtaining a further energy saving. This additional application is to be considered valid only under the main hypothesis that renewable energy (peaks of energy surplus) is used to power the electrolyser. Lastly, the water effluent from a wastewater treatment plant (WWTP) even could be used as source water for electrolysis. Although pre-treatments may be needed to assure the quality required an electrolyser.

Recently, a rapid strategy to enrich hydrogenotrophic methanogens in order to avoid the unbalance during the *in-situ* upgrading was proposed (**Chapter 5**), the effectiveness of the enrichment process was achieved, but it was also indicated that a ratio $H_2/CO_2$ above the stoichiometric should be adopted to further increase $CO_2$ conversion. Among previous studies performed under mesophilic conditions, treating sewage sludge in CSTR, only two reported almost pure methane in the gas output (98.9% and 100%). In the first Wang, et al., (2013) utilized synthetic coke oven gas (SCOG) composed by $H_2$ and CO in ratio $H_2$/CO: 92/8, thus considering also $CO_2$ derived from the biogas *in-situ* produced, an overall $H_2/CO_2$ ratio that varied from 3.3:1 to 5.4:1 was adopted, but synthetic coke oven gas differs from real biogas from organic substrate degradation. The other high methane content achievement was presented by Agneessens et al., (2017). In their study, 100% methane content was obtained operating in semi-batch adopting a 6:1 ratio. But this is referred to the biogas

produced from $H_2$ consumption, before mixing it with the biogas present in the reactor headspace before $H_2$ injection. Thus the extra methane corresponded to the stoichiometric conversion 4:1 of $H_2$ to $CH_4$ until the pH was below 8.18±0.05. Above this pH value, methane from $H_2$ was only 58±9% and the rest was converted into acetate, which increased 10-folds respect to control reactor. Furthermore, $CO_2$ content was indeed 16.8% and lastly, this achievement was obtained after only 5 consecutive days of $H_2$-pulse injections. Thus, further researches aiming to clarify the relevance of the $H_2/CO_2$ on the methane enrichment are yet to be addressed.

The purpose of this study was to evaluate biogas biological upgrading *in-situ* in continuous-mode with two CSTR parallel lab-scale reactors (11L each) with pH controlled to 7.4. To underline the relation between biogas composition and the $H_2/CO_2$ ratio, this was varied from 0.5:1 up to 7:1. Overall reactors performance and other process parameters such as alkalinity and volatile fatty acids (VFAs) speciation composition and COD balance were measured.

## 7.2. Materials and method

### Experimental set up

Two lab-scale CSTR reactors (Vtot=16L; Vliq=11 L), namely R1, R2 were inoculated with digestate taken from a full-scale mesophilic anaerobic digester of municipal WWTP (Bresso–Seveso Sud, Milan, Italy). R1 and R2 operated in parallel, were daily fed, with 0.5 L of primary and secondary sludge mix (VSS/TSS = 66%). One of the two identical reactor configuration used in this experiment is reported in (**Figure 1**).

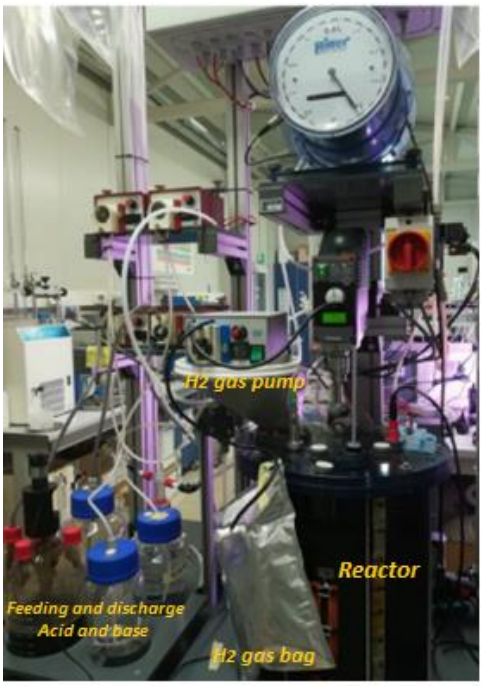


**Figure 1 One of two identical reactor's configuration of the *in-situ* biogas upgrading.**

The organic loading rate (OLR) referred to the solely sludge mix was on average 1.5 gCOD/(L·d) with an HRT of 23 days. The two independent reactors (from Umwelt GmbH) were equipped with peristaltic pumps for loading and discharge, acid and base dosing (HCl 0.5M and NaOH 1M) to maintain pH controlled to 7.4±0.2 set-point. Peristaltic pumps were also utilised to provide pure $H_2$ into the reactors through aluminium (Ø=6mm) bar with sinking at 3/4 height of total reactor depth (21.5 cm). Vigorous mixing at 120 rpm with vertical shaft agitators was assured. Reactors with a diameter of 0.3m had a surface-to-height ratio of the sludge of 32.9 cm. External heating jackets allowed an internal constant temperature of 37°C. The biogas flow was measured by a gas meter (RITTER Apparatebau GmbH & Co. KG) and collected in 5L gas bags, was automatically analysed, in terms of $CO_2$ (range 0-100%), $CH_4$ (range 0-100%), $O_2$ (in the range 0-25%) and $H_2S$ (up to 1500 ppm) by pressure and infrared compensation methods, except for $H_2S$ that was supplied by an electrochemical sensor. $H_2$ was analysed twice per week by using gas chromatography (DANI Master GC Analyser equipped with two columns HayeSep Q and Molesieve 5A).

## Experimental plan

The experimental plan consisted of VIII phases. In the first period (start-up) both reactors were under conventional anaerobic fed on sewage sludge only. Then, an enrichment period of 4 phases in which both systems worked under the hydrogenotrophic stoichiometric ratio and two periods in which a ratio above the stoichiometric was adopted. More in detail, Phase I, the AD start-up, lasted 4 times the HRT (97 days). Both reactors were fed only with mixed sludge 0.5L/d (OLR = 1.5 g COD/L·d) and no $H_2$ was injected. In Phase II to Phase VIII: 0.5L/d of mix sludge plus $H_2$ feeding applying an increasing $H_2/CO_2$ ratio was provided to reactors. Characteristics of the experimental phases are reported in **Table 1**.

**Table 1, Experimental plan characteristics for each phase: duration, OLR from the organic feed, OLR from $H_2$ + feed; $H_2/CO_2$ ratio adopted.**

| | Ph-I | Ph-II | Ph- III | Ph-IV | Ph- V | Ph- VI | Ph- VII | Ph- VIII |
|---|---|---|---|---|---|---|---|---|
| **HRT** | 23 | 23 | 23 | 23 | 23 | 23 | 23 | 23 |
| **Duration (days)** | 80[(1)] | 20 | 7 | 8 | 8 | 26 | 12 | 33 |
| **$OLR_{sl}$(g COD $L^{-1}$ $d^{-1}$)** | 1.5±0.4 | 1.5±0.1 | 1.5±0.3 | 1.5±0.4 | 1.5±0.2 | 1.4±0.4 | 1.5±0.8 | 1.5±0.6 |
| **$OLR_{sl+H2}$(g COD $L^{-1}$ $d^{-1}$)** | 1.5±0.4 | 1.5±0.1 | 2.1±0.3 | 1.6±0.4 | 1.6±0.2 | 1.6±0.3 | 1.7±0.8 | 1.8±0.6 |
| **$H_2/CO_2$** | - | 0.5 | 1 | 2 | 3 | 4 | 6 | 7 |

[(1)]In all figures, only last 20 days of this phase are shown.

From Phase II forward, $H_2$ dosage to be daily fed, to each reactor, was calculated according to the following equation:

$$H_2 \left(\frac{mlH_2}{d}\right)_{phase\ i} = \left(\frac{H_2}{CO_2}\right)_{phase\ i} \cdot \left(\frac{mlCO_2}{d}\right)_{phase\ I}$$

Where $\left(\frac{H_2}{CO_2}\right)_{phase\ i}$ is the stoichiometric coefficient adopted in the Phase i, while $\left(\frac{mlCO_2}{d}\right)_{phase\ I}$ was the $CO_2$ flow average produced during Phase I representing the amount of $CO_2$ produced and released in biogas by the anaerobic digestion of organic substrate fed.

**Substrate characteristics and feedstock preparation**

CSTR sewage sludge mix feeding was composed by primary and waste activated sludge (WAS), collected from a municipal WWTP located in Bresso, northern Milan (Italy). All substrates characteristics are reported in **Table 2**. The sludge from the WWTP was stored at -20°C, in 5L tanks in two different stocks. Before its usage, it was thawed for 4 days in the fridge than hashed and sieved through a 2 mm net in order to prevent clogging of the pump's tube. A BMP of the mixed sludge already sieved and hashed was evaluated in 229 Nml$CH_4$/gSV. A further BMP of the organic feeding was performed at the end of the experiment utilizing as inoculum the effluent from reactors, having a value of 210 Nml$CH_4$/gSV.

**Table 2, Characteristics of the inoculum and substrate used. The sludge mixture was composed of primary and waste activated sludge.**

| Parameters | Unit | Sludge mixture | Inoculum |
|---|---|---|---|
| Total Solids (TS) | gTS/kg | 26±6[(1)] | 20±2 |
| Volatile Solids (VS) | gVS/kg | 18±5 | 12±3 |
| VS/TS | % | 70 | 59±1 |
| $COD_{tot}$ | g/kg | 12 | 5.5 |
| $VFA_{tot}$ | mgHac/L | 728 | 238 |
| Alkalinity | mg$CaCO_3$/L | 1629±261 | 5666±148 |

Notes: [(1)] ± Standard Deviation

**Analytical Methods**

Total solids (TS), volatile solids (VS) were measured according to APHA standard methods for the examination of water and wastewater (APHA, 2005). Biogas composition ($CO_2$, $CH_4$, $H_2$, $O_2$, $N_2$)

was also analysed twice per week by using gas chromatography (DANI Master GC Analyser equipped with two columns HayeSep Q and Molesieve 5A). Effluent digestate was, also twice per week, analysed for VFA, soluble COD and alkalinity expressed in $mgCaCO_{3eq}/L$ (Fos/Tac HACH LANGE). Volatile fatty acids speciation (acetic, propionic, isobutyric, butyric, isovaleric and valeric) concentrations were determined according to Standard Methods 5560 (APHA, 2005), using a gas chromatograph (DANI Master GC) coupled with a flame ionization detector (FID Nukol fused silica). Total COD was determined according to Method 5130 APAT/IRSA-CNR, Analytical Methods for Water by digestion with $K_2Cr_2O_7$ and $H_2SO_4$ 95-96% (Velp Scientifica, Heating Digester ECO6) and titration with FAS (ferrous ammonium sulfate). The instrument used for the determination of BMP is the automatic AMPTS (Bioprocess Control). The tests were conducted using the UNI EN ISO 11734:2004 standard as a reference, by direct measurement over time of the production of biogas by volumetric method.

**Calculations**

The hydrogen volume correspondent to daily dose in reactors R1 and R2 during the i-th experimental phase $(H_2)_{phase\ i}$ (from II to VIII), expressed as $mLH_2 \cdot d^{-1}$, was calculated according to the following formula:

$$(H_2)_{phase\ i} = (H_2/CO_2)_{phase\ i} \cdot\ Q(CO_2)_{phase\ I}$$

Where: $(H_2/CO_2)_{phase\ i}$ is the hydrogen to carbon dioxide molar ratio adopted in phase "i", while $Q(CO_2)_{phase\ I}$ is carbon dioxide rate produced and released in biogas during the phase I and expressed in $mLCO_2 \cdot d^{-1}$.

Following indexes were calculated along the experiment on the basis of the result obtained. The amount of utilized $H_2$ was calculated by multiplying the amount injected $(H_2)_{phase\ i}$ per the $H_2$ % utilization efficiency according to the following formula:

$$H_2\ eff\ (\%) = \frac{\left(\frac{mlH_2}{d}\right)_{phase\ i} - \left(\frac{mlH_2}{d}\right)_{gas\ output\ phase\ i}}{\left(\frac{mlH_2}{d}\right)_{phase\ i}}$$

Where $(mlH_2/d)_{phase\ i}$ represents the $H_2$ dosed at phase i, and $(mlH_2/d)_{gas\ output\ phase\ i}$ is the $H_2$ unconverted, measured in the output gas phase.

Theoretical methane production:

$$Theroetical\ CH_4\ \left(\frac{mlCH_4}{d}\right) = [\left(\overline{\frac{mlCH_4}{gVS}}\right)_{phase\ I} \cdot \left(\frac{g\ VS}{d}\right)_i] + \frac{(\mathrm{H_2})_{\mathrm{phase\ i}}}{4} \cdot\ H_2eff(\%)$$

Where $\left(\overline{\frac{mlCH_4}{gVS}}\right)_{phase\ I}$ represents the specific $CH_4$ flow average from the pre-$H_2$ phase, $\left(\frac{g\ VS}{d}\right)_i$ represent volatile solid load from the sludge feeding, while $(mlH_2/d)_{dosed\ i} \cdot H_2eff(\%)$ is the $H_2$ fed and utilized in each phase divided by the equivalent factor on molar bases to convert it into methane (4:1 $H_2/CH_4$).

Volatile fatty acids/total alkalinity (VFA/TA) ratio was calculated as follows:

$$\frac{VFA}{TA} = \frac{VFA(mgCOD/L)}{TA\ (mg\frac{CaCO_3}{L})}$$

to monitor the stability of the anaerobic digestion process. Conversion factors for VFAs, ethanol, and VS are reported in the following **Table 3**:

**Table 3. Conversion factors of VFA ethanol and volatile solids (VS) used for COD conversion.**

| | **Acetic** | **Propionic** | **Iso-butyric** | **Butyric** | **Iso-valeric** | **Valeric** | **Ethanol** | **VS** |
|---|---|---|---|---|---|---|---|---|
| **gCOD/g** | 1.07 | 1.51 | 1.82 | 1.82 | 2.04 | 2.04 | 2.08 | 1.5 |

Moreover, VS removal for all periods in terms of % according to the following formula was calculated:

$$VS\ (\%) = \frac{VS_{in}(\frac{gVS}{l}) - VS_{out}(\frac{gVS}{l})}{VS_{in}(\frac{gVS}{l})} \cdot 100$$

This index was used to monitor the effect of $H_2$ injection over the organic substrate anaerobic degradation process. Furthermore, the COD mass-balance was monitored for both reactors. The influent $COD_{in}$ load as the sum of the COD of the organic feed plus the $H_2$ injected according to the following formula:

$$COD_{in} = COD_{feed,VS} + COD_{gas,H2}$$

The effluent $COD_{out}$ load as the sum of COD outputs of non-degradable volatile solids (VS) plus output gas contributions in terms of $CH_4$ and un-converted $H_2$:

$$COD_{out} = COD_{digestate,VS} + COD_{gasCH4} + COD_{gasH2}$$

Thus, COD closure in % was further calculated as follows:

$$COD_{closure}\% = (COD_{in} - COD_{out})/COD_{in}$$

All contribute were expressed in terms of COD equivalent: for $CH_4$ a factor of 2.857 mgCOD/Nml, for $H_2$ 0.7 mgCOD/Nml were used and for VS 1.5 gCOD/gVS.

## 7.3. Results and discussion

### Reactor performance

During the start-up period, reactors were operated as conventional anaerobic digesters fed with organic substrate only. An initial slight variability of methane yields, caused by the acclimatization of systems to the new operative conditions, in both reactors was observed **(Figure 2)**. The steady-state was obtained after operation for above three HRTs (80 days) when methane yields were comparable to the substrate BMP value (0.229 $NLCH_4$/gVS) and were respectively 0.26 and 0.28 $NLCH_4$/gVS for R1 and R2 as reported in **Table 3.**

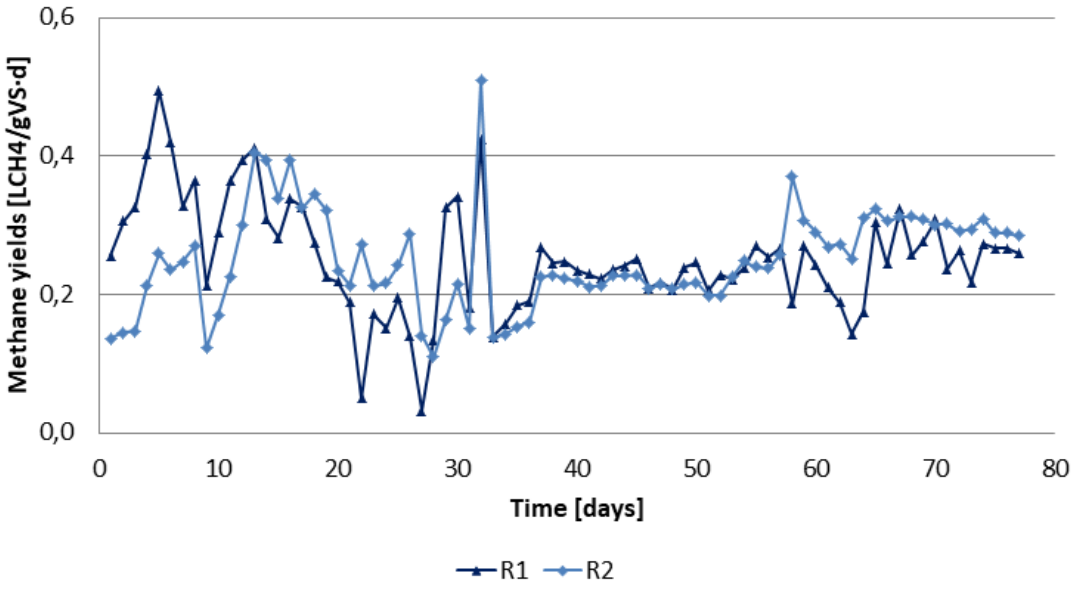



**Figure 2. Methane yields during the start-up phase without $H_2$ addition, for both R1 and R2 reactors.**

At steady-state, total volatile fatty acids (TVFA) were mainly composed by acetic acid (1 gCOD/L), alkalinity was about 4.3 $gCaCO_3$/L and biogas composition was, on average for both reactors,

71.5% $CH_4$ and 22.5% $CO_2$ (**Table 4**). In **Figure 3** and **Figure 4** methane flow rate observed and the theoretical methane curves are reported for R1 and R2. In these graphs, also TVFA and Ethanol trends are reported, to allow simultaneous visualization of the trend of typical indicators of anaerobic degradation chain performance (TVFA) and other soluble organic compounds, in this case, ethanol, which was detected during the experiment.

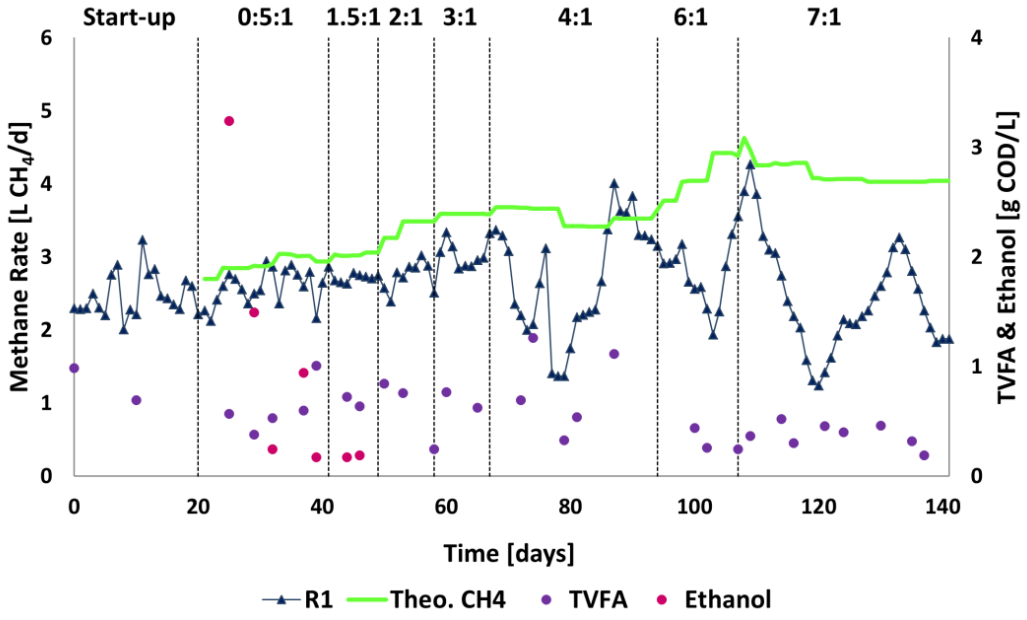



**Figure 3. Methane rate [L$CH_4$/d] for the in-situ (R1) biogas upgrading reactor and TVFA + Ethanol [gCOD/L] trends during the whole experiment.**

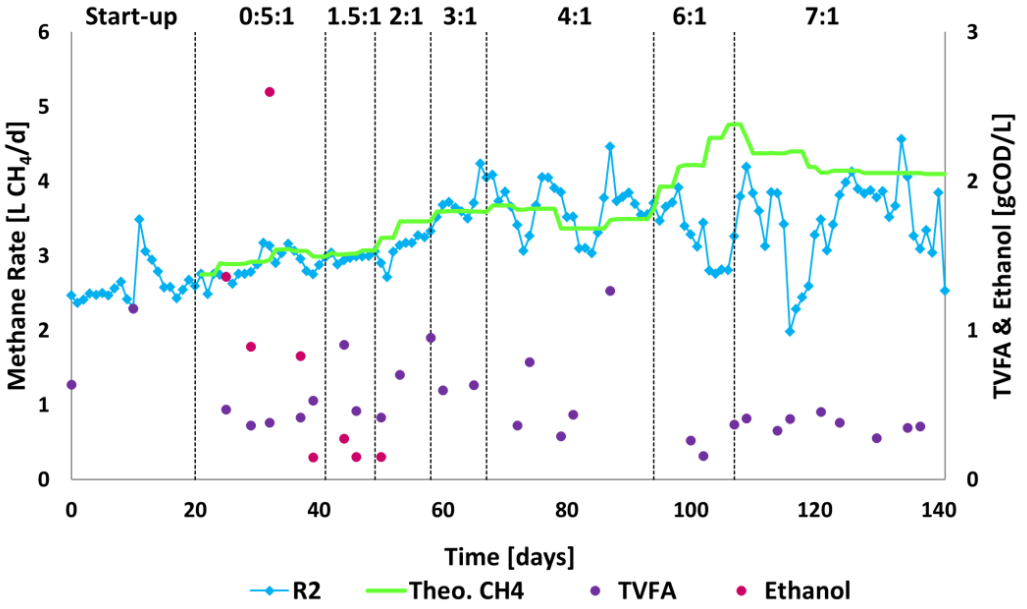



**Figure 4. Methane rate [L$CH_4$/d] for the in-situ (R2) biogas upgrading reactor and TVFA + Ethanol [gCOD/L] trends during the whole experiment.**

During Phase II (days 20-40) hydrogen was provided with an $H_2/CO_2$ ratio (0.5:1). In R2 the methane rate production increased following very closely the theoretical curve, similarly in R1 but higher fluctuating and a lower correspondence with the theoretical was observed.

This outcome is in accordance with the previous study, confirming that hydrogenotrophic methanogens usually work under their $H_2$ rate consumption capacity (Kern et al., 2016) and therefore able to respond immediately to increased availability of $H_2$. Although Liu et al., (2016) reported that around 40% of $H_2$ provided is converted through homoacetogenesis plus acetoclastic methanogenesis, that homoacetogens half-velocity constant (Ks) for $H_2$ is estimated to be 10-folds higher than that of hydrogenotrophic methanogens (Agneessens, 2018) from the registered dynamics of acetate levels in the current study (stable on 0.6 gCOD/L in R1 and on 0.4 gCOD/L in R2), it was not possible to detect homoacetogenesis + acetoclastic methanogenesis pathway. Despite methane rate increments, as soon as $H_2$ was injected, in both reactors ethanol picks of 1.2 gCOD/L were registered, as can be seen in **Figure 3** and **Figure 4** but also more clearly in **Figure 5** were total VFAs speciation plus alcohols levels, on average for each period, are presented.

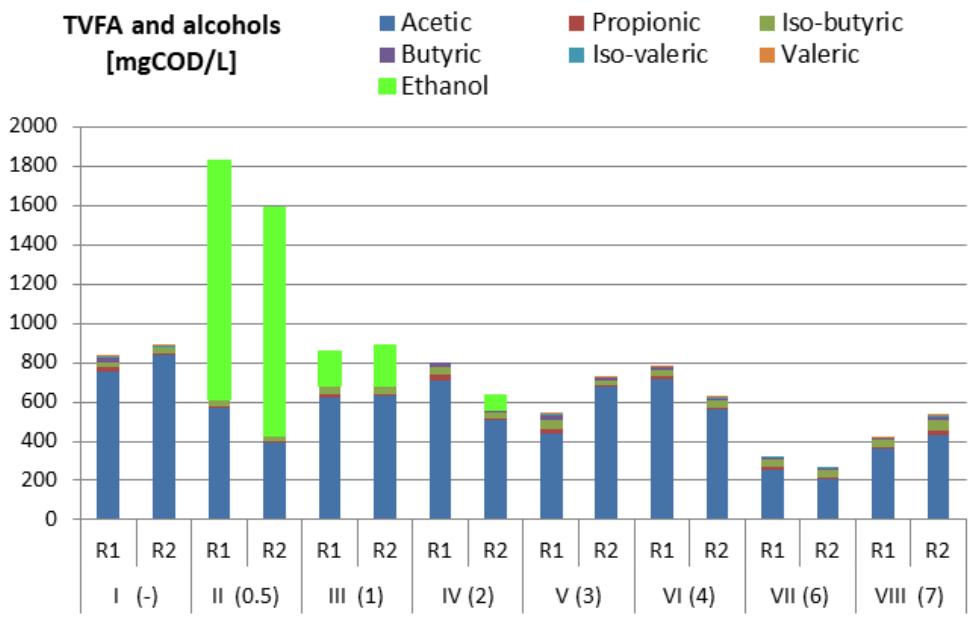



**Figure 5. TVFA speciation concentrations and alcohols of the in-situ (R1) and (R2) biogas upgrading reactors on average for each experimental phase; in brackets the $H_2/CO_2$ ratio adopted.**

This significant ethanol accumulation, to the best of our knowledge, it has not yet been reported on previous studies on biological *in-situ* biogas upgrading. The cause of this accumulation can be explained by the $H_2$ regulatory role in the normal running of anaerobic digestion in the interspecies hydrogen transfer. In an anaerobic environment, ethanol is formed during acidogenesis step from

sugars (Demirel & Scherer, 2008) plus syntrophic bacteria (Conrad et al., 1999). Hydrogenotrophic species, with their activity, stimulates reactions of hydrogen-producing bacteria (Dolfing, 1988). As stated above, low $H_2$ concentrations allow thermodynamic degradation of alcohols and fatty acids by $H_2$-producing syntrophic bacteria (Conrad at al. 1999), thus the amount of extra hydrogen provided to sludge not acclimatized influenced alcohols oxidation, leading ethanol accumulation. According to this, Kaspar and Wuhrmann (1977) observed that an $H_2$ partial pressure of 0.07 atm caused the ethanol conversion to stop in consortia not acclimatized. Nevertheless, even if the $H_2$ supply was never interrupted, the ethanol accumulations in both reactors were reduced to 0.2 gCOD/L in period III ($H_2/CO_2$ 1:1), and almost completely consumed in period IV. This fact can indicate that $H_2$-scavenging microorganisms (i.e. hydrogenotrophic methanogens) increased their activity, promptly reducing the exogenous $H_2$ and keeping its concentration as low as to enable alcohols degradation.

From period III to V (days 40-67) $H_2/CO_2$ ratio was progressively increased from 0.5:1 up to 3:1 every 8-9 days. An obvious methane rate increment was observed in both reactors, with an unvaried biogas composition except a slight $CO_2$ content decrement and an $H_2$% on average below 1% (**Figure 6** and **Table 3**).

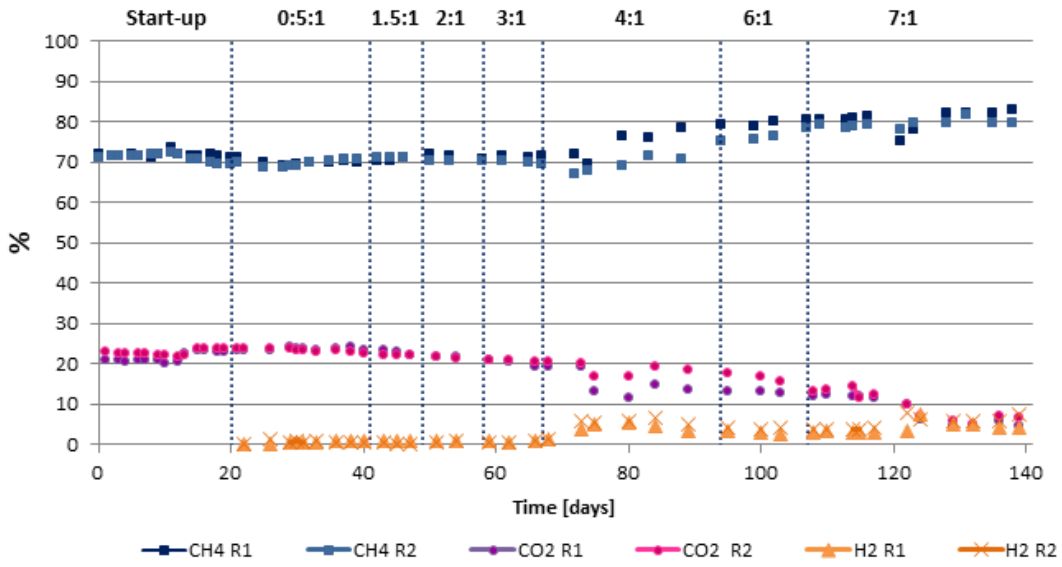



**Figure 6. Output gas composition of the in-situ (R1) and (R2) biogas upgrading reactors for each experimental phase; on top the $H_2/CO_2$ ratio adopted.**

**Table 4 Summary of reactors performance, for all experimental phases.**

| | Ph-I | | Ph-II | | Ph-III | | Ph-IV | | Ph-V | | Ph-VI | | Ph-VII | | Ph-VIII | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 | R1 | R2 |
| Gas feed ($H_2$ 100%) [NmL/d] | 0 | | 496 | | 794 | | 1538 | | 2011 | | 3438 | | 4403 | | 5236 | |
| $CH_4$ rate [NL/d] | 2.5 | 2.6 | 2.7 | 3 | 2.8 | 3.1 | 3 | 3.4 | 3.4 | 4.3 | 3.9 | 4.2 | 3.5 | 3.7 | 3.3 | 4.5 |
| $CH_4$ yield [NL/gVS] | 0.26 | 0.28 | 0.24 | 0.27 | 0.26 | 0.28 | 0.23 | 0.28 | 0.27 | 0.34 | 0.35 | 0.4 | 0.32 | 0.3 | 0.35 | 0.54 |
| $CO_2$ [%] | 22 | 23 | 24 | 23 | 22 | 22 | 21 | 21 | 19 | 21 | 13 | 18 | 12 | 15 | 5 | 6 |
| $CH_4$ [%] | 72 | 71 | 70 | 71 | 71 | 71,08 | 71,35 | 70 | 71 | 70 | 79 | 73 | 80 | 78 | 82 | 80 |
| $H_2$ [%] | 0 | 0 | 1 | 1 | 1 | 0 | 1 | 1 | 1 | 1 | 4 | 5 | 3 | 4 | 6 | 6 |
| $H_2$ conv efficiency [%] | | | 94 | 95 | 95 | 99 | 98 | 98 | 97 | 97 | 95 | 91 | 96 | 95 | 97 | 94 |
| Alkalinity [mg$CaCO_3$/L] | 4364 | 4329 | 4042 | 4067 | 4044 | 4014 | 3955 | 4024 | 3992 | 4369 | 3361 | 4064 | 3480 | 3918 | 2160 | 3631 |
| VFA/TA [mgAceq/mg$CaCO_3$] | 0.18 | 0.19 | 0.28 | 0.23 | 0.17 | 0.18 | 0.14 | 0.16 | 0.16 | 0.12 | 0.21 | 0.14 | 0.08 | 0.06 | 0.15 | 0.09 |
| VS removal [%] | 39% | 37% | 44% | 42% | 39% | 40% | 45% | 47% | 41% | 42% | 35% | 32% | 39% | 34% | 37% | 32% |

From a careful examination of the biogas composition (**Figure 6**), it can be observed that from the start-up period, the sum of the components $CO_2$ and $CH_4$ (and later $H_2$ plus $CO_2$ and $CH_4$) did not correspond to 100%. The missing share is due to the presence of nitrogen, other trace compounds not detectable. It was decided not to recalculate all components taking into account this missing amount, otherwise, there would have been final percentages in the biogas of maximum of 88% of methane, 6% of $CO_2$ and 6% $H_2$. In R2 methane rate trend rather matched the theoretical production curve, whilst R1, despite showing an increment, remained below the expected value. During these periods, VFAs levels progressively decreased and no other significant ethanol accumulation was registered (**Figure 5**).

COD soluble, the sum of all organic-soluble compounds, VFAs aggregated with ethanol, for both reactors, are reported for comparison in **Figure 9**. During the start-up period between CODs and VFAs + ethanol, there is a difference of approximately 1-1.5 gCOD/L. Then, their gap first tended to decrease, i.e. where ethanol was present at high concentration and then reaming in the order of 3.5±0.3 gCOD/L. Moreover, always during the enrichment mixed liquor volatile solids (MLVS) concentration reported an increment in both reactors, from a constant start-up phase value of about 12 g/L up to 13 g/L (**Figure** 7). All these factors lead to a partial explanation during the enrichment periods of the discrepancy between methane rates observed respect to the expected. In particular, the low concentration of VFA and ethanol, but a high concentration of CODs, leads to deduce the presence of other intermediates not detected or the same dissolved $H_2$. Moreover, the increase of MLVS can mean microbiological growth, especially of those microorganisms that are affected by the presence of an extra organic load in the form of $H_2$.

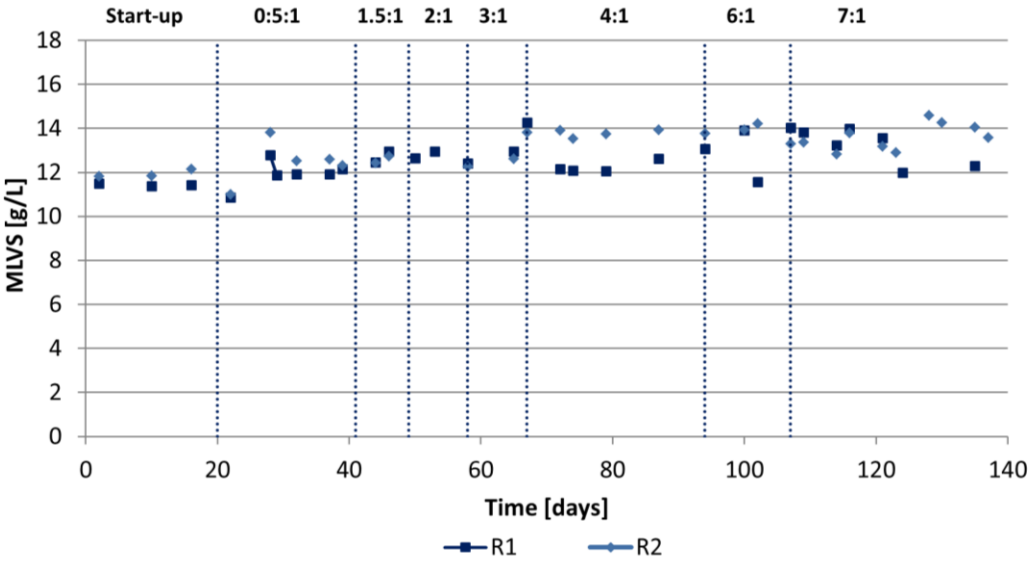



**Figure 7. Mixed liquor volatile solids (MLVS) trends for both reactors (R1) and (R2) for all experimental periods.**

During phase VI (days 67-94), the hydrogenotrophic methanogenesis stoichiometric value ($H_2/CO_2$ 4:1) was adopted. Both reactors showed more fluctuating trends if compared to their previous periods. R1 incurred in a period of high instability in which methane rate varied from a minimum of 1400 $NmlCH_4/d$ up to a maximum of 4000 $NmlCH_4/d$ as can be seen in **Figure 3**. The methane production was significantly lower than the theoretical one up to day 86 and then increased and reaching the theoretical value of 3500 $NmlCH_4/d$. The first point of the minimum is partly explained by a slight increase in volatile fatty acids, that once rapidly reduced brought up again the methane production. Also, the second minimum point is partially connected to intermediates accumulation and possibly related to due to the high variability of VS from organic substrate load as reported in **Figure 8**.

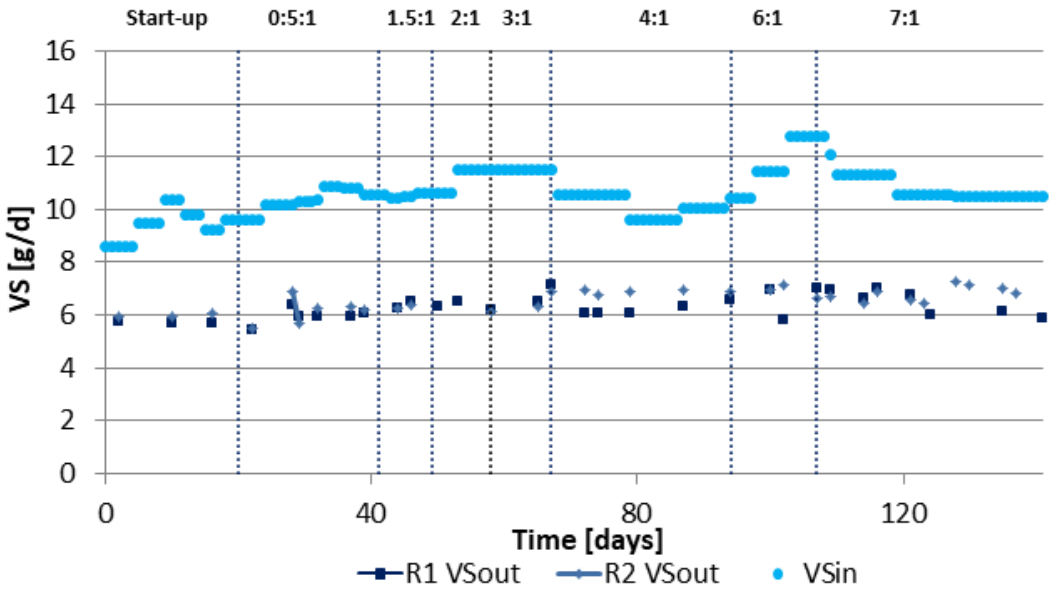



**Figure 8. Influent and effluent volatile solids load for both reactors (R1) and (R2).**

Afterward, a new decrease between CODs and TVFA was observed in the $H_2/CO_2$ 6:1 ratio period. In these two proximal periods (4:1 and 7:1), the discrepancy between CODs and the sum of VFAs + ethanol highlights the possible accumulation of other undetected intermediates, which can then explain fluctuation observed in methane production. Among the organic substances that may not have been analytically detected Shink (1997) reported that a hydrogen accumulation as well as causing accumulations of butyric and propionate, due to the inhibition of their syntrophic oxidation, can affect glycolate-fermenting bacterium which converted glycolate to glyoxylate and hydrogen. Other compounds can be other volatile fatty acids, formate, dissolved $H_2$ and alcohols except for ethanol. Furthermore, during this phases, MLVS of R1 tended to be more variable, between 12 and

14 mgVS/L (**Figure 8**). However, $CO_2$ content in the gas phase decreased to 13% and the methane rose up to 79%. R2 in comparison showed a general lower instability, the methane rate varied only from a minimum of 3000 Nml$CH_4$/d up to a maximum of 4460 Nml$CH_4$/d as can be seen in **Figure 4**. In this case, both minimum methane rate points can be explained by a slight increase in volatile fatty acids. However, despite the higher stability, $CO_2$ content in the gas phase showed a lower reduction to 18% while methane increases its content up to 73% as reported in **Figure 6**.

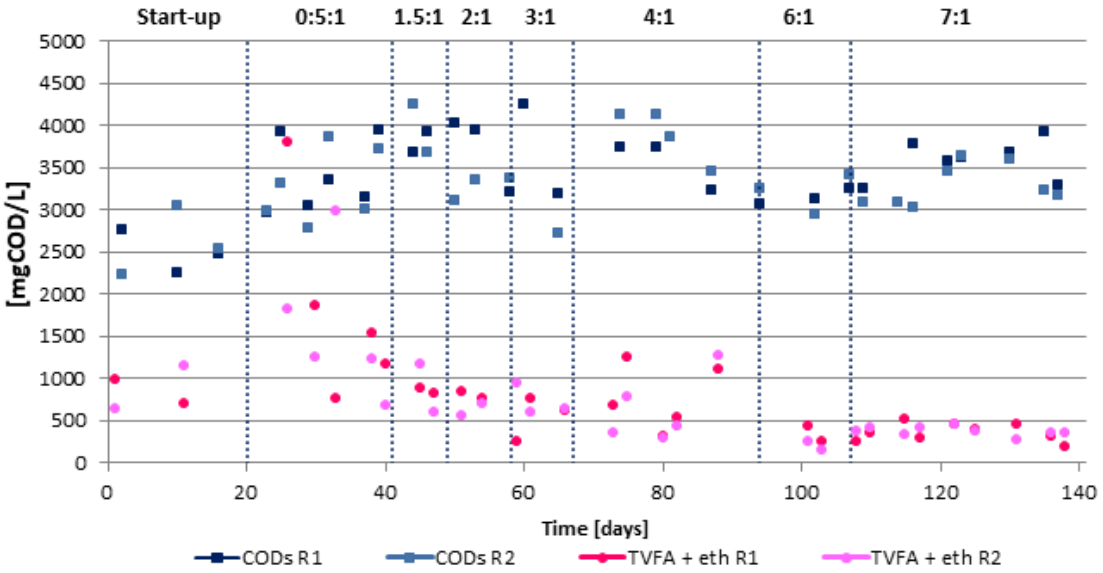



**Figure 9. CODs and TVFAs + ethanol levels for both reactors and all experimental phases expressed as mgCOD/L.**

As a ratio $H_2/CO_2$ of 6:1 (Phase VII) was adopted, methane rate decreased in the beginning and then rise to values close to the theoretical curves as a result of acclimation to the new operative conditions in both reactors. R1 reached a relative minimum of 1900 Nml$CH_4$/d. Reasons for these drastic reductions as mentioned above, cannot be found in total VFAs concentrations, **Figure 3 and Figure 4**, nor from their composition (**Figure 5**), they reached the lowest concentration levels of all experimental phases. In this period the concentration of the influent sludge and the OLR (considering only the sludge) was increased from 1.44 to 1.92 gCOD/Ld. Hence the aforementioned methane rate reduction may arise also from unwanted VS load increment as reported in **Figure 7**, probably caused by a non-homogeneous sludge mixture sieving and blending operations. CODs and VFAs + ethanol reduced their difference in this period with an average value of 2.5 gCOD/L. It can be also observed that in the second part of the period (6:1) the methane production increases and we approach the theoretical, indeed R1 reaches it while R2 does not. The effect of the increase in OLR seems positive on the production of biogas and therefore probably also of hydrogenotrophic

methane production. Noteworthy, it was registered a further $CO_2$ conversion and thus higher methane content, in R1 12%-80% and in R2 15%-76%.

Since, right after R1 and R2 slightly fluctuating trends, VFAs were very low and methane rate was recovered reaching the theoretical production (R1 up to 4250 Nml$CH_4$/d and R2 up to 4190 Nml$CH_4$/d), $H_2/CO_2$ ratio was further increased up to 7:1. At the very beginning of this period, both R1 and R2 followed the theoretical production but then decreased progressively to a minimum value of 1300 and 1970 Nml$CH_4$/d. Here again, there's no evidence of inhibition from total VFAs and ethanol that were below 0.5 gCOD/L, and also no evident picks of CODs can be detected from **Figure 9.** At the end of this period, R2 showed a methane rate very close to the theoretical and registering on average a value of 4500 NmL$CH_4$/d (**Figure 4**) while R1 a methane production well below the theoretical. In order to better understand the causes of these different trends between R1 and R2, the specific COD mass-balance only for this period is presented in **Figure 11**. In detail, in R1 for three points the COD balance didn't close for a significant 20-27%. With a constant percentage of effluent VS, the share missing seems to be methane. In R2, on the other hand, only for one point, there is a 23% COD output lower than the input, while usually, the closure is positive by about 10%. These results, with R1 almost always in negative, could suggest an underestimation of the biogas flow rate by the gas counter. However, in R1 reached a minimum content of $CO_2$ of 5% was reached and final methane content of 83% while in R2 final biogas output was composed by 6% $CO_2$ and methane 80% (**Figure 6**).

During the whole experiment, volatile solids reduction efficiency was considered as organic matter degradation. Influent and effluent organic load trend (gVS/d) is reported for both reactors in **Figure 8**, while the efficiency summarized in **Table 3**. Organic load removal remained fairly constant over time, except a few values that were higher than start-up phase, specifically form a 38% VS degradation on average of the start-up period, to a value of 44% and 42% were registered in R1 and R2 both in period II; 45% and 47% in the IV period, and 41% and 42% in period V. Organic substrate degradation was therefore not affected by the exogenous injection of $H_2$, but, especially in low amount (from $H_2/CO_2$ of 0.5:1 up to 3:1) could have had a positive effect on the whole anaerobic consortium to degrade the organic substrate more efficiently. To evaluate this aspect, a second organic substrate BMP test was carried out (day 137), using a mix of reactor sludges as the inoculum of the test. However, a value of 210 Nml$CH_4$/gVS was obtained, very close to the original BMP of 229 Nml$CH_4$/gVS. No evidence of this hypothesis was obtained, this aspect should be further investigated, carrying out a test batch similar to a BMP able to simulate what could actually happen: a possible synergistic effect on substrate degradation via $H_2$ injection of a sludge acclimatized.

In **Figure 10** the alkalinity trend in both reactors and a straight line with the average value of Phase I is reported as a reference (4.3 $gCaCO_3/L$). As can be noticed, from phase II, an alkalinity consumption tendency was observed along with the progressive $H_2/CO_2$ increasing ratio. This outcome is the direct result of $CO_2$ depletion in the liquid phase, in accordance with a previous study (Wang et al., 2013). The overall consumption, at the end of the experiment, was significantly higher in R1 (50%) than in R2 (17%) (**Table 3**) as confirmed by the higher $CO_2$ reduction registered in R1. Thus, in this study, it did not result in an excessive loss in buffer capacity. Indeed, considering that VFA/TA value, often used to assess the stability of the AD process, remained always below 0.4 (**Table 3**), indicating process stability (Browne et al., 2014). However, this aspect is to be considered an important factor in view of a full-scale upgrading process application and to be evaluated case by case depending on the organic substrate characteristics and its degradability.

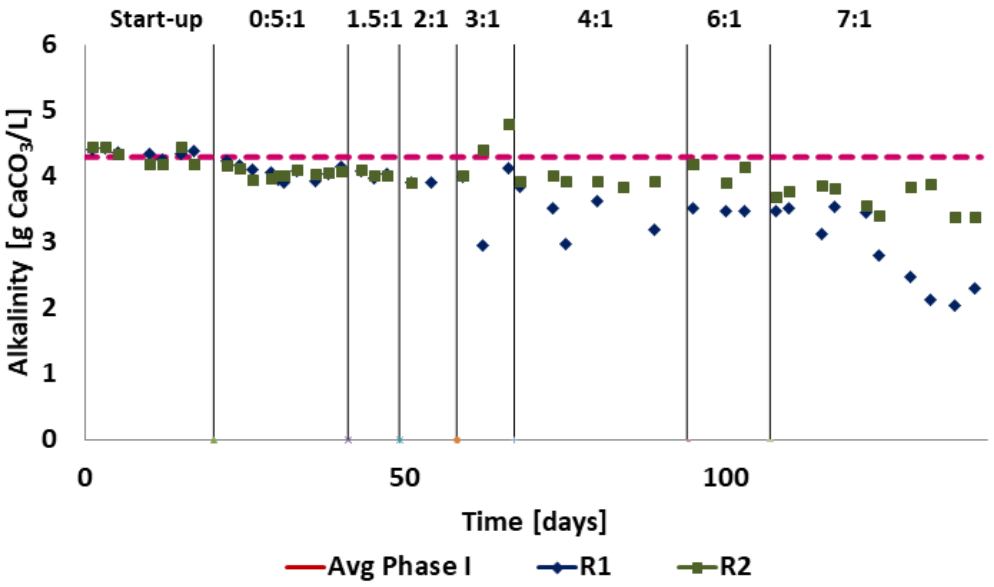



**Figure 10. Alkalinity concentrations for both *in-situ* (R1) and (R2) biogas upgrading reactors during the whole experiment.**

From COD mass-balances shown in **Figure 11**, it can be noticed that the closing error, was around 10% allow considering data results from this experimentation quite reliable, except two period for R2 in which the closure was -16 and -15% possibly due to measurement errors.

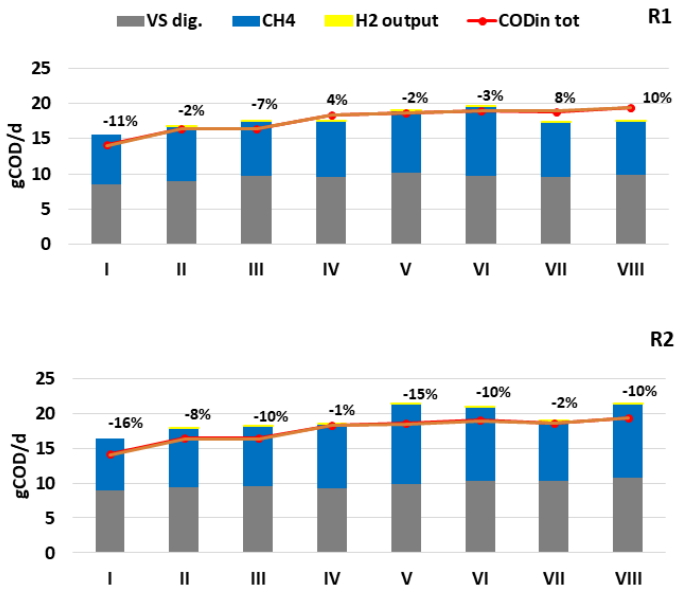



**Figure 11. COD mass-balance for both *in-situ* upgrading reactors (R1) and (R2) for all the experimental periods; % is calculated as follows (CODin–CODout)/CODin.**

Moreover, to better describe the last experimental phase I which especially R1 showed the highest instability, a more detailed COD-mass balance was calculated and presented in **Figure 12**.

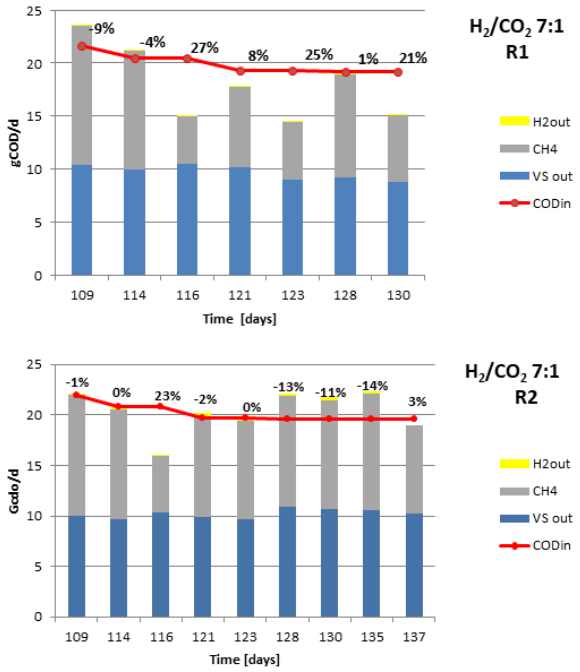



**Figure 12. COD mass-balance for both *in-situ* upgrading reactors (R1) and (R2) for the last experimental period, % is calculated as follows (CODin–CODout)/CODin.**

In this graph, it can be clearly seen that the missing share of COD amount, point by point, but that on average closed the balance of the period for (-10% in R1 and +10% in R2) was the output

methane. This outcome can be explained with the CODs as the sum of the organic compounds present in the liquid phase.

## 7.4. Conclusions

In the present study, biological biogas upgrading in-situ was evaluated for 220 days on an anaerobic municipal sludge not acclimatized in two parallel reactors (11L). After a start-up period, with the only organic substrate as feeding, pure $H_2$ gas was provided with an increasing dosage from an $H_2/CO_2$ ratio of 0.5:1 up to 7:1. During the very first $H_2$ step (II and III periods) corresponding to an $H_2/CO_2$ ratio extremely low (0.5:1 and 1:1), in both reactors a significant ethanol accumulation occurred (2.5–3 gCOD/L). Even if the $H_2$ supply was never interrupted, but increased, these accumulations were consumed, thus indicating progressive biomass acclimatization. At the end of each incremental $H_2$ period, R2 showed to reach an equilibrium point achieving the theoretical methane production, except for $H_2/CO_2$ of 6:1. Conversely, R1 that showed to incur in a more frequent period of instability, a stable condition were reached for all periods except the last ($H_2/CO_2$ 7:1). No clear effect was observed on the organic substrate *in-situ* anaerobic degradation that remained almost stable (40% VS destruction). This outcome indicates the high adaptability of anaerobic mixed culture during continuous $H_2$ injections. Moreover alkalinity consumption of 50% and 17% respectively R1 and R2 were observed, indicating the high $CO_2$ depletion in the liquid phase. This aspect indicates that an excessive $CO_2$ depletion in the liquid phase, when pH is controlled, could lead to a lower limit of operability if the influent doesn't reintegrate the alkalinity. Major effects detected were: increment of CODs, an increase in methane production. Finally, biogas was upgraded reaching a $CO_2$ minimum content of 6%, corresponding to maximum methane of 88% and $H_2$ 6%, if the sum of only these 3 component is considered, operating above the stoichiometric value ($H_2/CO_2$ 7:1).

### *Acknowledgments*

The authors wish to thank Dott. Nadia Margariti for her valuable support in the analysis, and Master students Ilaria Centemero and Michele Rosito for their support in the laboratory activities.

## References

Agneessens, L. M., Ditlev, L., Ottosen, M., Vinther, N., Lund, J., Jonge, N. De, Holst, C., Vedel, M., and Kofoed, W. (2017) Bioresource Technology In-situ biogas upgrading with pulse H 2 additions : The relevance of methanogen adaption and inorganic carbon level. Bioresource Technology, 233, 256–263. http://dx.doi.org/10.1016/j.biortech.2017.02.016.

Alfaro, N., Fdz-Polanco, M., Fdz-Polanco, F., and Díaz, I. (2018) Evaluation of process performance, energy consumption and microbiota characterization in a ceramic membrane bioreactor for ex-situ biomethanation of H2and CO2. Bioresource Technology, 258(December 2017), 142–150. https://doi.org/10.1016/j.biortech.2018.02.087.

Angelidaki, I., Luo, G., and Kougias, P. (2006) Simultaneous hydrogen utilization and biogas upgrading by anaerobic microorganisms. , 2–5.

Angelidaki, I., Treu, L., Tsapekos, P., Luo, G., Campanaro, S., Wenzel, H., and Kougias, P. G. (2018) Biogas upgrading and utilization: Current status and perspectives. Biotechnology Advances, 36(2), 452–466. https://doi.org/10.1016/j.biotechadv.2018.01.011.

APHA 2005 Standard Methods for the Examination of Water and Wastewater 2005 21st edn, American Public Health Association/American Water Works Association/Water Environment Federation, Washington DC, USA.

Awe, O. W., Zhao, Y., Nzihou, A., Minh, D. P., and Lyczko, N. (2017) A Review of Biogas Utilisation, Purification and Upgrading Technologies. Waste and Biomass Valorization, 0(0), 0. http://link.springer.com/10.1007/s12649-016-9826-4.

Bassani, I., Kougias, P. G., Treu, L., Portè, H., Campanaro, S., and Angelidaki, I. (2017) Optimization of hydrogen dispersion in thermophilic up-flow reactors for ex-situ biogas upgrading. Bioresource Technology, 234(March), 310–319. http://dx.doi.org/10.1016/j.biortech.2017.03.055.

Bauer, F., Hulteberg, C., Persson, T., and Tamm, D. (2013) Biogas upgrading – Review of commercial ( Biogasuppgradering – Granskning av kommersiella tekniker ).

Browne, J. D., Allen, E., and Murphy, J. D. (2014) Assessing the variability in biomethane production from the organic fraction of municipal solid waste in batch and continuous operation. Applied Energy, 128, 307–314. http://dx.doi.org/10.1016/j.apenergy.2014.04.097.

Conrad, R. (1999) Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils. FEMS Microbial Ecology, 28, 193–202.

Corbellini, V., Kougias, P. G., Bassani, I., and Treu, L. (2018) Hybrid biogas upgrading in a two-stage thermophilic reactor. Energy Conversion and Management, 168, 1–12.

Demirel, B. and Scherer, P. (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Reviews in Environmental Science and Biotechnology, 7(2), 173–190.

EBA statistical report 2017 (http://european-biogas.eu/2017/12/14/eba-statistical-report-2017).

Gomec, C. Y., Eroglu, V., and Wilderer, P. A. (2008) Granular Sludges Treating Synthetic Sewage By. , 17(212), 135–153.

Guandalini, G., Robinius, M., Grube, T., Campanari, S., and Stolten, D. (2017) Long-term power-to-gas potential from wind and solar power: A country analysis for Italy. International Journal of Hydrogen Energy, 42(19), 13389–13406.

Kaspar, H. F. and Wuhrmann, K. (1977) Product inhibition in sludge digestion. Microbial Ecology, 4(3), 241–248.

Kern T, Theiss J, Röske K, Rother M. Assessment of hydrogen metabolism in commercial anaerobic digesters. Appl Microbiol Biotechnol 2016:1–12. doi:10.1007/s00253-016-7436-5.

Kohl AL, Nielsen RB. Gas Purification (5th). Hous- ton, TX: Gulf Pub, 1997.

Kougias, P. G., Treu, L., Peñailillo, D., Boe, K., Campanaro, S., and Angelidaki, I. (2017) Bioresource Technology Ex-situ biogas upgrading and enhancement in different reactor systems. Bioresource Technology, 225, 429–437.

Liu R., Hao X. & Wei J. (2016). Function of homoacetogenesis on the heterotrophic methane production with exogenous H2 / CO2 involved. Chemical Engineering Journal, 284:1196–1203.

Luo, G. and Angelidaki, I. (2012) Integrated biogas upgrading and hydrogen utilization in an anaerobic reactor containing enriched hydrogenotrophic methanogenic culture. Biotechnology and Bioengineering, 109(11), 2729–2736.

Luo, G. and Angelidaki, I. (2013a) Co-digestion of manure and whey for in situ biogas upgrading by the addition of H2: Process performance and microbial insights. Applied Microbiology and Biotechnology, 97(3), 1373–1381.

Luo, G. and Angelidaki, I. (2013b) Hollow fiber membrane based H2 diffusion for efficient in situ biogas upgrading in an anaerobic reactor. Applied Microbiology and Biotechnology, 97(8), 3739–3744.

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., and Angelidaki, I. (2012) Simultaneous hydrogen utilization and in situ biogas upgrading in an anaerobic reactor. Biotechnology and Bioengineering, 109(4), 1088–1094.

Mulat, D. G., Mosbæk, F., Ward, A. J., Polag, D., Greule, M., Keppler, F., Nielsen, J. L., and Feilberg, A. (2017) Exogenous addition of H 2 for an in situ biogas upgrading through biological reduction of carbon dioxide into methane. Waste Management, 68, 146–156.

Muñoz, R., Meier, L., Diaz, I., and Jeison, D. (2015a) A review on the state-of-the-art of physical/chemical and biological technologies for biogas upgrading. Reviews in Environmental Science and Biotechnology, 14(4), 727–759.

Rachbauer, L., Voitl, G., Bochmann, G., and Fuchs, W. (2016) Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Applied Energy, 180, 483–490.

Ryckebosch, E., Drouillon, M., and Vervaeren, H. (2011) Techniques for transformation of biogas to biomethane. Biomass and Bioenergy, 35(5), 1633–1645. http://dx.doi.org/10.1016/j.biombioe.2011.02.033.

Savvas, S., Donnelly, J., Patterson, T., Chong, Z. S., and Esteves, S. R. (2017) Biological methanation of CO2in a novel biofilm plug-flow reactor: A high rate and low parasitic energy process. Applied Energy, 202, 238–247.

Schink B., Phelps T.J., Eichler B.and Zeikus J.G. (1985), Comparison of Ethanol Degradation Pathways in Anoxic Freshwater Journal of General Microbiology 131, 651-660.

Schink, B. (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and molecular biology reviews : MMBR, 61(2), 262–280.

Shen, Y., Linville, J. L., Urgun-Demirtas, M., Mintz, M. M., and Snyder, S. W. (2015) An overview of biogas production and utilization at full-scale wastewater treatment plants (WWTPs) in the United States: Challenges and opportunities towards energy-neutral WWTPs. Renewable and Sustainable Energy Reviews, 50, 346–362.

Speece, R. E. (1983) Anaerobic biotechnology for industrial wastewater treatment. Environmental Science and Technology, 17(9), 416A–427A.

Starr, K., Gabarrell, X., Villalba, G., Talens, L., and Lombardi, L. (2012) Life cycle assessment of biogas upgrading technologies. Waste Management, 32(5), 991–999.

Strevett, K. A., Vieth, R. F., and Grasso, D. (1995) Chemo-autotrophic biogas purification for methane enrichment: mechanism and kinetics. The Chemical Engineering Journal and the Biochemical Engineering Journal, 58(1), 71–79.

Wang W, Xie L, Luo G, Zhou Q, Angelidaki I. Performance and microbial community analysis of the anaerobic reactor with coke oven gas biomethanation and in-situ biogas upgrading. Bioresour Technol 2013;146:234–9. doi:10.1016/j.biortech.2013.07.049.

Zinder, S. H. and Koch, M. (1984) Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Archives of Microbiology, 138(3), 263–272.

# 8. Microbial analysis of the *in-situ* biological biogas upgrading reactors

## 8.1. Introduction

In AD process a numerous of series or parallel reactions take place (biochemical and physicochemical) that are attributed to a complex microbial consortium, interconnected in a trophic chain, which degrades the introduced organic substrate, to a number of metabolites and different fermentation end-products (Rittmann & Holubar, 2014). Usually, in an anaerobic reactor, a core community is present and another fraction of microorganisms can be found depending on both operative conditions and substrates treated (Bassani, 2017). Among biochemical reactions, four main have been identified as relevant to describe the whole process: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Batstone et al., 2002). In the first phase, fermenting and hydrolytic bacteria excrete enzymes which hydrolyse complex organic matters into simpler molecules down to monosaccharides, amino acids, long chain fatty acids (LCFA) and alcohols (Pavlostathius & Giraldo-Gomez, 1991). This step is carried out by anaerobic bacteria such as *Bacteroides*, *Clostridium*, and *Streptococcus* (Liu & Whitman 2008). Hydrolytic simpler soluble compounds are then fermented or anaerobically oxidized into short-chain fatty acids, hydrogen, carbon dioxide and ammonia by acidogens. In the third phase, syntrophic acetogenic and obligate $H_2$-producing bacteria oxidize to acetate short-chain and long fatty acids. Syntrophic acetogenesis is thermodynamically favourable only at low $H_2$ pressures (<10 Pa) (Dolfing, 1988) and can occur only in syntrophic relation with $H_2$-scavenging microorganisms. The reverse syntrophic reaction, reductive homoacetogenesis is performed mostly by *Clostridium* and *Acetobacterium* genera which grow chemo-litho-autotrophically on $H_2$ plus $CO_2$ as energy and cell carbon source to produce acetate. These microorganisms do not compete well with hydrogenotrophic methanogens for hydrogen consumption; they outcompeted only in some environments (Liu & Whitman 2008) at a high level of $H_2$. The final anaerobic trophic chain is methanogenesis in which acetate and carbon dioxide plus $H_2$ are converted to methane respectively by acetoclastic and hydrogenotrophic methanogenic strains. In this phase, the accumulation of $H_2$ by hydrogen-utilising methanogens and short chain fatty acids by acetoclastic methanogens is therefore avoided by their conversion to $CH_4$ (Pavlostathius & Giraldo-Gomez, 1991). For a detailed examination of these complex processes, diverse molecular approaches used so far are based on the analysis of small subunit ribosomal RNA. Furthermore, changes in the microbial community may occur, even without an effect on process performance due to high microbial diversity in contributing to being a stable ecosystem (Talbot et al., 2008). Thus, especially in a biological *in-situ* biogas upgrading process, it is crucial to

deepen the knowledge on the effect of H2 injection in shaping a consortium able to accomplish and maximise $CO_2$ conversion without losing the capacity to convert anaerobic substrate for the biogas production derived from the organic substrate feeding.

In this study, the $H_2$ injection effects on microbial community dynamics of mesophilic reactors, described in Chapter 7, during the enrichment phases and related to periods working above an $H_2/CO_2$ ratio were assessed. Correlations to process parameters were analysed by 16S rRNA gene amplicon sequencing taken during several experimental points.

## 8.2. Material & methods

### DNA extraction and 16S rRNA gene analysis

8x2 samples (11 mL each) from R1 and R2 reactors were taken for microbial analysis. In brief, the samples were corresponding to the end of Period IV (R1-4 and R2-4) and V (R1-5 and R2-5), 2 collection points during Period VI (R1-6 and R2-6), one collection point at the end of Period VII (R1-7 and R2-7) and 3 collection points in period VIII (R1-8_a; R1-8_b; R1-8_c; R2-8_a; R2-8_b; R2-8_c) (**Figure 1**).

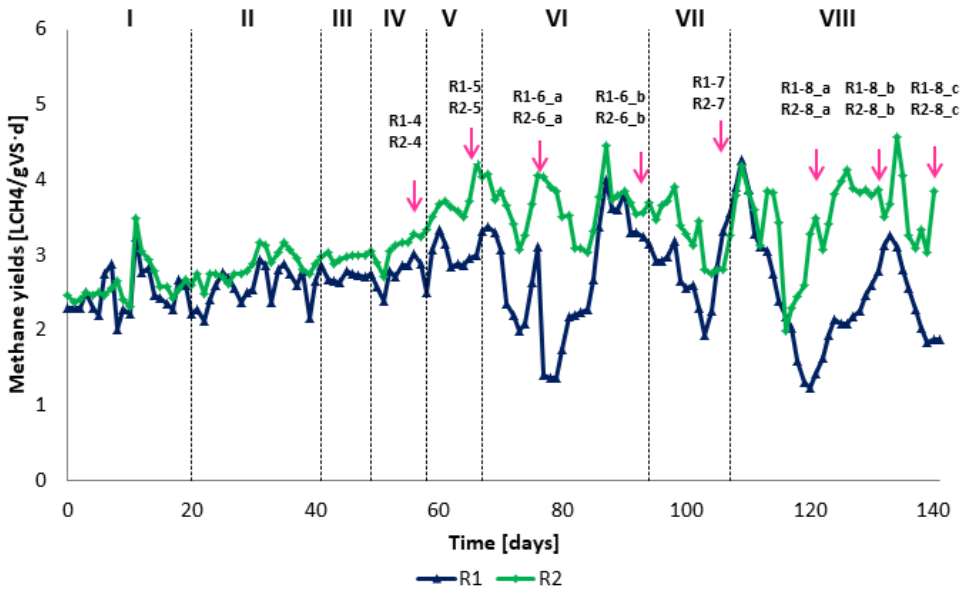



**Figure 1. Collections points of DNA extraction and 16S rRNA gene analysis for the two biogas upgrading reactors.**

Centrifugation of the samples (7000 rpm, at 4°C for 10 min) was conducted to obtain around 2 g of cell pellet. The total microbial DNA extraction (DNA isolation and purification) using FastDNA

Spin for Soil kit (MP Biomedicals, Solon, OH) according to the manufacturer's protocol, and quality of extracted DNA was evaluated electrophoretically.

The 16S rRNA gene was first amplified by PCR using different DNA dilutions to verify possible inhibition phenomena. Two sets of specific primers were used to amplify the bacterial DNA (27f and 519r) the archaea DNA (IA_349F and IA_571R). The bacterial PCR was performed in 20 μL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI) and 1 μM of each primer, while de archaeal PCR was performed in 25 μL volume with Phusion high fidelity polymerase (Thermo Scientific) and 2 μM of each primer. After this inhibition test, the bacterial V5-V6 hypervariable regions of the 16S rRNA gene were PCR-amplified using 783F and 1046R primers, while for the archaeal communities a fragment of the 16S rRNA gene was PCR-amplified using the IA_349F-IA_571R primers (Gagliano et al., 2016). The multiplexed libraries were prepared using a dual PCR amplification protocol.

The bacterial PCR was performed in 2 × 50 μL volume reactions with GoTaq® Green Master Mix (Promega Corporation, Madison, WI) and 1 μM of each primer and the cycling conditions were: initial denaturation at 98°C for 30 s; 20 cycles at 98°C for 10 s, 47°C for 30 s, and 72°C for 5 s and a final extension at 72°C for 2 min. The archaeal PCR was performed in 4 × 25 μL volume reactions with Phusion high fidelity polymerase (Thermo Scientific) and 2 μM of each primer and the cycling conditions were: initial denaturation at 96°C for 4 min; 10 cycles at 96°C for 30 s, 68°C for 30 s, and 72°C for 25 s; then 30 cycles at 96°C for 30s, 58°C for 30 s and 72 °C for 25 s; and a final extension at 72°C for 5 min. After the amplification, DNA quality was evaluated spectrophotometrically and DNA was quantified using Qubit® (Life Technologies, Carlsbad, CA). The sequencing was carried out at Consorzio per il Centro di Biomedicina Molecolare (CBM) (Trieste, Italy). Reads from sequencing were de-multiplexed according to the indexes and then quality filtered. Quality-filtered reads were assembled into error-corrected amplicon sequence variants (ASVs) using DADA2 v1.4.0 (Callahan at al., 2016), which represent unique bacterial/archaeal taxa. Assembled ASVs were assigned taxonomy (phylum to species) using the Ribosomal Database Project (RDP). Rarefaction curves, as well as non-metric multidimensional scaling NMDS analysis to underlined microbial results over environmental variables, were performed using PAST3 software. STAMP software was used to assess PCA the dissimilarity among the samples and make a comparison between R1 and R2 among all periods. Comparison of the bacterial and archaeal abundance between the samples and reactors performed was calculated as a percentage of the total community for each sample. Results discussion is focused on the most abundant bacteria and archaea in the community (>0.5% of relative abundance for archaea and >1% for bacteria).

## 8.3. Results and discussion

### Quality indexes of microbial analysis results

Results of 16S rRNA gene sequencing, of bacteria and archaea after filtering, generated more 1 million of ASVs. Rarefaction curves of the 16 samples for archaea sequencing are depicted in **Figure 2.** All samples, except R1-8_b and R2-8_b, reached the plateau, indicating that the number of ASVs covered the sample richness.

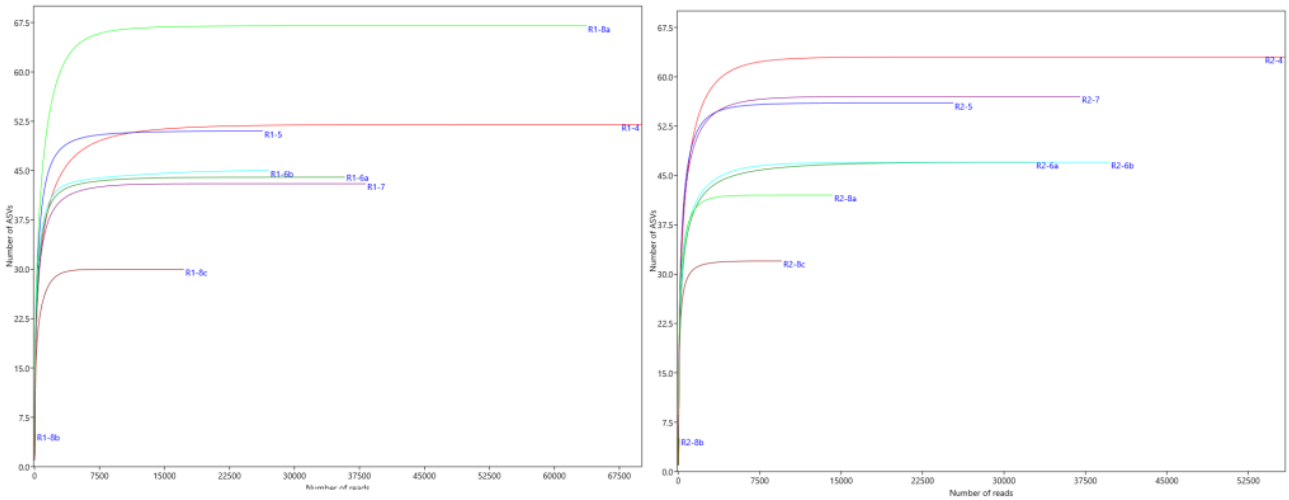



**Figure 2. Rarefaction curves of all archaea samples (R1 on the left and R2 on the right).**

In **Figure 3** rarefaction curves of the 16 samples for bacteria sequencing are reported. For all samples number of ASVs covers the sample richness.

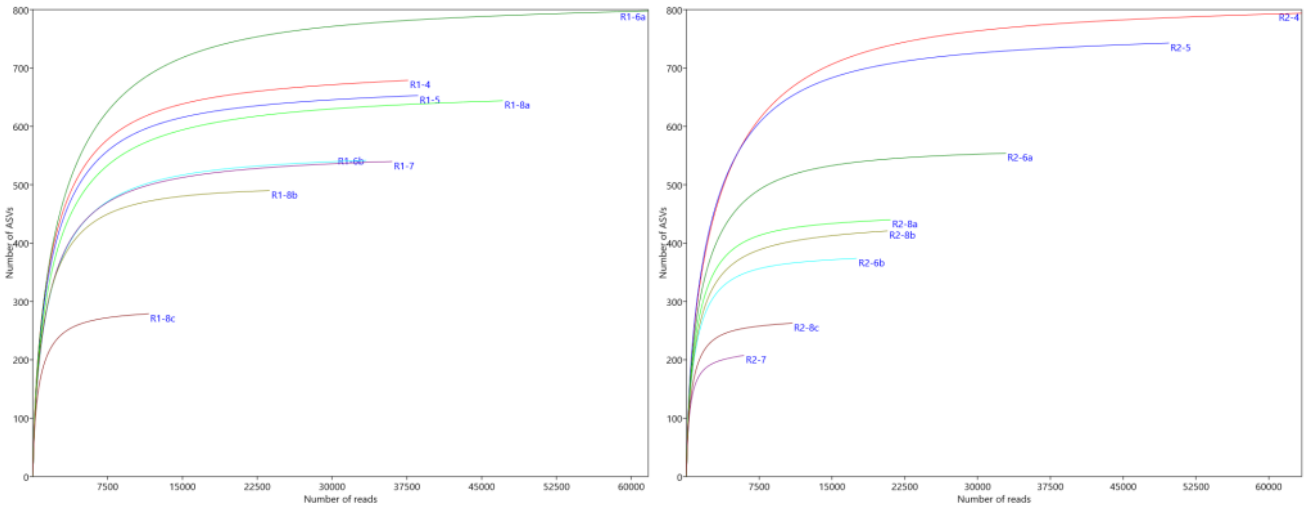



**Figure 3. Rarefaction curves of all bacteria samples (R1 on the left and R2 on the right).**

**Figure 4** presents the beta diversity for archaeal samples of the two reactors displayed in the Principal Component Analysis (PCA) plot. Samples from the same period from both reactors were put together (i.e. in P4 are included all points from period IV). It can be observed that samples of the archaeal community all clustered together within R1 and R2 in one group except for P28 and P18 that seems to be more different.

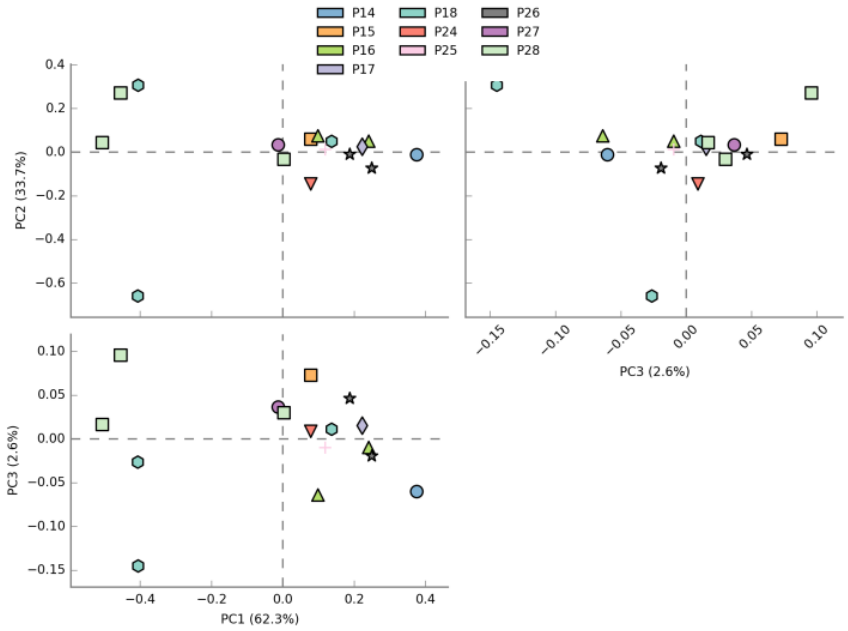



**Figure 4. Beta diversity analyses for all archaeal samples of R1 and R2.**

In **Figure 5** the beta diversity for bacterial samples of the two reactors displayed in the PCA plot. Despite, bacterial community, samples among components which display higher diversity PC1 and PC2 tend to cluster all together except for R2-4. More in detail it can be observed that all samples from period 8 from both reactors are more similar to the proximal temporal period of R2-7, while R1-6 and R2-6 samples are more clustered with the initial samples R1-4.

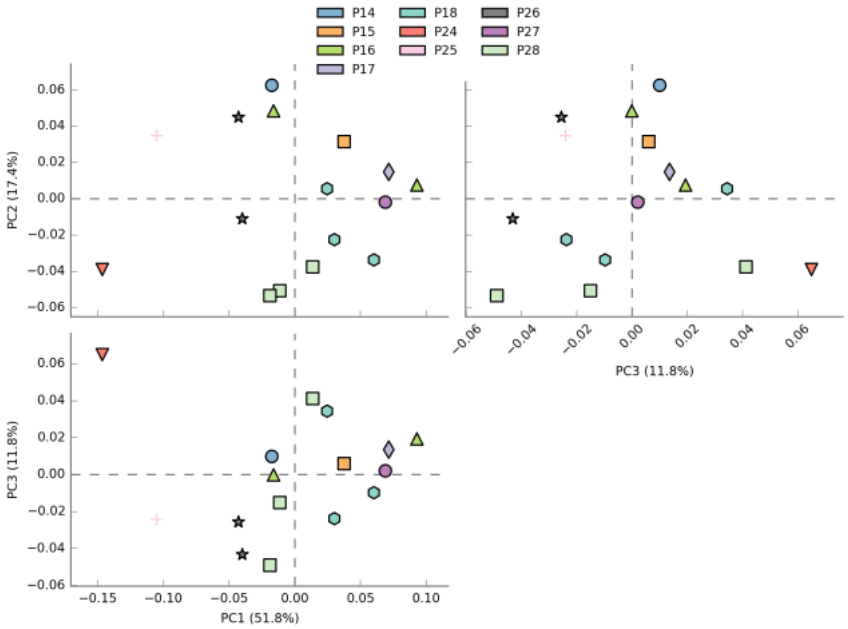



**Figure 5. Beta diversity analyses for all bacterial samples of R1 and R2.**

## Bacterial dynamic analysis

The taxonomic classification of the bacterial community showed that the most abundant phyla in R1 and R2 were similar: *Firmicutes* (28% and 23%), *Proteobacteria* (25% and 23%), *Actinobacteria (20* and *16%), Unclassified_bacteria* (12% and 13%), *Bacteroidetes (8%* and *12%), Cloacimonetes (3%* and *19%), Synergistetes (1%* and *2%), lastly Planctomycetes, Candidatus_Saccharibacteria* and *Chloroflexi* were all 1% abundant (**Figure 6**).

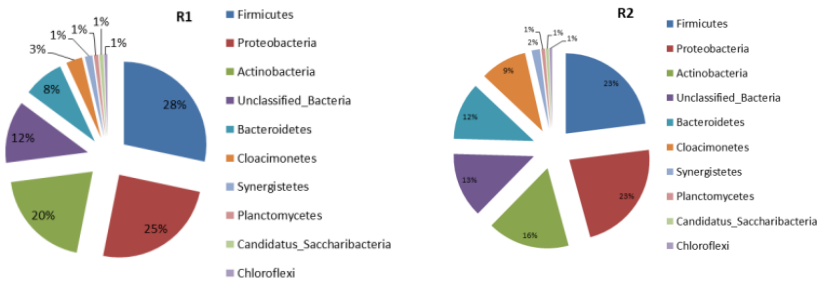



**Figure 6. Most abundant phyla among the bacterial community of R1 (on the left) and R2 (on the right).**

All further discussions on microbial analysis results will be focused only on the most abundant ASVs, having a relative abundance >1%. In particular, 36 ASVs represent the most abundant members both of R1 and R2 and can be considered representative of all the samples. In **Figure 7** and **Figure 8**, the relative abundance and fold change of the identified ASVs are represented for all collection points from the two reactors. For each sample, the relative abundances of ASVs are represented as the rainbow heat map. The comparisons between consecutive phases and of last phase (VIII) compared to the first (IV), was shown by the green-red heat map where green represents the microbes' abundance decreases and red represents the microbe's abundance increases.

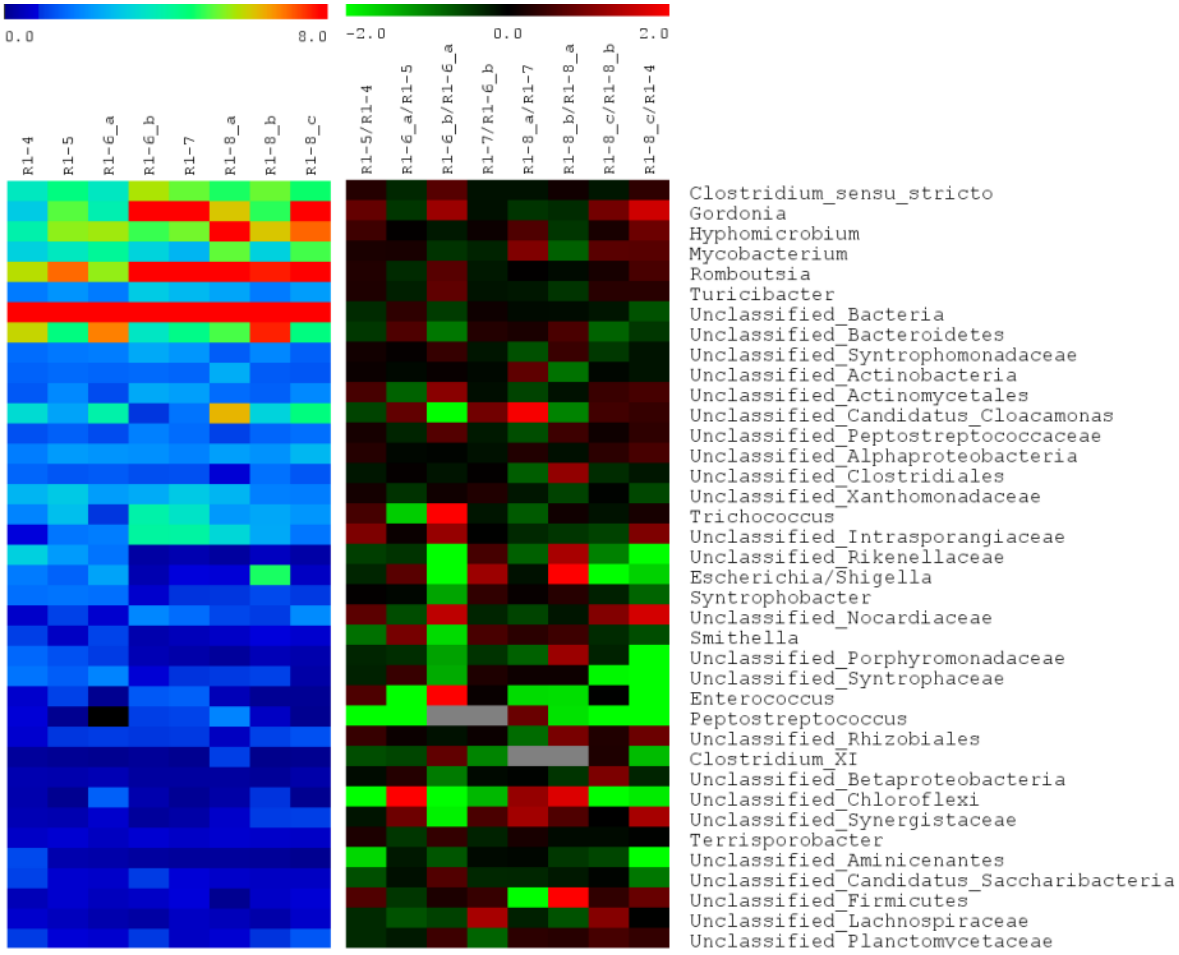



**Figure 7. Heat maps of relative abundance (>1%; left part of the panel) and folds change (log2; right part of the panel) of the most abundant bacterial ASVs of R1.**

From **Figure 7**, it can be observed that except *Unclassified_bacteria* always present (on average 11% of relative abundance), samples can be distinguished into two main clusters. The first 3

collection points can be put together in the first one cluster, and a second group can be formed with the remaining samples (from R1-6_b up to R1-8_c). To the first group belong one collection point of high instability (R1-6_a in **Figure 1**) and others more stable such R1-4 and R1-5. In this cluster, the second higher abundant $ASV_S$ were assigned to *Unclassified_Bacteroidetes* known to be involved in polysaccharides and proteins hydrolysis step during AD process (Bassani et al., 2015), in R1-5 a pick of *Romboutsia* (14.5% of relative abundance) known to be an homoacetogens (Agneessens, 2018) indicated a possible shift of $H_2$ consuming towards acetic acid, even if it was not confirmed by high acetic acids levels (**Chapter 7, Figure 5)**. To the other cluster, most abundant bacterial ASVs were taxonomically assigned, at genus level, to *Hyphomicrobium* (increased from 3.9% to 7.1%) which is known to co-operate in mixture with Methanosarcina in simultaneous denitrification and methanogenesis (Fesefeldt et al., 1998), the aforementioned *Romboutsia* (from 5.84% up to 8.71% relative abundance) indicating a constant $H_2$ consuming also by homoacetogenesis. Furthermore, *Gordonia* genus that showed to increase, except two first collection point of period VII, during the experiment (from 2.97% up to 9.19% relative abundance), which is able to degrade environmental pollutants such as polycyclic aromatic hydrocarbons (PHAs) (Drzyzga, 2012) but also to cause foaming issue in sludge digesters for its filamentous nature (Kougias et al., 2014). This second cluster can be more related to the upgrading process (while in the first (enrichment phase) more bacteria linked to the anaerobic degradation process and homoacetogens were present.

Among the most abundant genus, only limited fold chance was detected, as can be seen from the right panel **Figure 7**, except for *Unclassified_bateroidetes* aforementioned to be in charge of organic matter degradation. Noteworthy, among the less abundant bacteria, the *Unclassified_Candidatus _Cloacamonas* showed a higher variation. This genus might be a possible syntrophic bacterium that oxidizes propionate and ferment sugars and amino acids to produce $H_2$ and $CO_2$ (Bassani et al., 2015).

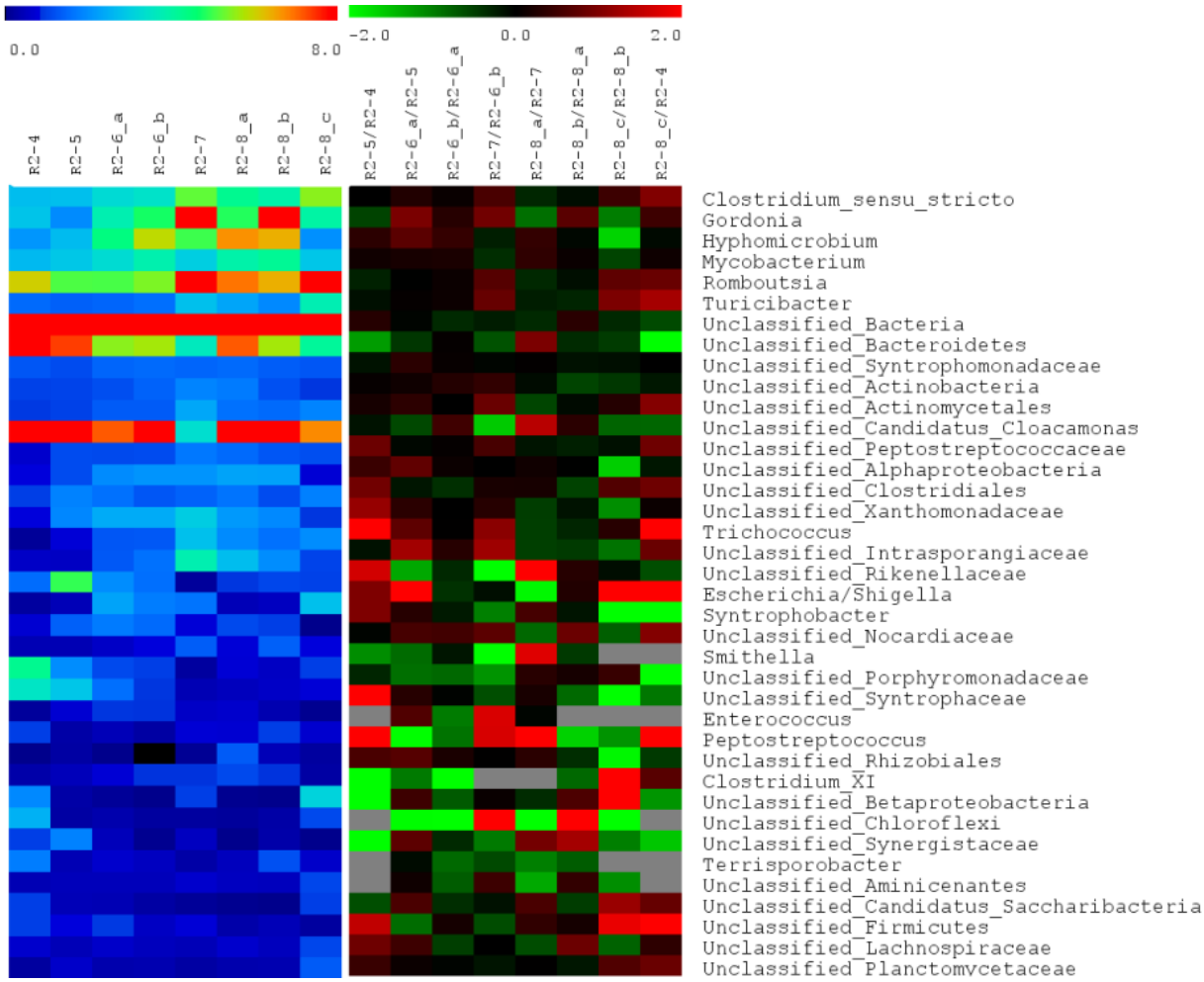



**Figure 8. Heat maps of relative abundance (>1%; left part of the panel) and folds change (log2; right part of the panel) of the most abundant bacterial ASVs of R2.**

In reactor R2, as it can be observed in **Figure 8,** samples can be grouped in three different clusters: the first, composed by R2-4 up to R2-6_b, the second of R2-7 alone, while third put together all samples from last experimental period. The first comparison with R1, is that clusters are more defined by temporal proximity, and this, given the much more regular trends of R2 (**Chapter 7, Figure 4**) compared to those of R1 (**Chapter 7, Figure 3**), allows to assume an evolution of a microbial community in the desired direction following the increasing $H_2/CO_2$ ratio imposed. Thus, this second cluster could represent a microbial community in which bacteria and methanogens efficient co-operate in order to upgrade biogas. Among them, the aforementioned *Gordonia* (which increased from 3% up to 8.9% relative abundance), *Hyphomicrobium* (increased from 2.1% up to 6%), *Romboutsia* (increased from 6% up to 10.7%) and *Unclassified_Candidatus _Cloacamonas*.

**Archaeal community dynamics**

Regarding the archaeal community two phyla were identified: Euryarchaeota (98%) and Woesearchaeota (1.5%). Among the Euryarchaeota, in particular, 12 ASVs represent the most abundant members both of R1 and R2 and can be considered representative of all the samples. Among those, 5 can be considered as the core community displaying more than 90% abundance of each sample.

The first, most abundant from phase 4 to phase 8_b, was assigned at genus level to *Methanolinea.* From the BLASTn search against 16S rRNA sequences (Bacteria and Archaea) database was found to be 100% similar to *Methanolinea mesophila* sp. a mesophilic hydrogenotrophic methanogen recently isolated from rice field soil, highly abundant (on average: 60% in R1 and 46% in R2) but significantly decreased in last collection points in both reactors (**Figure 9** and **Figure 10**).

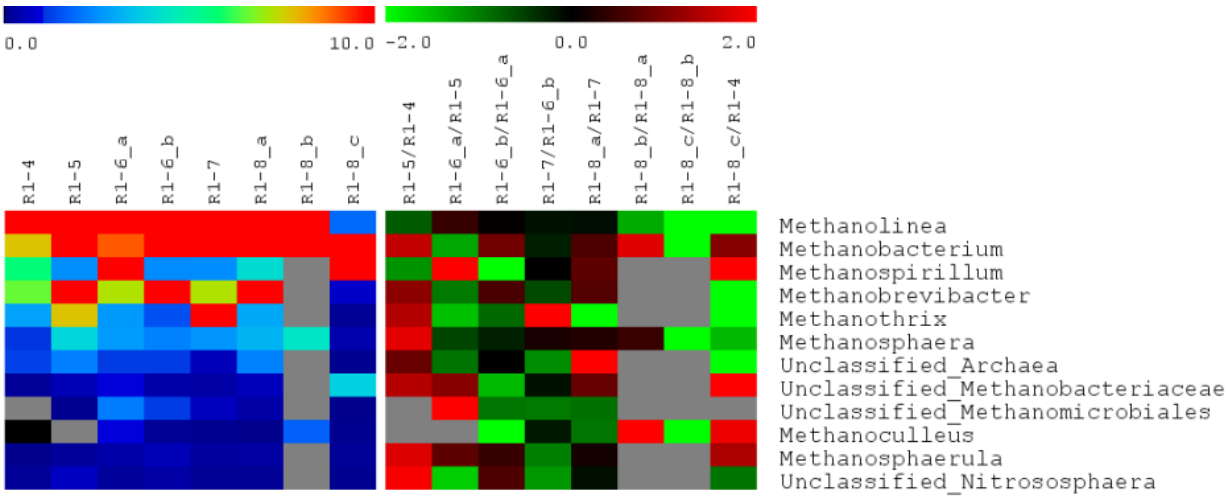



**Figure 9 Heat maps of relative abundance (>0.5%; left part of the panel) and folds change (log2; right part of the panel) of the most abundant archaeal ASVs of R1.**

The second most abundant species was assigned to *Methanobacterium* and representing on average 15% in R1 and 20% in R2 in almost samples, except the last two collection points where it was the most abundant (44% and in R1 and 62% in R2). Based on the results from the BLASTn search, *Methanobacterium* was found to be 100% similar to *Methanobacterium palustre* which can utilize $H_2/CO_2$, formate, as well as 2-propanol for growth and/or methane production (Zellner et al., 1989), thus indicating dominance of hydrogenotrophic methanogens.

The other three abundant species were: *Methanobrevibacter* (10% relative abundant in both reactors) *which* BLASTn search revealed to be similar 100% to *Methanobrevibacter smithii* known to be another $H_2$-consuming methanogen (Pavlostathis et al., 1990); *Methanospirillum* (5% in R1

and 10% in R2 till 8_a) and *Methanothrix* (5.5% in R1 and 4.5% in R2 till 8_a). A comparison with the NCBI database for those last two species indicated 100% similarity with *Methanospirillum hungatei* which is a methanogen that produces methane by reducing formate or $H_2/CO_2$ (Demirel at al., 2008) and 100% similarity to *Methanothrix soehngenii* which is an acetoclastic methanogen (Dolfing & Bloeman., 1985). Noteworthy it can be observed that while *Methanospirillum* was not detected (due to probably a technical issue) in both reactors in sample 8_b, it was found to be respectively the most abundant and highly abundant in the last collection points, 76% in R1 and 20% in R2. Despite, *Methanothrix* strongly decreased in the last points representing lower than 1% of the archaeal community of both reactors (**Figure 9** and **Figure 10**), also this strongly confirmed the dominance of hydrogenotrophic methanogens among the archaeal community.

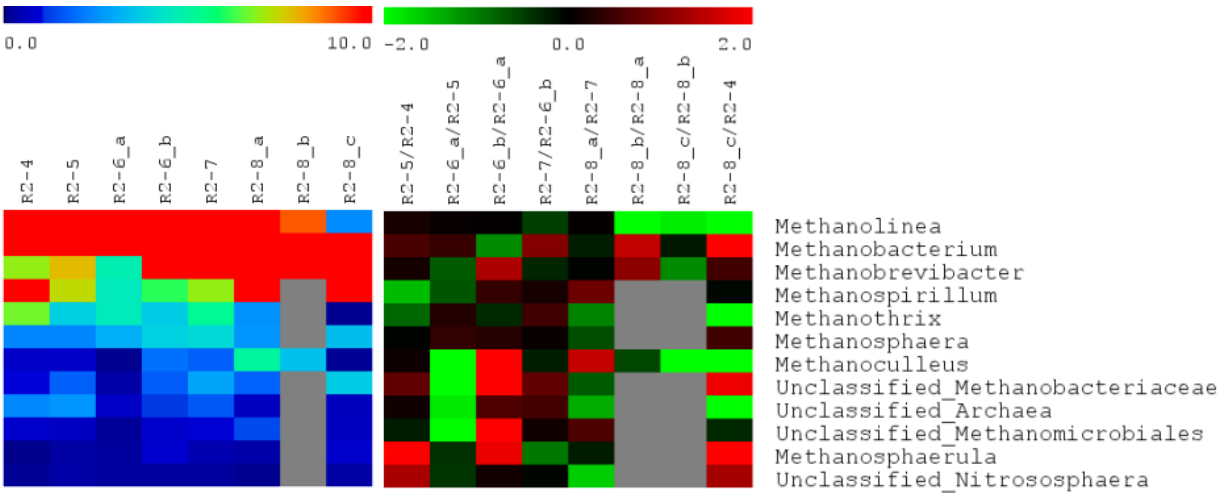



**Figure 10. Heat maps of relative abundance (>0.5%; left part of the panel) and folds change (log2; right part of the panel) of the most abundant archaeal ASVs of R2**

A Non-metric Multidimensional Scaling (NMDS) analysis computed for the archaea in R1 and R2 is depicted respectively in **Figure 11** and **Figure 12.**

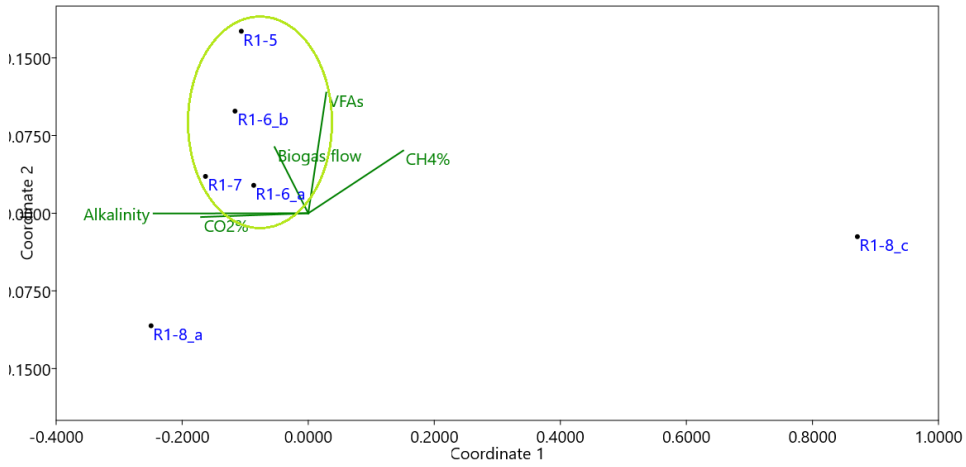



**Figure 11 Non-metric Multidimensional Scaling (NMDS) of archaeal community in R1.**

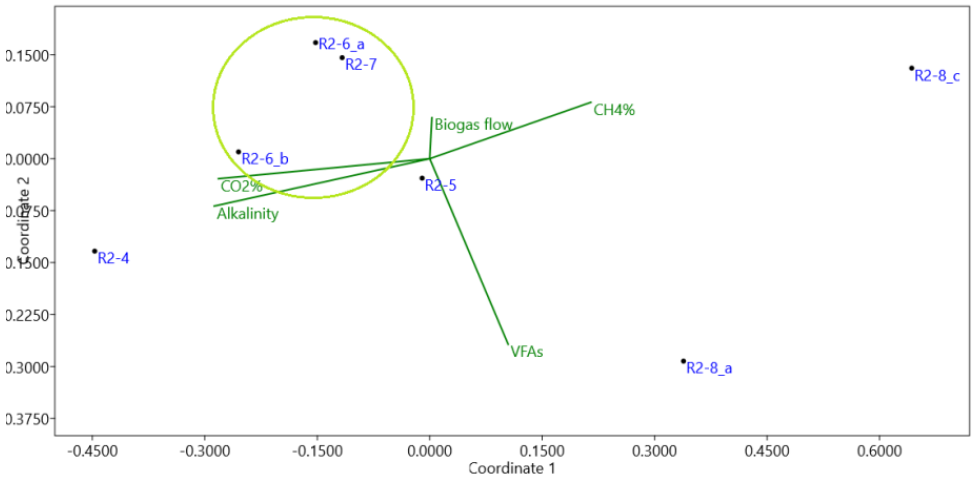



**Figure 12 Non-metric Multidimensional Scaling (NMDS) of archaeal community in R2.**

Regarding R1, the green arrows indicate that the $H_2/CO_2$ ratio of phases V and VII (3:1 up to 6:1) significantly enhanced biogas production and alkalinity reduction and were correlated also to positive VFAs content variations. It is noteworthy the fact that R1-8_a and R1-8_c samples seems inconsistent with others. In R2, increasing $H_2/CO_2$ ratio from phases VI and VII (4:1 up to 6:1) significantly enhanced biogas production and alkalinity reduction while VFAs was not correlated. Here again, R2-8_a and R2-8_c seem less correlated with others samples to the reactor performances considered in this analysis.

## 8.4. Conclusions

In this study, a detailed microbial investigation analysis of an un-acclimatized inoculum to exogenous $H_2$, both during the enrichment phases and during the operation of the biological

upgrading process was carried out. Major results revealed an evolution to a new shape of the core microbial community able in co-operating to the parallel organic substrate degradation and $CO_2$ conversion to extra methane. More in detail the anaerobic consortia accounted on a slight varied bacterial community including homoacetogens, and to an archaeal community mostly composed by hydrogenotrophic methanogenic species in which only one acetoclastic methanogenic was observed. Among bacteria the core community was composed by: *Unclassified_bacteria*, *Gordonia Hyphomicrobium Romboutsia,* and *Unclassified_Candidatus _Cloacamonas* were highly abundant. Among Euryarchaeota, the archaeal core community was mostly composed by hydrogenotrophic methanogens such as *Methanobacterium, Methanobrevibacter, Methanospirillum, Methanolinea* and only one acetoclastic methanogen *Methanothrix.*


### *Acknowledgments*

The authors wish to thank Arianna Catenacci and Micol Bellucci for their valuable support in the microbial analysis, and all DISAT laboratory from the Bicocca University of Milan.


## References


Agneessens, L. M. M. (2018) The Power of the Small : Microbial Conversion of Hydrogen to Methane during Anaerobic Digestion., (October).

Bassani I, Kougias PG, Treu L, Angelidaki I. (2015) Biogas Upgrading via Hydrogenotrophic Methanogenesis in Two-Stage Continuous Stirred Tank Reactors at Mesophilic and Thermophilic Conditions. Environ Sci Technol; 49:12585–93. doi:10.1021/acs.est.5b03451.

Bassani, I., Angelidaki, I., and Kougias (2017) Hydrogen assisted biological biogas upgrading. Kgs. Lyngby, (June).

Batstone, D.J., Keller, J., Angelidaki, I., Kalyuzhnyi, S.V., Pavlostathis, S.G., Rozzi, A., Sanders, W.T.M., Siegrist, H. and Vavilin, V.A. (2002). The IWA Anaerobic Digestion Model No.1 (ADM1). Wat. Sci. Technol., 45(10), 65–73

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., and Holmes, S. P. (2016) DADA2: High-resolution sample inference from Illumina amplicon data. Nature Methods.

Demirel, B. and Scherer, P. (2008) The roles of acetotrophic and hydrogenotrophic methanogens during anaerobic conversion of biomass to methane: A review. Reviews in Environmental Science and Biotechnology, 7(2), 173–190.

Drzyzga O. The strengths and weaknesses of Gordonia: A review of an emerging genus with increasing biotechnological potential. Crit Rev Microbiol 2012;38:300–16. doi:10.3109/1040841X.2012.668134.

Fesefeldt A, Holm NC, Gliesche CG. Genetic diversity and population dynamics of Hyphomicrobium spp. in a sewage treatment plant and its receiving lake. Water Sci Technol 1998;37:113–6. doi:10.1016/S0273-1223(98)00092-4.

Gagliano, A. L., Tagliavia, M., D'Alessandro, W., Franzetti, A., Parello, F., and Quatrini, P. (2016) So close, so different: geothermal flux shapes divergent soil microbial communities at neighbouring sites. Geobiology, 14(2), 150–162.

Pavlostathius SG, Miller TL, Wolin MJ. Cellulose fermentation by continuous cultures of Ruminococcus albus and Methanobrevibacter smithii. Appl Microbiol Biotechnol 1990;33:109–16. doi:10.1007/BF00170581.

Pavlostathius, S. G. and Giraldo-Gomez, E. (1991) Kinetics of anaerobic treatment. Wal. Sci. Tech. Vol. 24, No.8, pp. 35-59, 1991., 24(8), 35–59

Rittmann, S. and Holubar, P. (2014) Rapid extraction of total RNA from an anaerobic sludge biocoenosis. Folia Microbiologica, 59(2), 127–132.

Zellner G., Bleicher K., Braun E., Kneifel H., Tindall B. J., Conway de Macario E. and Winter J. Characterization of a new mesophilic, secondary alcohol-utilizing methanogen, Methanobacterium palustre spec. nov. from a peat bog 1989:1–9. doi:10.1007/BF00444660.

# 9. General conclusion and perspectives

The innovative biogas upgrading via a biological pathway of the enhancement of hydrogenotrophic methanogenesis has gained more and more attention in the last decade. Even if the process of methane bio-production by $CO_2$ reduction by $H_2$ was known from 1936 (Zinder & Koch 1984), the interest in the application of this reaction rose when Strevett et al., (1995) proposed this process to enhance biogas purification using the chemo-autotrophic *Methanobacterium thermoautotrophicum*. After this first study, in very recent years, many others researchers have challenged themselves on this route assaying different substrates, reactor types, and operating conditions (Luo & Angelidaki 2012; Luo et al., 2012; Ako et al., 2008; Lee et al., 2012; Rachbauer et al., 2016; Navarro et al,. 2016; Agneessens, 2018). Two main applications to achieve high methane-rich output gas set-ups were mainly studied at lab-scale: *in-situ* and *ex-situ*. *In-situ* concept consists of adding $H_2$ directly into the anaerobic digester where organic substrate is digested and provide the direct $CO_2$ source, whilst the *ex-situ* set-up involves the injection of $H_2$ and $CO_2$ (from biogas or other industrial sources) into a reactor which is contained an enrichment (or pure) culture of hydrogenotrophic methanogens (Luo & Angelidaki 2013; Bassani et al., 2017; Kougias et al., 2017). Among these configurations, only the *ex-situ* showed to be ready for scaling-up (Jensen et al., 2018) even if still some issue related to the $H_2$ transfer limit is still unsolved.

The general aim of this thesis was the further develop and study the innovative biological biogas upgrading *in-situ* via the enhancement of hydrogenotrophic methanogenic activity fostering its applicability also in WWTPs field. More in detail, several experimental trials were performed each with the intention of achieving results that would advance the development of this process towards its wider application. The following main conclusions can be drawn from the experimental activities.

The first study was focused on proposing a simple manometric test protocol to measure the specific hydrogenotrophic methanogenic activity (SHMA) towards a standardization method not yet established among the scientific community. The simple method was tested on 3 different municipal sludges in combination with 2 different manometric measurement devices, both manual and automatic apparatus. Some major results can be reassumed as follows:

- Test conditions of the proposed protocol, time (6 h), the ratio between headspace and liquid volume (1:1), inoculum concentration (5gVS/L), initial pressure P ($H_2/CO_2$ 1.2 atm), and rpm (150) were found to be optimal conditions for the measurement of the SHMA.
- automatic tests showed worse performance in terms of both pressure tightness and thus reproducibility of the replicas, compared to the manual set-up;

- the standard deviation between apparatus on SHMA value of approximately 1 except for one sludge (st.dev = 8), indicated that the process can be easily described only from P measurement trends;
- Statistical non-reproducibility between replicates may not lead to an error in the estimation of the kinetics;

Results indicated that further assessment should be done by means of testing the SHMA protocol proposed on other anaerobic sludges (treating: industrial- animal- agricultural- wastes) in order to extend and validate the operating conditions characteristics to allows its applicability as a standard measurement protocol.

A novel hydrogenotrophic enrichment procedure allowing a rapid start-up of the *in-situ* biogas upgrading process was designed and tested. Major parameters monitored during the experimental trial to demonstrate the effectiveness of the proposed strategy were: process stability (pH, VFA), biogas composition, methane production $H_2$ utilization. The novel proposed approach was tested with 2+1 control lab-scale CSTRs filled with anaerobic sludge collected from a full-scale WWTP. The experimentation lasted 50 days and was divided into 5 phases: the anaerobic digestion start-up followed by four $H_2$ injection phases ($H_2/CO_2$ ranging from 1:1 to 4:1 on a molar basis). Major results can be summarized as follows:

- a temporary slight increase in VFAs after 4 days of $H_2$ injections during phase II (2.56 $gHac \cdot L^{-1}$), and in phase III were observed; a close TVFA dynamics monitoring was confirmed to be a proper tool to follow the running of anaerobic degradation chain but also anaerobic consortia acclimation to an increasing hydrogen dose;
- a mild pH increment from 7.3 to 7.4 was registered indicating the expected $CO_2$ depletion;
- during all phases $H_2$ was utilized up to 98%;
- the time duration of one week for each $H_2$ increasing step seemed to be appropriate for the effective adaptation of the hydrogenotrophic methanogenic culture;
- during the final phase, a methane content percentage of 81% was achieved in one of the two reactors;
- SHMA tests demonstrated the effectiveness of hydrogenotrophic enrichment;
- the enrichment procedure here proposed could be an effective tool for the start-up of a pilot and full-scale reactors to be used for *in-situ* biological biogas upgrading applications.

The need to operate adopting or exceeding the $H_2/CO_2$ ratio of 4:1 in a longer trial was underlined and a longer acclimation time is needed when achieving or exceeding the stoichiometric $H_2/CO_2$ ratio.

During the research period stay ad DTU (Denmark), an innovative biological upgrading set-up, called Hybrid, was designed and assessed in a continuous experiment for 4 months. The proposed configuration consists of a double-stage reactor composed of a CSTR, working as a conventional anaerobic digester and where the $H_2$ is injected (in-situ biogas upgrading), and an up-flow reactor, receiving the upgraded biogas from the CSTR, together with the unutilized $H_2$. The principal aim was to evaluate the performance of the Hybrid system with respect to the conversion efficiency and final methane content in the output gas. It was also assessed whether the hybrid technology was 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.

Major outcomes demonstrate some advantages of the hybrid concept but and also identify specific issues that need to be addressed for further process optimization:

- an adaptation period must be taken into consideration as the immediate addition of $H_2$ led to a remarkable accumulation of VFA, in agreement with the previous enrichment experiment;
- couple the *in-situ* and the *ex-situ* in one operation unit, allowed maximum methane content in the final output of 95%, $CO_2$ was decreased by 57% and 98% of the injected $H_2$ was utilized;
- since the upgrading process was achieved in two stages, the $CO_2$ removal slightly increased the pH but maintaining it within the range of optimal methanogenesis.

Further assessment should be performed in order to verify if a pre-enrichment period could avoid the high unbalance observed in the *in-situ* reactor.

Continuous *in-situ* operation at increasing $H_2/CO_2$ ratio (from 0.5 up to 7:1) in a longer trial was assessed in a biological biogas upgrading *in-situ* experiment carried out for more than 7 months. This study was conducted with two CSTR parallel lab-scale reactors (V=11L each) with pH controlled to 7.4. To underline the relation between biogas composition and the $H_2/CO_2$ ratio, this was varied from 0.5:1 up to 7:1. Overall reactors performance and other process parameters such as alkalinity and volatile fatty acids (VFAs) speciation composition and COD balance were measured. Major results are listed below:

- During the very first $H_2$ step ($H_2/CO_2$ 0.5:1 and 1:1), in both reactors, a significant ethanol accumulation occurred (2.5 – 3 gCOD/L), not highlighted in other studied, possible occurred for inhibition of ethanol-oxidizer bacteria due to $H_2$ partial pressure increment;
- The behavior of the reactors was different: at the end of each incremental $H_2$ period, R2 showed to reach an equilibrium point achieving the theoretical methane production, except

for $H_2/CO_2$ of 6:1. Conversely, R1 that showed to incur in a more frequent period of instability, a stable condition were reached for all periods except the last ($H_2/CO_2$ 7:1).

- No clear effect was observed on the organic substrate in-situ anaerobic degradation that remained almost stable (40% VS destruction). This outcome indicates the high adaptability of anaerobic mixed culture during continuous $H_2$ injections.
- High alkalinity consumption (50% and 17% respectively R1 and R2) was observed, indicating the high $CO_2$ depletion in the liquid phase. This aspect indicates that an excessive $CO_2$ depletion in the liquid phase, when pH is controlled, could lead to a lower limit of operability if the influent doesn't reintegrate the alkalinity.
- The increment of CODs, not only due to VFA and ethanol measured, may probes that also other intermediates were accumulated during incremental $H_2$ injections;
- Finally, biogas was upgraded reaching a $CO_2$ minimum content of 6%, corresponding to maximum methane of 88% and $H_2$ 6% if the sum of only these 3 component is considered, operating above the stoichiometric value ($H_2/CO_2$ 7:1).
- A detailed microbial investigation analysis revealed a new shape of the core microbial community able in co-operating to the parallel organic substrate degradation and $CO_2$ conversion to extra methane. More in detail the anaerobic shaped consortia accounted on a slight varied bacterial community including homoacetogens and to an archaeal community mostly composed by hydrogenotrophic methanogenic species and only one acetoclastic methanogenic.

Further study and the support of a modeling-approach could allow identifying the optimal operating condition of maximum $CO_2$ conversion but considering the effect on the stability of the simultaneous process of organic substance degradation. As for the effects of $H_2$ injection in a complex anaerobic system, in addition to the accumulation of ethanol, the increase of CODs not linked to typical intermediates (VFA, alcohols) underline the need to identify other substances so as to investigate other metabolic processes, such as intermediates oxidation occurring in steps preceding methanogenesis, for a more in-depth comprehension of the effect of an exogenous $H_2$ injections. The use of radio-labeled substances or measurement systems that extend to other organic acids usually not measured could help in this purpose. The results obtained have shown that with careful acclimatization it is possible to reach a maximum methane content of 88% with a stoichiometric ratio of 7:1 ($H_2/CO_2$) without leading to the biochemical imbalance of the digestion of the organic substance of sewage sludge.

In the future, a technical-economic evaluation study should be carried out to identify a maximum target of sustainable methane % achievable from the *in-situ* biological plant, taking into account the costs of the (renewable) $H_2$ to be used, the energy recovery from the upgraded biogas, and also the possible pure $O_2$ recovery from the electrolysis process, to be used in the aeration phase in a WWTP. Moreover, if the objective is to obtain a complete biogas upgrading, a technical-economical assessment could be extended by evaluating a possible integration to the biological biogas upgrading with a post-treatment utilizing commercial systems.

# References


Agneessens LM, Ditlev L, Ottosen M, Vinther N, Lund J, Jonge N De, et al. Bioresource Technology In-situ biogas upgrading with pulse H 2 additions : The relevance of methanogen adaption and inorganic carbon level. Bioresour Technol 2017;233:256–63. doi:10.1016/j.biortech.2017.02.016.

Ako OY, Kitamura Y, Intabon K, Satake T. Steady-state characteristics of acclimated hydrogenotrophic methanogens on inorganic substrate in continuous chemostat reactors. Bioresour Technol 2008;99:6305–10. doi:10.1016/j.biortech.2007.12.016.

Jensen, M. B., Kofoed, M. V. W., Fischer, K., Voigt, N. V., Agneessens, L. M., Batstone, D. J., and Ottosen, L. D. M. (2018) Venturi-type injection system as a potential H2 mass transfer technology for full-scale in situ biomethanation. Applied Energy, **222**(April), 840–846.

Lee JC, Kim JH, Chang WS, Pak D. Biological conversion of CO2 to CH4 using hydrogenotrophic methanogen in a fixed bed reactor. J Chem Technol Biotechnol. 2012;87:844–77

Navarro SS, Cimpoia R, Bruant G, Guiot SR. Biomethanation of Syngas Using Anaerobic Sludge : Shift in the Catabolic Routes with the CO Partial Pressure Increase 2016;7:1–13. doi:10.3389/fmicb.2016.01188.

Rachbauer L, Voitl G, Bochmann G, Fuchs W. Biological biogas upgrading capacity of a hydrogenotrophic community in a trickle-bed reactor. Appl Energy 2016;180:483–90

Zinder SH, Koch M. Non-aceticlastic methanogenesis from acetate: acetate oxidation by a thermophilic syntrophic coculture. Arch Microbiol. 1984;138(3):263–72

Luo, G., Johansson, S., Boe, K., Xie, L., Zhou, Q., and Angelidaki, I. (2012) Simultaneous hydrogen utilization and in situ biogas upgrading in an anaerobic reactor. Biotechnology and Bioengineering, **109**(4), 1088–1094.