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# DOTTORATO DI RICERCA IN
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CICLO XXXI

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*TOWARDS ANAEROBIC BIOREFINERIES – IMPROVEMENT OF THE ANAEROBIC DIGESTION OF THE ORGANIC FRACTION OF MUNICIPAL SOLID WASTE (OFMSW) USING SUBSTRATE PRETREATMENTS AND TWO-STAGE DIGESTION TECHNOLOGY*

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# DECLARATION

I hereby declare that this submission is my own work and, to the best of my knowledge and belief, it contains no material previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at University of Florence or any other educational institution, except where due references are provided in the thesis itself.

Any contribution made to the research by others I have been working with is explicitly acknowledged in the thesis.

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*March 2019*

# ABSTRACT

In 2016, the household waste production in EU-28 amounted to about 215 million tons. As for the most industrialized countries, the annual generation overtakes 500 kg per capita with organic residues representing the predominant fraction (European Commission, 2018a). In Italy, in 2016, domestic waste production reached 30 million tons and the content of Organic Fraction of Municipal Solids Waste (OFMSW) accounted for approximately 20% (ISPRA, 2017). According to future predictions, this percentage is estimated to increase due to the improvement of separate collection systems. Achieving high rates of separation requires a strengthening of the recovery of the OFMSW (Ranieri et al., 2018). In response, the recent European environmental policy is twofold: reduction of landfill disposal and valorisation of organic residues. European and national legislations have focused on diverting OFMSW from landfilling due to potential environmental impacts and risks (i.e. odour and greenhouse gas emissions, groundwater contamination by leachate) and promoting of technologies able to transform a waste to be disposed of in a valuable product. These are the milestones of the European Union action plan for the circular economy (European Commission, 2015) aiming at developing a sustainable future built on alternative sources of energy and materials. A world where products at the end of their life are turned into resources for new purposes (Webster, 2013).

In this context, the biorefinery concept fits well with this perspective. A biorefinery is a facility where biomass is converted into bioenergy, biofuels or bioproducts for further industrial or

commercial applications (Alibardi and Cossu, 2016). Due to the production of energy and bioproducts (digestate), Anaerobic Digestion (AD) is considered one of the first examples of biorefinery (Sawatdeenarunat et al., 2016). AD has been in use for many decades. To date, it has been primarily aimed at stabilising organic waste, ranging from high solid feedstocks (i.e. animal manure, food waste and agro-industrial biomass), as well as municipal wastewaters. Nowadays, a more open mind is required to look beyond these original applications. According to the biorefinery concept, AD is not regarded as a final disposal treatment but is considered the centrepiece of a larger process with multiple functions such as the production of energy, fuels, heat and biobased materials (i.e.: biopolymers and agricultural fertilizers) and the remediation and stabilization of organic waste (Surendra et al., 2015).

The aim of the present work is to overcome the traditional AD of the OFMSW through the study of two lines of research: the application of substrate pretreatments and the adoption of a two-stage digestion technology.

Pretreatments of OFMSW can be used to solubilize organic matter prior to AD in order to improve the overall process in terms of faster rates and degree of substrate degradation, thus increasing methane production (Cesaro and Belgiorno, 2014). Several methods have been assessed including mechanical, chemical, biological, thermal, hydrothermal and microwave treatments (Ariunbaatar et al., 2014). The present research focuses its attention on these two latter methods. Autoclaving (A) was tested since is able to release the cellulosic materials enmeshed in lignin resulting in an increase of smaller molecules available for further processing (Heerah et al., 2008; Papadimitrou et al., 2010). Similarly, Microwaving (MW) is an optimal method to solubilize organic solids and as such is a suitable candidate to treat OFMSW (Shahriari et al., 2013).

With the aim to further improve AD efficiency, the two-stage process has been identified as a promising method because it allows a better reduction of organic load and increases the overall energy conversion efficiency by generating two gases with high combustion power (Liu et al., 2013). The traditional AD is separated into two reactors connected in series. While the first fermentative phase produces a hydrogen rich biogas and releases volatile fatty acids (VFAs) in the liquid solution, the second phase converts VFAs and the residual biodegradable matter into methane and carbon dioxide (De Gioannis et al., 2013). European Union (European Commission, 2003) promotes hydrogen production, as it is a sustainable energy source with no greenhouse gases emissions from its combustion and high-energy yield. Moreover, the significant generation of organic acids during the fermentative stage can be used to produce polyhydroxyalkanoates

(PHAs), a class of bio-polyesters completely biodegradable. Their chief property is the mechanical behaviour that make them comparable to common plastics (Colombo et al., 2017).

The dissertation consists of four main chapters. Chapter 1 provides a background of the current OFMSW disposal, introduces the concepts of circular economy and anaerobic biorefinery and defines the objectives of the research. Chapter 2 and Chapter 3 are the core elements of the dissertation since they respectively present the study on substrate pretreatments and the study on the two-stage technology. In these chapters, the state of the art of the two lines of research, the materials and methods used, the results and their discussion are provided. Both investigations follow a scale up strategy. Laboratory batch tests play the role of preliminary experiments where process parameters are varied in order to find the optimum condition to be tested on pilot scale semi-continuous trials. Chapter 4 is the final section where conclusions and future developments of the research are stated.

The dissertation summarizes the work of three years of research where laboratory equipment changed and evolved over time. The first year (2016) was dedicated to the study of substrate pretreatments. The second and the third year (2017 and 2018) were devoted to the study of the two-stage process and the composition of the doctorate thesis. This line of research was supported by the Bio2Energy project (Pecorini et al., 2017), a project funded by MIUR-Regione Toscana DGRT 1208/2012 and MIUR-MISE-Regione Toscana DGRT 758/2013 PAR FAS 2007-2013 in sub-programme FAR-FAS 2014 (Linea d'Azione 1.1), which provided new equipment to deeply study the topic.

# INDEX

# ABBREVIATIONS

| | |
|---|---|
| A | Autoclaving |
| AcoD | Anaerobic co-Digestion |
| AD | Anaerobic Digestion |
| AS | Activated Sludge |
| APAT | Agenzia per la Protezione dell'Ambiente e per i servizi Tecnici |
| BHP | Biochemical Hydrogen Potential |
| BMP | Biochemical Methane Potential |
| CHP | Combined Heat and Power |
| COD | Chemical Oxygen Demand |
| CSTR | Continuous Stirred Tank Reactor |
| DF | Dark Fermentation |
| DOE | Design Of the Experiment |
| EB | Energy from Biogas |
| EBA | European Biogas Association |
| EC | European Commission |
| ED | Energy Demand |
| EEA | European Environment Agency |
| EQ | Energy in the form of heat |
| EU | European Union |
| F/M | Food-to-Microorganism ratio |
| FW | Food Waste |
| GPR | Gas Production Rate |
| GS | Gas production Sum |
| HCB | Hydrogen Consuming Bacteria |
| HPB | Hydrogen Producing Bacteria |
| HRT | Hydraulic Retention Time |
| IA | Intermediate Alkalinity |
| IPCC | Intergovernmental Panel on Climate Change |
| ISPRA | Istituto Superiore per la Protezione e la Ricerca Ambientale |
| LCFA | Long Chain Fatty Acids |
| LHV | Lower Heating Value |
| MW | Microwaving |

| | |
|---|---|
| OFMSW | Organic Fraction of Municipal Solid Waste |
| OLR | Organic Loading Rate |
| PA | Partial Alkalinity |
| PHAs | Polyhydroxyalkanoates |
| SGP | Specific Gas Production |
| SHP | Specific Hydrogen Production |
| SMP | Specific Methane Production |
| TAN | Total Ammonia-Nitrogen |
| TA | Total Alkalinity |
| TKN | Total Kjeldhal Nitrogen |
| TOC | Total Organic Carbon |
| TS | Total Solids |
| TVS | Total Volatile Solids |
| VFAs | Volatile Fatty Acids |
| WWTP | WasteWater Treatment Plant |

# CHAPTER 1 - TOWARDS ANAEROBIC BIOREFINERIES

## 1.1 Introduction

In 2016, the household waste production in EU-28 amounted to about 215 million tons. As for the most industrialized countries, the annual generation overtakes 500 kg per capita with organic residues representing the predominant fraction (European Commission, 2018a). In Italy, in 2016, domestic waste production reached 30 million tons and the content of Organic Fraction of Municipal Solids Waste (OFMSW) accounted for approximately 20% (ISPRA, 2017). OFMSW production has gradually increased in recent years due to the improvement of separate collection systems (Figure 1). According to the future predictions, this trend will not stop in the coming years, thus requiring a greater capacity of treatment.

Anaerobic digestion (AD) is an organic waste treatment that has gained interest during the last years. Currently, in Italy, more than the 40% of the OFMSW is processed using AD (Figure 2). It is right to consider that this percentage is going to increase due to an overall better ecological and energetic footprint of AD compared to composting (Mata-Alvarez et al., 2000). One of the main final products of AD is biogas that can be used in factory boilers and in engine generator sets to produce electricity and heat, whereas composting is a net energy consumer. Secondly, composting produces larger and uncontrolled emissions of volatile compounds, such as ketones, aldehydes and ammonia that negatively affect its ecological sustainability (De Baere, 2000). Nevertheless, the

feasibility of the anaerobic treatment is closely related to the composition of the OFMSW. The seasonal variability and the presence of high percentages of lignocellulosic fractions may be relevant constraints for AD, which can then be discarded in favor of composting.

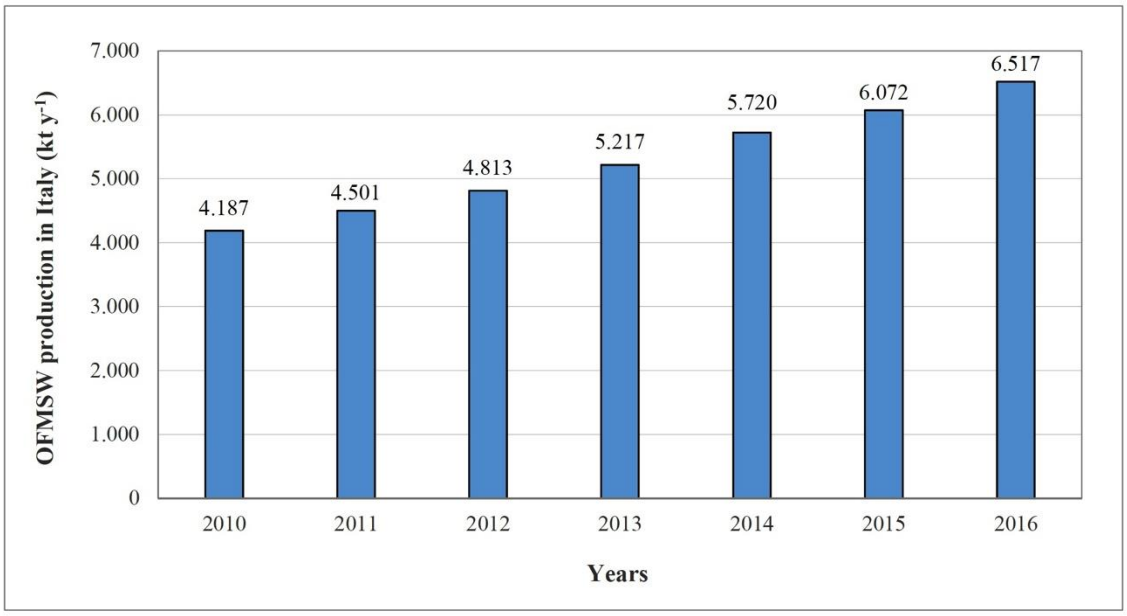


**Figure 1: OFMSW production in Italy (ISPRA, 2017).**

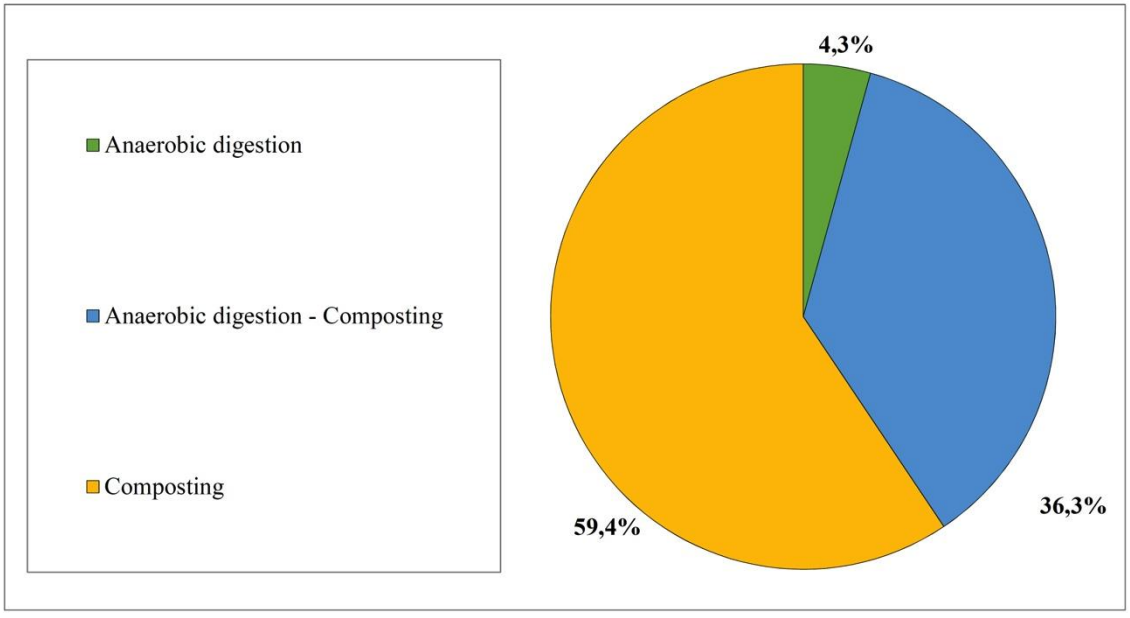


**Figure 2: Treatment of the OFMSW in Italy, 2016 (ISPRA, 2017).**

The OFMSW is a heterogeneous waste based on composition, source and structure (Abdullah et al., 2008). Typically, the OFMSW includes food waste (FW), leaves and yard waste. FW represents a significant proportion of organic material: it can originate from residential and commercial kitchens (i.e., restaurants and hospitals) or come from distribution and retail agents.

Leaves and yard waste consist of lignocellulosic based materials, such as green grass clippings and thatch, trimmings, weeds, brush, and tree prunings, whose production varies widely through the year. A further, although minor, contribution to lignocellulosic content of organic MSW is provided by soiled paper and vegetables (Sundberg et al., 2011). With reference to Italy, De Gioannis et al. (2007) assessed that OFMSW could be modelled as composed by 10 w/w% meat, 65 w/w% fruit and vegetables, 10 w/w% bread and 15 w/w% pasta and rice. Similarly, Alibardi and Cossu, 2015 studied OFMSW composition investigating five fractions: meat-fish-cheese (0.3 – 12 w/w%), fruit (12.7 - 24.8 w/w%), vegetables (18.2 - 42.3 w/w%), pasta-bread (1.3 - 12.3 w/w%), undersieve (13.0 – 17.5 w/w%), rejected materials as paper and cardboard, kernels, etc (17.0 – 22.2 w/w%). This latter categories and yard waste are typical lignocellulosic fractions, which are significant parts in Tuscan OFMSW (Pecorini et al., 2013).

The factors affecting OFMSW composition are multiple. Among them, seasonality, lifestyle and economic situation, such as available population income and consumer behaviour (Belgiorno et al., 2011) can be recognized as well as waste management strategies, with particular reference to the collection system provided for this fraction. Different choices about the way of separating municipal solid waste fractions significantly affect material composition and characteristics (Cecchi et al., 2013). In many countries, green waste is part of the collected biowaste: in this case, the lignocellulosic portion of the OFMSW is higher than the one belonging to a collection system mainly implemented for household waste and commercial activities. As for the collection system, often a door-to-door collection is the most suitable way to access high quality OFMSW.

As the composition changes, the presence of carbohydrates, proteins, lipids and lignin in the waste varies as well, thus affecting the anaerobic process of the whole substrate. Each compound is indeed characterised by a particular biodegradation kinetic and biogas yield (APAT et al., 2005). For instance, wood fibre of yard waste typically comprises around 25-30% hemicellulose and 45% cellulose, on a dry weight basis (Perez et al., 2002). The encasing of cellulose and hemicelluloses in lignin may considerably restrict anaerobic degradation (Delgenès et al., 2003). Conversely, carbohydrate-rich and lipid-rich substrates, such as FW, result in relevant biogas productions (Mata-Alvarez et al., 2000).

Aiming at overcoming the main issues of AD, such as the failure of exploiting lignocellulosic substrates, and increasing the performances of the treatment, recent scientific efforts have been carried out in studying AD as centrepiece of a larger process with multiple functions such as the production of energy, fuels, heat and bio-based materials (i.e.: biopolymers and agricultural fertilizers). According to this concept, AD is transformed in anaerobic biorefinery. Despite the

future potential, one of the main challenges of this new approach is the definition of the best treatment chain in function of the substrate composition (Surendra et al., 2015). Indeed, carbohydrate-rich, protein-rich, lipid-rich and lignin-rich substances can be exploited differently for producing a wide range of valuable products, from biopolymers and biofertilizers to bioenergy and biofuels.

Under this perspective, the present work studies the improvement of the traditional AD of the OFMSW through two lines of research: the application of substrate pretreatments and the adoption of a two-stage digestion technology. The overall goal is the improvement of the anaerobic process in terms of biogas production, biogas composition and volatile solids removal. Nevertheless, their applicability is function of the composition of the incoming OFMSW.

Substrate pretreatments are evaluated in literature to solubilize recalcitrant organic matter prior to AD aiming at improving the process in terms of faster rates and degree of degradation, thus increasing methane production (Cesaro and Belgiorno, 2014). They are generally applied to low biodegradable sludge (Appels et al., 2013; Kuglarz et al., 2013; Eskicioglu et al., 2007; Wilson and Novak, 2009) or lignocellulosic agro-industrial waste (Heerah et al., 2008; Marchesi et al, 2013; Pellera and Giradakos, 2018). Moreover, substrate pre-treatments have been shown to be a useful step to enhance aerobic biodegradation processes as composting (Ibrahim et al., 2011) and for pathogens destruction (Ariunbaatar et al., 2014). Several methods have been assessed including mechanical, chemical, biological, thermal, hydrothermal and microwave treatments (Ariunbaatar et al., 2014). The present research focuses its attention on these two latter methods. Autoclaving (A) was tested since is able to release the cellulosic materials enmeshed in lignin resulting in an increase of smaller molecules available for further processing (Heerah et al., 2008; Papadimitrou et al., 2010). Similarly, Microwaving (MW) is an optimal method to solubilize organic solids and as such is a suitable candidate to treat OFMSW (Shahriari et al., 2013).

Conversely, the two-stage technology aims at improving the AD process from carbohydrate-rich substrates such as FW (De Gioannis et al., 2013; Ghimire et al., 2015). Due to their simple molecular structure, carbohydrate-rich substances can be easily degraded by fermentative bacteria to produce hydrogen (Alibardi and Cossu, 2016). Therefore, the two-stage technology mainly aims at increasing the overall energy conversion efficiency by generating two gases with high combustion power (Liu et al., 2013). The traditional AD is separated into two reactors connected in series. While the first fermentative phase produces a hydrogen rich biogas and releases VFAs in the liquid solution, the second phase converts VFAs and the residual biodegradable matter into methane and carbon dioxide (De Gioannis et al., 2013). The two gas flow can be used either by

itself or mixed in a mixture that simulates the composition of hythane (Chinellato et al., 2013). Additionally, the significant generation of organic acids during the fermentative stage can be used to produce polyhydroxyalkanoates (PHAs), a class of bio-polyesters completely biodegradable. Their chief property is the mechanical behaviour that make them comparable to common plastics (Colombo et al., 2017).

The following introductive section aims at presenting the state of the art of the anaerobic process, the future possibilities of the anaerobic biorefinery and the objectives of the present research.

## 1.2 Anaerobic digestion

AD is a biological technology that converts organic substrates in absence of oxygen into biogas, a renewable source of energy, and digestate, a valuable fertilizer and soil conditioner (Iacovidou et al., 2012; Tambone et al., 2010). In this process, bacteria convert organic carbon ($C_{org}$) to its most oxidized state ($CO_2$) and its most reduced state ($CH_4$). As the result of the removal of carbon, organic bound non-carbon compounds are released to their soluble inorganic form (Angelidaki et al., 2006). The main objectives of the treatment are: mass and volume reduction of waste, mineralization of biodegradable compounds, sanitization and energy recovery from biogas uptake (Mata-Alvarez et al., 2000). The use of biogas is increasing rapidly today. Over 14,000 commercial AD plants are already in operation in Europe, while Germany alone has more than 8,000 plants (EBA, 2014). The produced biogas is used for combined heat and power (CHP) generation, and/or upgraded to biomethane to be used as transportation fuels, or injected into natural gas grid (Sawatdeenarunat et al., 2016).

### 1.2.1 Anaerobic digestion phases

Biogas is mainly composed of carbon dioxide (30-45% v/v) and methane (55-75% v/v) and minor quantities (usually less than 1% v/v) of other products such as nitrogen, nitrogen oxides, hydrogen, ammonia, hydrogen sulphide. Its generation and final composition depends on four interrelating biochemical phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The first three phases are often presented under a sole macro-phase called fermentation. The four phases are depicted in Figure 3 and act as follows:

1. Hydrolysis: it is a reaction with water and it is the first and often the rate-limiting step. It can be defined as the breakdown of complex organic substrates into smaller products that can subsequently be taken up and degraded by bacteria. During hydrolysis (Reaction 1), macro-pollutants polymers such as lipids, proteins and carbohydrates are depolymerized to glycerol and long chain fatty acids, to amino acids and to oligosaccharides and monosaccharides, respectively.

   Reaction 1: biomass + $H_2O \rightarrow$ monomers

2. Acidogenesis: simultaneously to the hydrolysis of the complex organic material, the acidogenic process converts organic oligomers and monomers, carbohydrates, fatty acids and

amino acids to short chain volatile acids (mainly butyric and propionic acids, Reaction 2), ketones (glycerol and acetone) and alcohols (ethanol and methanol, Reaction 3).

Reaction 2: $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$

Reaction 3: $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$

3. Acetogenesis: the acidogenesis intermediates are attacked by acetogenic bacteria. The products from acetogenesis include acetic acid, $CO_2$, and $H_2$. Several bacteria contributes to acetogenesis, including: *Syntrophobacter wolinii* (propionate decomposer), *Syntrophomonos wolfei* (butyrate decomposer) and *Clostridium spp., peptococcus anaerobes, lactobacillus,* and *actinomyces* (acid formers). The Reactions 4-7 occur during acetogenesis.

   Reaction 4: $CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO_3^- + 3H_2$

   Reaction 5: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$

   Reaction 6: $CH_3CH_2OH + 2H_2O \rightarrow CH_3COO^- + 2H_2 + H^+$

   Reaction 7: $2HCO_3^- + 4H_2 + H^+ \rightarrow CH_3COO^- + 4H_2O$

4. Methanogenesis: methane's production is the final step of the anaerobic digestion process. Two main different reactions characterize this phase: the first produces methane thanks to hydrogenotrophic bacteria that anaerobically oxidize hydrogen and carbon dioxide (Reaction 8) while the second transforms acetic acid into methane and carbon dioxide (Reaction 9). While the first reaction releases approximately the 28% of the final methane content, the second one accounts for approximately the 72% (APAT, 2005). Other minor reactions allow to produce methane from ethanol (Reaction 10), methanol (Reaction 11) or other end products such as hydrogen sulphide (Reaction 12) or ammonium (Reaction 13). Several bacteria contribute to methanogenesis, including: *methanobacterium, methanobacillus, methanococcus,* and *methanosarcina.*

   Reaction 8: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

   Reaction 9: $CH_3COOH \rightarrow CH_4 + CO_2$

   Reaction 10: $2CH_3CH_2OH + CO_2 \rightarrow 2CH_3COOH + CH_4$

   Reaction 11: $CH_3OH \rightarrow CH_4 + H_2O$

   Reaction 12: $CH_3COO^- + SO_4^{2-} + H^+ \rightarrow 2HCO_3 + H_2S$

   Reaction 13: $CH_3COO^- + NO^- + H_2O + H^+ \rightarrow 2HCO_3 + NH_4$

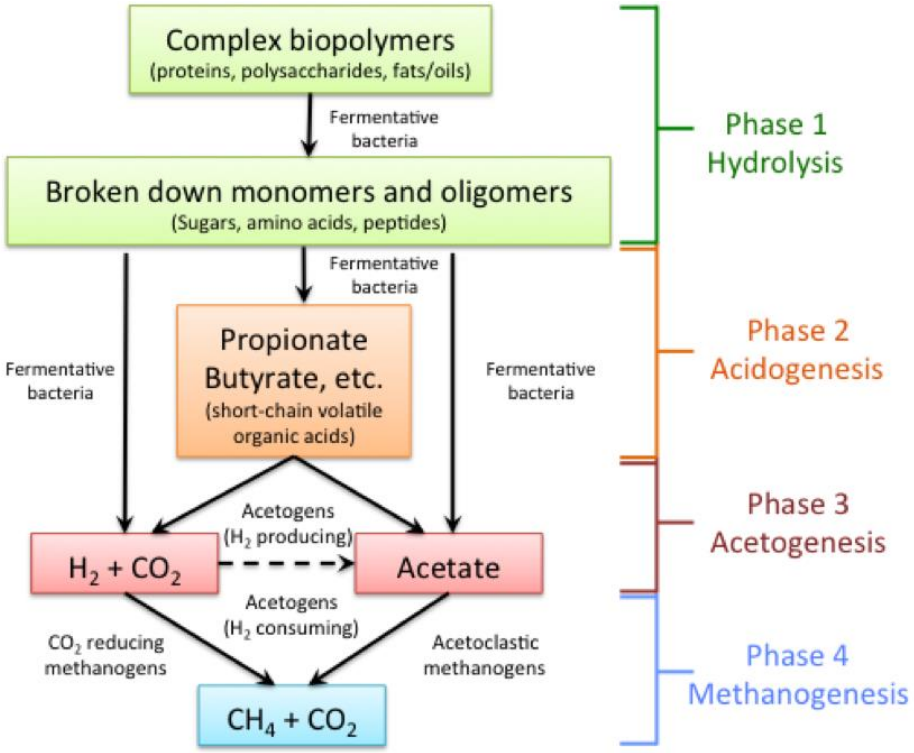


**Figure 3: Anaerobic digestion phases (APAT, 2005).**

## 1.2.2 Process indicators

Several physical, chemical and physiological factors affect the anaerobic degradation of organic compounds. These factors can be summarized in two categories: process stability indicators and performance indicators. While the former aim at monitoring the proper operation of the process, the latter aim at evaluating its efficiency.

### 1.2.2.1 Process stability indicators:

These include: pH, VFAs, alkalinity, ammonia concentration. In addition, some features of the feeding substrate, such as the carbon to nitrogen ratio (C:N) and the amount of nutrients, are relevant prerequisites to obtain a stable process:

- pH: maintaining pH in the proper range is required for efficient anaerobic digestion. The generally accepted values are in the neutral range, between 6.5 and 8. As abovementioned, AD requires the joint work of several groups of microorganisms, from which methanogens are the most sensitive to low pH. Changes in digester operating conditions or introduction of toxic substances may result in process imbalance and accumulation of VFAs. Unless the system contains enough buffer capacity (alkalinity), the pH will drop below optimal levels

and the digester will become "sour". Depending on the pH magnitude and the duration of the drop, the biogas production will decrease to a point where it may completely cease.

- Alkalinity: The buffering capacity of an anaerobic digester is determined by the amount of alkalinity present in the system. Alkalinity can be defined as the total proton accepting capacity and is equal to the stoichiometric sum of the bases in solution. The main alkalinity sources are the carbonate/bicarbonate system ($HCO_3^-$) and non-protonated forms of VFAs. The concentration of $HCO_3^-$ in solution is related to the percent of carbon dioxide in the gas phase. In a typical digester with a pH 7.4 and a percent $CO_2$ of 35%, the bicarbonate alkalinity is about 5,500 mg$CaCO_3$ $L^{-1}$ (APAT, 2005). Such alkalinity is predominant and usually provides enough buffering capacity to withstand moderate loads of VFAs and maintaining the system's pH in the proper range. Conversely, the alkalinity related to the non-protonated forms of VFAs is directly dependent to VFA concentration. Aiming at measuring these two contributions, two end-point titration methodologies are usually adopted. These methods lead to obtain the measurement of total alkalinity (TA) and partial alkalinity (PA). The former includes both VFA and bicarbonate alkalinity, while the latter is roughly related only to bicarbonate alkalinity. The difference, defined as intermediate alkalinity (IA), is related to VFA and other anions alkalinity (APAT, 2005; Martín-Gonzalez et al., 2013). Several studies have included alkalinity ratios as monitoring parameters and the ratio intermediate/partial alkalinity (IA $PA^{-1}$) is generally used to monitor VFAs/alkalinity ratio. According to Martín-González et al., 2013, an IA $PA^{-1}$ ratio below 0.3 is recommended to achieve stable reactor performance. Other secondary sources of alkalinity are related to ammonia and hydroxide ions that are generally released during the degradation of proteins (Jung et al., 2011).
- Volatile Fatty Acids (VFAs): VFA concentration is probably the most sensitive parameter to monitor. They can be inhibitory of the digestion process. VFAs encompass a group of six main compounds: acetate, propionate, butyrate, valerate, caproate and enanthate, from which acetate is predominant. In a correctly designed and well-operated digester, the concentration of total VFA is typically below 500 mg $L^{-1}$ as acetic acid. However, if the digester is undersized for the organic load this concentration can be higher. At VFA concentrations over 3,500 mg $L^{-1}$, biogas production might be limited by inhibition (Martín-González et al., 2013). However, rather than a specific concentration, it is a sudden and steady increase of VFAs in the effluent what can be a sign of a digester upset. Thus, it

is essential to monitor VFAs periodically in order to detect problems on time, and make the necessary operational changes before digester failure occurs.

- Total Ammonia-Nitrogen (TAN): ammonia is produced during the degradation of protein-rich substrates and its role in AD is multiple. Optimal levels of ammonia (up to 200 mg $L^{-1}$) ensure adequate supply of nitrogen as nutrient substance for anaerobic biomass and increase system's buffer capacity, counteracting acidification lead by VFA production (Polizzi et al., 2018). On the contrary, ammonia concentration exceeding certain critical thresholds is detrimental to the process due to its toxic effect. Concentrations over 1,500 mg $L^{-1}$ have been reported to be inhibitory for the digestion process (APAT, 2005).
- C:N ratio: on the contrary of the previous indicators, the C:N ratio monitoring does not concern the material inside the digester but has to be measured on the feeding substrate. In particular, a C:N ratio for optimum digestion performance is in the range of 20-30 (Iacovidou et al., 2012; Jain et al., 2015). A C:N ratio over 30 is usually a cause for instability, mainly due to nutrient deficiency which severely affects microorganism activity resulting in lower substrate removal and methane production (Kayhanian and Hardy, 1994), whereas C:N ratios lower than 6 negatively affect the digestion process, mostly due to low carbon availability in combination with high ammonia generation (Kayhanian and Hardy, 1994).
- Nutrients: the major nutrients required for the bacteria in the digester are C, $H_2$, $O_2$, $N_2$, P and S. Out of all these nutrients $N_2$ and P are always found in short supply and therefore to maintain proper balance of nutrients an extra raw material rich in P and $N_2$ should be added to maximize biogas production (Jain et al., 2015).

#### 1.2.2.2 Performance indicators

These include: biogas production, biogas composition and total volatile solids removal.

- Biogas production: it is the most important parameter to monitor in anaerobic digesters. Biogas production should be fairly stable over time. If it drops below the average daily values, it is most likely that other indicators, as discussed above, have changed as well, and it is an indication of a digester upset. It can be expressed on the basis of daily feeding of organic matter or digester volume. The former is represented by the specific gas production (SGP, NL $kgTVS_{IN}^{-1}$ $d^{-1}$) while the latter is represented by the gas production rate (GPR, NL $L_r^{-1}$ $d^{-1}$).

- Biogas composition: as abovementioned, biogas is almost completely composed of methane and carbon dioxide, but it also includes traces of ammonia nitrogen, hydrogen, hydrogen sulfide, and other gases. Methane is the final product of anaerobic digestion, and its production is a measure of how well the digester is performing (expressed on volume basis, % v/v). The amount of methane produced during the digestion is directly related to the amount of organic matter that has been degraded. More importantly, the more methane is produced, the more energy (electricity and heat) can be generated. In a well-operated digester a $CH_4$ percentage about 55-75% v/v, high biogas production and low VFA concentration should be registered. An increase of $CO_2$ content and VFA concentration mean the onset of a predominant acidogenic phase while a decrease of biogas production could lead to a process inhibition.
- Total volatile solids (TVS) removal: volatile solids provide a measure of the organic content of waste. The difference between the TVS concentration in the influent and the TVS concentration of the effluent indicates the amount of waste that has been degraded through the digestion process. The extent of organic matter stabilization primarily depends on the system configuration and the substrate's physicochemical characteristics. It is generally expressed as percentage on weight basis (% w/w).

### 1.2.3 Digester operation

There are some operational characteristics that classify anaerobic digesters in several types.

A first distinction in anaerobic digestion technologies can be made on the basis of the dry matter content of the incoming substrate. The process can be defined wet, semi-dry or dry. The influent of a wet process has a total solids (TS) content lower than 10%. The semi-dry process works with a dry matter content in the range of 10-20% TS while a dry influent has at least 20% of dry solids (Angelonidi and Smith, 2015). The amount of water required for the desired process is added to the waste substance before it is fed to the reactor.

Another distinction can be made based on the feeding method. A batch process works with cycles in which the digester is fed with new feedstock on a regular basis, for example every day or once a week. Conversely, continuous processes have constant input of waste. The main continuous systems concern plug-flow digesters and continuous stirred tank reactors (CSTR). In plug-flow digesters, the substrate is not completely mixed, but move as a plug through the reactor from the feed port to the exit. This type of reactor maintains at least 20% TS in the tank and

requires heavy process equipment that can handle dry and viscous material that does not flow freely. At lower solids content, sediment can quickly accumulate in the reactors. CSTR are basically tanks in which substrate is heated and mixed with an active mass of microorganism. The digester can be continuously or intermittently mixed (semi-CSTR). The mixing in CSTR is mechanically done and is important to achieve an efficient operation of a digester, avoiding sedimentation, and breaking up the scum layer (Silva and Naik, 2007). Stirring is a relevant topic in CSTRs. Since bacteria in the digester have very limited reach to the food, it is necessary that the slurry is properly mixed and bacteria get their food supply. It is found that slight mixing improves the fermentation; however, a violent slurry agitation retards the digestion (Jain et al., 2015). Complete mix digesters work best when substrate contains 5-8% TS. Digester size can be an issue at lower solids concentrations since lower solids mean greater digester volume. Although continuous processes have dominated the marketplace, they have not established themselves for the processing of lignocellulosic biomass. The primary advantages of batch reactors are their relative simplicity, minimum maintenance requirements, low parasitic energy loss, and, above all, minimum capital cost. In a typical batch system, organic waste or biomass ranging from 30% to 40% TS is digested in a gas-tight container or room. The dry stackable waste is inoculated with finished digestate from the previous batch. To reduce the amount of inoculum needed, leachate can be collected from the reactor and reapplied to the material. Systems that recycle leachate into the reactor vessel are called percolation systems. Leachate recycling enables the colonization of the bacteria throughout the digester by providing a passive transport mechanism for microbial communities. This reduces the amount of digested material needed to inoculate the fresh feedstock before loading the digester. The leachate may also be mixed with fresh material to directly inoculate it without any digested solids being added. This provides the operational benefit of reduced handling costs and higher reactor volume utilization. However, the continual reapplication of leachate will produce a wetter digested product than systems using recycled digestate. The digested material may need to be processed after digestion to reduce the moisture content and stabilize the product. Concluding, batch processes requires less pre-treatment and less mixing equipment and are therefore considered cost-effective (Li et al., 2011; Muzenda, 2014). On the contrary, a dry batch process produces 20% less methane than continuous processes and a batch reactor requires more volume than a continuous reactor.

Temperature has a strong influence on AD since it is a crucial variable in controlling the rate of microbial metabolism. It affects survival and growth of microorganisms and it also influences their metabolic activities. AD is applied under two different temperature ranges: mesophilic (25-

40 °C) and thermophilic (45-65 °C). Besides this, there is another temperature range, which is psicrophilic (20°C). The optimum temperature for growth is usually close to the upper limit of its range and a change of only a few degrees already has a strong effect on the activity of the microorganisms. Due to the lower operating temperature, mesophilic systems require less external energy inputs than thermophilic processes. Conversely, gas production and the operating speed are lower (Li et al., 2011). In addition, operating at thermophilic conditions provides the added benefit of increased pathogen reduction (Jain et al., 2015).

Further distinction can be made between one-phase and two-phase digesters. In a one-phase digester, all four processes happen in the same reactor. This reactor has conditions suitable for all phases, not specifically for one. In a two-phase system, the first reactor has optimal conditions to perform fermentation (set of hydrolysis, acedigenesis and acetogenesis). After the first reactor the methanogenesis happens in the second reactor. Both reactors are designed with optimal conditions for the reactions that have to happen (Li et al, 2011). Two-phase processes are most suitable for flows with highly biodegradable wastes such as separately collected organic waste, since this process can handle higher loads in the digester (Forster-Carneiro et al., 2004). However, one-phase systems are prevailing on an industrial scale (Mata-Alvarez et al., 2000). One-phase digesters are with 93% of total digesters most commonly used. This is mostly due to higher investment and operational cost for two-phase systems (De Baere and Mattheeuws, 2013).

A proper operating mode is strictly dependent on two related parameters: the organic loading rate (OLR) and the hydraulic retention time (HRT). The former indicates the amount of volatile solids fed daily to the digester and is generally expressed as kgTVS $m_r^{-3}$ $d^{-1}$. The latter is the average time that a given volume of material stays in the digester and it is generally expressed as days. These two parameters affect both the biochemical process and the economics of the digester. For a given volume of substrate, a smaller digester (lower capital cost) results in a shorter HRT. Conversely, this may not be long enough to reach the optimum result such as high biogas production, low emissions of odour and greenhouse gases and high degradation of organic matter and pathogens. Too low HRT and high OLR lead to an increase in acidogenic bacteria. High concentrations of acidogenic bacteria make the pH value drop. Methanogenic bacteria die at low pH values and without methanogenic bacteria there is no biogas production. So OLR and HRT have to be calculated and maintained carefully in order to prevent a system failure. Table 1 shows the optimum range of HRT and OLR for continuous one-stage digestion of OFMSW (APAT, 2005).

**Table 1: Hydraulic retention time and organic loading rate for continuous one-stage digestion of OFMSW (APAT, 2005).**

| | Mesophilic process | | | Thermophilic process | | |
|---|---|---|---|---|---|---|
| | Wet | Semi-dry | Dry | Wet | Semi-dry | Dry |
| HRT (d) | 12-18 | 12-18 | 17-25 | 8-16 | 10-16 | 12-16 |
| OLR (kgTVS $m_r^{-3}$ $d^{-1}$) | 2-3 | 3-4 | 4-6 | 2-5 | 4-10 | 6-9 |

### 1.2.4 Co-digestion

Co-digestion involves the digestion of two or more substrates together as a way to improve the digestion efficiency and increase the energy output (Iacovidou et al., 2012). The interest in the co-digestion of OFMSW and sewage sludge has recently increased as it is considered a solution to face two main issues: low biogas production from sludge digestion and the lack of OFMSW digesters. Indeed, AD is a widespread technology employed for the stabilisation of sewage sludge. Despite the positive potentials, most wastewater digesters face problems such as low biogas yields due to low biodegradability of sludge and low OLR due to oversized digesters (Cabbai et al., 2016). Anaerobic co-digestion (AcoD) of sludge and organic waste is a valuable solution improving the energy output using the spare digestion capacity at wastewater treatment plants (WWTPs) and reducing costs (Appels et al., 2011). It is a strategic and cross-sectorial solution based on water industry and waste management synergy, where the necessary infrastructures and human resources are provided (Cabbai et al., 2016; Iacovidou et al., 2012).

The improvement in methane yields from the addition of organic waste to sewage sludge has been widely reported in literature (Heo et al., 2003; Kim et al., 2003; la Cour Jansen et al., 2004; Mata-Alvarez et al., 2000). Methane yields were found to increase notably by increasing the fraction of organic waste in sewage sludge. It is also worth noting that the improved C:N ratio and kinetic reactions achieved by the addition of organic waste to sewage sludge were reported to be the prerequisites for improving methane yield (Dohányos et al., 2004). In general, sewage sludge is characterised by a low C:N ratio ranging from 6 to 9. Intrinsically, the addition of FW which is characterised by a high C:N ratio can improve the C:N ratio of sewage sludge (Heo et al., 2003; Kim et al., 2003). Organic waste provides essential carbon to sewage sludge digestion that is essential for the improvement of digestion performance, mainly because of its influence on the kinetics of the process (Fernández et al., 2010). Hydrolysis has been reported as the rate-limiting step in the AD of sewage sludge (Dohányos et al., 2004; Mottet et al., 2010). This is mainly because of the composition of sewage sludge, which is rich in proteins that have a lower hydrolytic potential, than carbohydrates and lipids (Wilson and Novak, 2008). Therefore, the addition of

easily degradable matter in the form of organic waste has been reported to improve the degradation efficiency and accelerate the hydrolysis of sewage sludge, because of the faster growth of anaerobic microorganisms (Dohányos et al., 2004; Kim et al., 2003; Mottet et al., 2010).

## 1.3 Circular economy, bio-economy, anaerobic biorefineries

### 1.3.1 European policy

The worldwide exploitation of fossil fuels and raw materials is driving earth towards energy emergency, environmental pressure and climate change (IPCC, 2014). In recent years, society began to recognize the necessity of a sustainable world built on alternative sources of energy and materials. The European Union action plan for the Circular Economy (EC, 2015) and the Bioeconomy Strategy (EC, 2018) are first answers to develop a sustainable, low carbon and resource efficient future. The Circular Economy Policy Package aims to close material loops through the recycling and reuse of products, effectively reducing virgin material use and associated environmental pressures. The Bioeconomy Strategy is a research and innovation agenda aimed at enhancing the exploitation of biomaterials in a sustainable way. The two strategies are closely interrelated since sustainable bioeconomy is the renewable segment of the circular economy turning bio-waste, residues and discards into valuable resources (EEA, 2018).

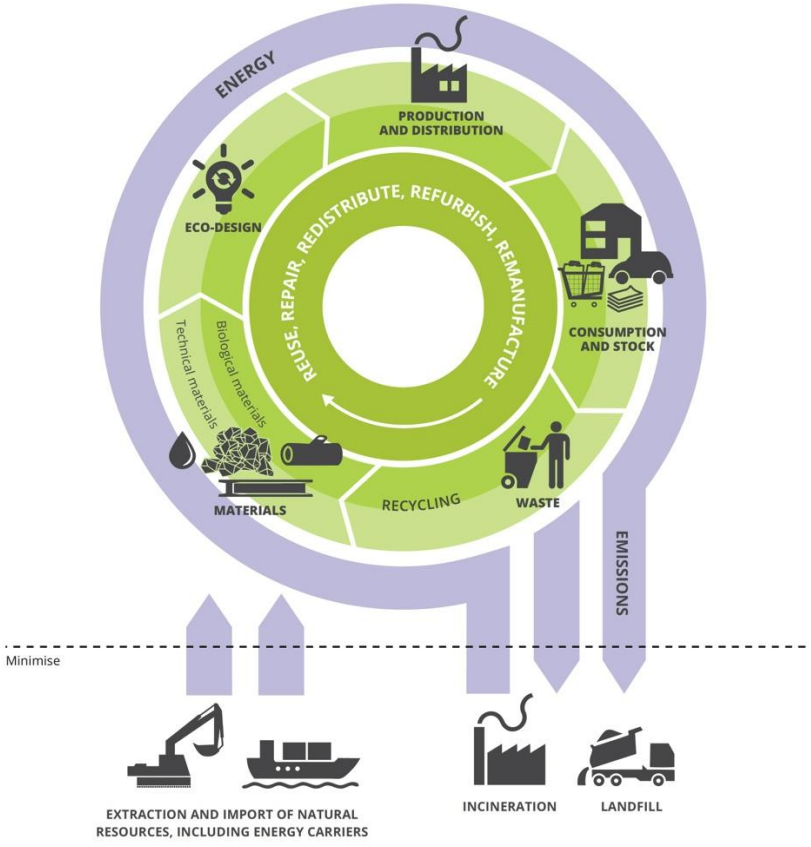


**Figure 4: Circular economy system diagram (EEA, 2018).**

This new approach focused on municipal waste and wastewater sectors as key fields that can be widely improved (Webster, 2013). The two main actions of the European policy are reduction of landfill disposal and valorisation of organic residues. European and national legislations have focused on diverting OFMSW from landfilling due to potential environmental impacts and risks and promoting of technologies able to transform a waste to be disposed of in a valuable product. In this context, the biorefinery concept fits well with this perspective. A biorefinery is a facility where biomass is sustainably processed into a spectrum of marketable food and feed ingredients, bio-based products (chemicals, materials) and bioenergy (biofuels, power and/or heat) (EEA, 2018). Biorefinery plants process a variety of bio-based raw materials, side streams and waste in highly integrated and resource-efficient processes. As such, they provide the opportunity for joining bio- and circular economy principles, especially when using second-generation feedstocks from outside the food and feed sector, including materials such as harvest residues and biowaste. AD of OFMSW is considered a key strategy of European bio- and circular economy due to its potential of valorising a second-generation feedstock. Moreover, due to the combined generation of bioenergy and bioproducts (digestate), AD is considered one of the first examples of biorefinery (Sawatdeenarunat et al., 2016).

### 1.3.2 Anaerobic biorefinery

To date, AD has been primarily used for stabilising organic waste and producing biogas. Nowadays, a more open mind is required to look beyond these original applications. According to the biorefinery concept, AD is not regarded as a final disposal treatment but is considered the centrepiece of a larger process with multiple functions such as the production of energy, fuels, heat and biobased materials (i.e.: biopolymers and agricultural fertilizers) and the remediation and stabilization of organic waste (Figure 5). The following section presents a wide range of options aiming at upgrading the traditional anaerobic process through both effluents' valorisation and biogas improvement.

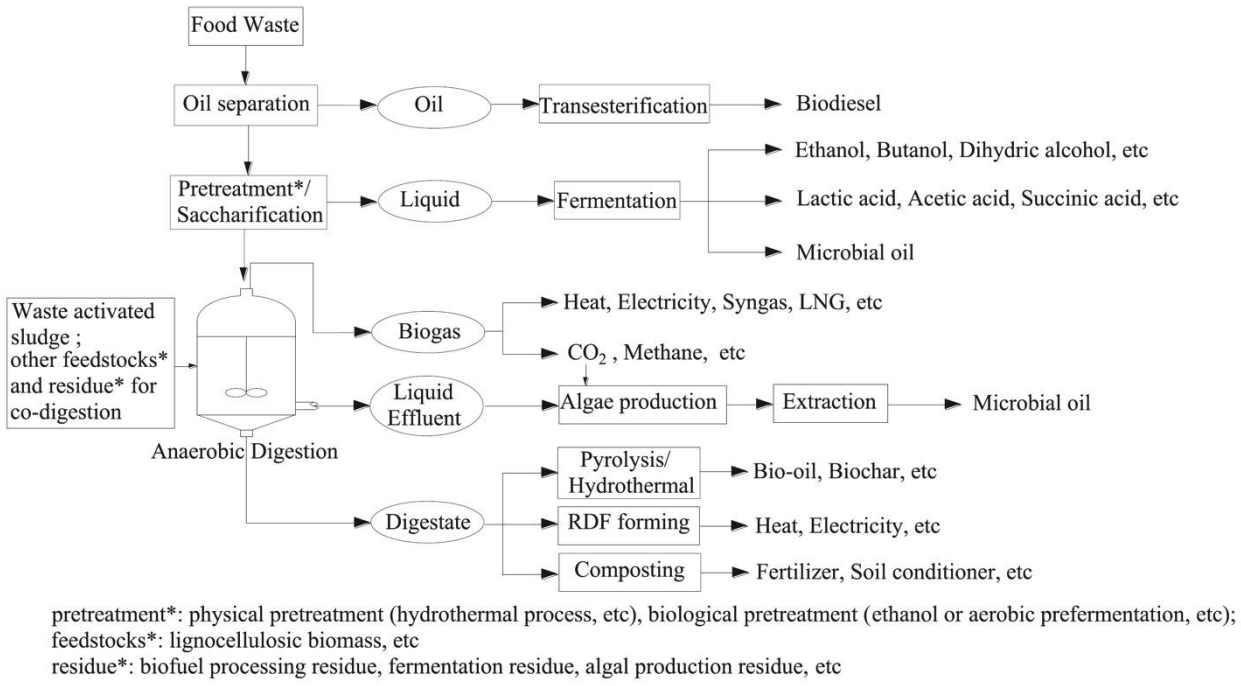


**Figure 5: Scheme of an anaerobic biorefinery (Ren et al., 2018).**

### 1.3.2.1 Effluents valorisation

The AD effluent is called digestate, which is a mixture of microbial biomass and undigested material. The prime use of digestate is as agricultural fertilizer or soil improver in compliance with local and national legislations and requirements (Iacovidou et al., 2012; Tambone et al., 2010). Its utility for agricultural purposes represents economic and environmental benefits, such as the substitution of chemical fertilizers. To date, digestate is generally mechanically separated into liquid and solid fractions that are stored separately for easy handling and transport. The liquid fraction contains high concentrations of nutrients (e.g.: nitrogen and potassium) and VFAs, whereas the solid fraction is composed of a large amount of residual lignocellulosic fibres (Monlau et al., 2013). Both fractions can serve as substrates for producing a wide variety of high-value bio-based products.

Lignocellulosic residues mainly composed of cellulose and lignin can be further exploited for recovering energy and resources.

As for cellulose, one option is to added enzymes to saccharify its fibres into soluble sugars. Such sugars, in the form of glucose, mannose, galactose, xylose, and arabinose are ideal substrates for producing different alcohols, organic acids and a wide range of biochemicals and bio-based products (Surendra et al., 2015). Bioethanol fermentation by yeast and biobutanol production by *Clostridium spp*. has been extensively studied (Bramono et al., 2011, Nanda et al., 2014). Sugars

can also be re-converted to short chain volatile fatty acids by using fermentative microorganisms. For example, succinic acid, with a global market demand of up to 50,000 t $y^{-1}$, can be used as platform chemical to produce high-value products such as 1,4-butanediol, tetrahydrofuran, and biopolymers (Jansen and van Gulik, 2014). Besides alcohol or acid fermentation pathways, dehydration of sugars to produce furans has been studied as an alternative conversion route. Dehydration of sugars has been applied to produce biomass-derived furfural and hydroxymethylfurfural, respectively. Furans are platform chemicals to produce a wide-range of chemicals, such as levulinic acids, furfural alcohol, and biopolymers (Bozell and Petersen, 2010). Instead of going through enzymatic saccharification, an alternative pathway for utilizing the cellulose present in digestate is to purify the cellulose by a delignification process. There is a wide range of derivatives from cellulose, such as polypropylene–micro crystalline cellulose composite, cellulose esters, and cellulose ethers, which have many industrial applications. For example, cellulose esters like cellulose acetate and cellulose nitrate have been commercialized in producing membrane filters for water treatment, food production, and medical supplies (Klemm et al., 2005).

As for lignin, thermochemical processes such as combustion, gasification, and pyrolysis are commonly adopted for its conversion. Besides the thermochemical pathway, a potential future technology uses an opposite approach, which aims to keep the polymerized structure of lignin intact instead of breaking it down to its monomers. Technological advancements to purify lignin for carbon fibres and biopolymers pose a potentially interesting opportunity for a lignin biorefinery. Bozell et al. (2007) reported a technology for lignin utilization, which produces aromatic compounds, including phenols, benzene–toluene–xylene and lignin monomers through catalysis and selective oxidation processes. Although the demands for such products are high, lack of technical experience and high energy costs have hindered the commercialization of lignin-based high-value chemicals production.

Besides the lignin and cellulose utilizations, the solid fibres after the solid–liquid separation of the digestate could be used for the production of bio-oil and biochar via pyrolysis. Bio-oil can also be produced by hydrous pyrolysis in which thermal decomposition takes place in the presence of water known as hydrothermal treatment. Biochar is mainly used as a soil conditioner, which has been found to be effective in carbon sequestration thereby reducing net carbon dioxide emissions (Sawatdeenarunat et al., 2016). Aiming at exploiting the remaining potential of lignocellulosic digestate, thermal treatment is applied in order to break the complex structure, thus transforming the digestate into a substrate that is again attractive for the AD process. Lissens et al. (2004) obtained an increase of methane yield of 35-40% by applying thermal wet oxidation, showing that

there is still a considerable amount of methane that can be harvested from digestate. Similarly, substrate pretreatments, topic of the first line of research of the present work, are applied to enhance hydrolysis and to solubilize organic matter prior to AD in order to improve the process in terms of faster rates and degree of substrate degradation.

As for the liquid fraction, it is characterized by relevant nutrient concentrations and VFAs.

Recently, algae farming using such nutrient-rich effluents has gained significant interest as an efficient approach for nutrient recovery (Van Den Hende et al., 2015). An algae farm using such a nutrient source not only offers nutrient recovery from the effluent, but also provides algal biomass that can be further processed into biofuels and biobased products. Microalgae (*Chlorella spp.*) are being widely adopted for this process due to their high nutrient removal efficiencies (Yan and Zheng, 2013). Algal biomass usually is rich in lipid and protein contents (Spolaore et al., 2006). Lipid from algal biomass can be extracted for producing biodiesel and glycerol. The protein-rich solid residue after lipid extraction canbe used in animal feed application or as a co-substrate for AD (Park and Li, 2012). The effluent following algal biomass separation can be recycled back as process water in AD plants or used for irrigation with less risk of ground and surface water contamination. Furthermore, nutrient-rich effluent from AD process can potentially be used to produce struvite ($MgNH_4PO_4$), which has potential application as a slow release bio-fertiliser. In particular, struvite formation takes place when the ratio of $Mg:NH_4:PO_4$ is greater than 1:1:1 on a molar basis and pH is high (Le Corre et al., 2009).

Concerning VFAs, they are produced during the acidogenic phase of AD and may be further biologically/chemically processed to obtain valuable bio-based products. They can be converted into alcohol-based fuels such as ethanol and butanol or other value-added products such as polyhydroxyalkanoates (PHA). PHAs are a class of bio-polyesters completely biodegradable. Their chief property is the mechanical behaviour that make them comparable to common plastics (Colombo et al., 2017). Other options are the direct use in microbial fuel cells to produce electricity, the use as carbon source in biological nutrient removal at WWTPs or the use for lipid production by oleaginous microorganisms for subsequent biodiesel production. Anyway, relevant concentration of VFAs can inhibit the AD process ($\geq$ 3,500 mg $L^{-1}$, Martín-González et al. 2013). The adoption of a two-stage technology can avoid problems of toxicity for methanogenic bacteria. The yield and composition of VFAs produced closely depend on operating conditions such as pH, OLR and HRT. Acidic conditions are favourable for VFA production. Propionate production is higher at lower pH (4.0-5.0) while the production of acetate and butyrate is favoured in the range of 5.5-6.5 (Yu and Fang, 2002). Since the acidogens are fast growing compared to the

methanogens, at a longer HRT methanogens are likely to metabolize VFAS into methane, resulting in lower VFAs yield. As such, VFAs production is maximised for lower HRT (Surendra et al., 2015). Conversely, VFAs production increases at increasing OLRs (Oktem et al., 2006).

#### 1.3.2.2 Biogas improvement

Currently, biogas is used by means of different technologies: direct use in internal combustion engine (ICE), gas turbine, organic Rankine Cycle or fuel cells. Among them, the most widespread technology is ICE, also offering the possibility of CHP generation. Electrical efficiencies of about 30–40% and thermal efficiencies of about 40% can be achieved with CHP (Murphy et al., 2004; Pöschl et al., 2010).

The most common biogas improvement is the upgrading to biomethane ($CH_4$ content ≥ 95% v/v). This technology is currently in use in Northern Europe (Backman and Rogulska, 2016) and is gaining increasing interest worldwide. Biogas is purified by removing impurities such as hydrogen sulphide ($H_2S$), ammonia ($NH_3$), particulates, moisture, and carbon dioxide ($CO_2$). Thanks to its high methane content, biomethane can be used for transportation, injection into natural gas grid, microturbines, and fuel cells (Murphy et al., 2004; Pöschl et al., 2010). Several processes are commercially available for biogas purification including physical and chemical absorption, adsorption and membranes. Among them, high-pressure water scrubbing is the most commonly adopted (Carnevale and Lombardi, 2015).

AD has also been demonstrated to be capable of producing hydrogen. This is already considered an important carrier for next-generation technologies, and much research is now focused on the best way to produce it in a clean and cost-effective way (European Commission, 2003). Biological hydrogen production from organic biomass fermentation is considered as one of the best options with the greatest potential (Kotay and Das, 2007) and is the topic of the second line of research of the present work. Hydrogen is produced during fermentation in the anaerobic digestion process, and a two-stage AD system can be exploited to produce both hydrogen and methane (Cavinato et al., 2012; Chinellato et al., 2013; De Gioannis et al., 2017). With such a scenario, the hydrogen could be used either by itself or to improve the combustion performance of methane, making a mixture that simulates the composition of hythane. Such a mixture, often described as biohythane, has a typical composition of 5-10% $H_2$, 30-40% $CO_2$, 50-65% $CH_4$ (Chinellato et al., 2013) and has been shown to give better efficiency and emissions performance than natural gas when used in a conventional internal combustion engine (Martinez-Perez et al., 2007; Porpatham et al., 2007; Rakopoulos and Michos, 2009).

Another option for biogas valorisation is the conversion to other higher-value chemicals such as methanol (Surendra et al., 2015). Methanol is the simplest alcohol and it has multiple industrial applications including in gasoline blends, biodiesel production, and as a carbon source for wastewater treatment. Currently methanol is being produced from petroleum-derived methane, which is first chemically oxidized to hydrogen and carbon dioxide. Finally, carbon dioxide is reduced to methanol. This process not only involves redundant steps but also is costly (Taher and Chandran, 2013). Thanks to the presence of methane and carbon dioxide, methanol can be produced from biogas. The process conversion includes both the chemical and the biological pathways. One of the biological options is the adoption of ammonia-oxidizing bacteria that are capable of reducing $CH_4$ to methanol by using ammonia as an energy source (Taher and Chandran, 2013).

## 1.4 Objectives and novelty of the research

The overall objective of the research is evaluating the improvement of the AD of the OFMSW adopting microwave and autoclave pretreatments and the two-stage process. As for the latter, its application has also been tested on the co-digestion of FW and activated sludge (AS).

Specific objectives of the research concern the identification of the optimal process conditions of the two lines of research. More specifically, with regard to autoclaving and microwaving: treatment duration, amount of substrate to be processed and adequacy of the treatment to substrate composition (e.g. lignocellulosic content). As for the two-stage technology: food-to-microorganism ratio, pH and pH control mode.

Both investigations follow a scale up strategy. Laboratory batch tests play the role of preliminary experiments where process parameters are varied in order to find the optimum condition to be tested on pilot scale semi-continuous trials. Thanks to their simpler and shorter procedures in comparison with continuous reactor experiments, batch tests are widely adopted in literature as preliminary tests for assessing the potential of a technology. Their main feature is the versatility, which make them optimal tools to test a wide range of process conditions. On the other hand, pilot scale semi-continuous trials are more complex and expensive experiments that represent the reality of a process in a deeper way and are usually employed for assessing the feasibility and adequacy of the technology transfer on an industrial scale. As such, specific objectives are studied through laboratory batch tests while the overall improvement of AD is finally assessed using semi-continuous trials.

The novelty of the work consists in the application of the two technologies to new substrates. Indeed, microwaving and autoclaving are generally employed in literature to treat low biodegradable sludge or recalcitrant agro-industrial waste while the two-stage technology is usually applied to carbohydrate-rich substrates such as FW. In this research, due to the similar characteristics of the substrates, autoclaving and microwaving have been studied on lignocellulosic OFMSW while the two-stage technology has been applied on the co-digestion of FW and AS after being assessed on the sole FW from OFMSW.

# CHAPTER 2 - SUBSTRATE PRETREATMENTS OF THE ORGANIC FRACTION OF MUNICIPAL SOLID WASTE: MICROWAVING AND AUTOCLAVING

## 2.1 Introduction

Although AD has both benefits of organic waste stabilisation and energy generation, it includes the inability of bacteria to completely degrade complex molecules such as lignin and highly insoluble organic polymers. Therefore, the organic matter is never completely degraded and a significant amount of recalcitrant substrate passes unaltered in the digestate without being exploited for energy recovery. This affects almost all substrates including agro-industrial waste (Pellera and Giradakos, 2018), FW (Bong et al., 2018), OFMSW (Cesaro and Belgiorno, 2014) and municipal wastewater sludge (Kuglarz et al., 2013). The former can be composed by a relevant amount of lignocellulosic fractions while sludge consists in microbial cells agglomerated by extracellular polymeric substances and cations resulting in low biodegradable flocks (Kuglarz et al., 2013). Another important factor that can affect the degradation rate is the particle size. More specifically, smaller particles are more easily degradable by anaerobic bacteria (Zhang et al., 2014).

Pretreatments of OFMSW can be used to solubilize organic matter prior to AD in order to improve the hydrolytic step and the overall process in terms of substrate degradation, methane production and energy recovery (Cesaro and Belgiorno, 2014). In addition, pretreatments have

been shown to be a useful step to enhance aerobic biodegradation processes as composting (Ibrahim et al., 2011) and for pathogens destruction (Ariunbaatar et al., 2014). Nevertheless, even if they enhance the AD process performance, these methods could be unsustainable in terms of energetic footprints due to relevant energy demand (Carballa et al., 2011).

Several methods have been assessed for their technical feasibility at treating residues. These can be generally divided in mechanical, thermal, chemical and biological (Ariunbaatar et al., 2014; Cesaro and Belgiorno, 2014; Jain et al., 2015). The following paragraphs and Table 2 aim at presenting advantages and disadvantages of the different treatments applied to OFMSW.

### 2.1.1 Mechanical pretreatments

Mechanical and physical pretreatments were the first studied methods (Mata-Alvarez et al., 2000). Screw presses, rotary drums and shredders are currently used at industrial scale with the purpose of separate non-wanted objects and reduction of particle size (Hansen et al., 2007).

Mechanical methods include grinding, physical separation and sonication:

- Grinding: it is generally performed using shredders, wet macerators or bead mills (Izumi et al., 2010; Davidsson et al., 2007; Hansen et al., 2007). Grinding solid particles releases cell compounds and increases the specific surface area. This provides a better contact between substrate and bacteria thus enhancing anaerobic performance (Ariunbaatar et al., 2014). Esposito et al. (2012) studied the reduction of particles size from 50 mm to 2.5 mm highlighting that at larger particle sizes, COD degradation and methane production were lower. Similar results were achieved by Izumi et al. (2010), who obtained an increase of methane production (+28%) and COD solubilisation (+40%) using bead mills. Nevertheless, excessive milling was not in favour of methane production because smaller particle substrates accelerated VFA accumulation.
- Physical separation: it is generally performed using disc screens, screw presses and rotary drums. Physical separation affects the quality of the substrate (content of plastic and particle size), dry matter content and chemical composition (Hansen et al., 2007). Nayono et al. (2010) highlighted that using press extruders prior to AD and composting was twofold beneficial. The separation of the surplus moisture from the OFMSW improved the composting process and reduced carbon dioxide emission. At the same time, the AD of the press water resulted in an increase of energy of about 15%. Bernstad et al. (2013) studied the performance of screw press technology on full-scale facilities in Sweden. The

pretreatment technology was able to discard 13-39%TS of refuses of the incoming waste concluding that the physical treatment was able to increase the overall digestion process. Concerning rotary drums, Subramani and Ponkumar (2012) obtained an increase of methane production (+37%) and volatile solids degradation (+11.5%) by rotating organic waste for 1 day prior to AD. Hansen et al. (2007) compared the effect of screw press, disc screen and shredder + magnet. The screw press resulted in a larger selective effect than the disc screen by routing to AD more water and easy degradable organic matter, whereas slowly degradable organics and unwanted materials, such as plastics and papers, were effectively removed. The shredder + magnet did not have any sorting effect, except for removing unwanted magnetic metal from the organic waste.

- Sonication: it is considered a mechanical treatment since it is able to disrupt the cell structure of substrates (Cesaro et al., 2012). It is usually applied at a frequency of 20 kHz, for a time interval in the range of 5–60 min., on 250-800 mL of material and electric powers between 130 and 1,000 W (Cesaro et al., 2012; Elbeshbishy and Nakhla, 2011; Deepanraj et al., 2017). There are two mechanisms linked with ultrasound treatment: cavitation (which happens at low frequencies) and chemical reactions (due to the release of $OH^-$, $HO_2$, $H^+$ radicals). The results of the experimental activity of Cesaro et al. (2012) showed that sonication was useful to enhance the solubilisation of the organic matter, thus allowing the improvement of the anaerobic process. More specifically, the ultrasound pretreatment resulted in an increase of soluble COD (60%), which determined an increase of biogas production of about 24%. Similarly, Elbeshbishy and Nakhla (2011) and Deepanraj et al. (2017) obtained increases in biogas production of 31.2% and 11.2%, and in volatile solids degradation of 22.2% and 10.8%, respectively.

### 2.1.2 Thermal pretreatments

Thermal pretreatments are used in literature to increase the solubilisation of organic matter by thermal breakdown of large organic matter into simple forms that are more readily accessible to bacteria (Bong et al., 2018). The efficiency of the treatment is dependent on the type of waste, temperature and time, which are the main factors in determining whether the improvement in biogas production outweighs the energy consumption. A moderate temperature in combination with moderate heating time generally results as the best combination (Bong et al., 2018). Normally, thermal treatments are a better strategy for increasing organic matter solubilisation than increasing biogas production (Cesaro and Belgiorno, 2014).

Thermal pretreatments include conventional heating, microwaving and autoclaving:

- Conventional heating: the temperature range for conventional heating has been reported to be between 50 and 250°C (Jain et al., 2015). Liu et al. (2012) heated 1.5 kg of kitchen waste at 175°C for 60 min prior to AD. Results showed a relevant decrease in viscosity and an improvement in the release of soluble COD, soluble sugars and soluble proteins. More specifically, 59.7% of the organic compounds were released in the liquid phase after the treatment. On the other hand, a decrease of methane production of approximately 8% was observed due to the formation of melanoidin. Similarly, Quiao et al. (2011) obtained a reduction of methane yield of about 7.5% after performing conventional heating at 170°C, for 60 min, on 1 kg of FW. Better results were obtained adopting lower temperatures. Kuo and Cheng (2007) achieved a hydrolytic efficiency of 27.3% and lipid removal of 37.7% performing conventional heating at 60°C on kitchen waste. This condition yielded significant COD removal efficiencies and higher gas production rates than the control (+86%). Ariunbaatar et al. (2014b) highlighted that the kinetics of the anaerobic process were accelerated by substrate heating at low temperature (<120°C). The best result was obtained at 80°C for 90 min. In this case, the enhanced methane production (+52%) could cover the energy demand of the treatment.
- Microwaving: it is an electromagnetic radiation with a wavelength between 0.001 and 1 m, corresponding to an oscillation frequency of 300–0.3 GHz (Appels et al., 2013; Eskicioglu et al., 2007). Domestic "kitchen" microwave ovens and industrial microwave generators are generally operating at a frequency of 2.45 GHz with a corresponding wavelength of 0.12 m and energy of 1.02·10-5 eV (Appels et al., 2013; Beszédes et al., 2008). It considers the direct interaction between a heated object and an applied electromagnetic field to increase heat thus combining both thermal and non-thermal effects generated in aqueous environment. The movement of ions and the vibration of polar molecules give rise to heat and extensive intermolecular collisions, which accelerate chemical, physical and biological processes. When microwave is used to treat complex molecules, it selectively heats the more polar part and this unique heating feature results in an improved distribution of the recalcitrant structures. Regarding non-thermal effects, the electromagnetic field helps to accelerate the destruction of crystalline structures and changes the super molecular structure of lignocellulosic material improving its reactivity. MW is an alternative method to conventional heating as it is able to synthesize organic molecules (Cesaro and Belgiorno, 2014). The cell

liquor and extracellular organic matter within polymeric network can release into the soluble phase increasing the ratio of accessible and biodegradable component. This effect could be manifested by different ratio of soluble and total COD and the increased rate of biogas production (Beszédes et al., 2008). The main factors influencing the treatment are temperature, power and irradiation time. Literature reports a range of application of the power between 440-500 W (Elagroudy and El-Gohary, 2013; Rani et al., 2013; Sólyom et al., 2011) and 1,250 W (Coelho et al., 2011; Eskicioglu et al., 2007; Marin et al., 2010). The applied temperature covers a wide range of values: from 30°C (Kuglarz et al., 2013) to 175°C (Marin et al., 2010). The irradiation time is generally found to be in the order of few minutes (1-10 min) even if some works present irradiation time higher than 40 min (Marin et al., 2010; Shahriari et al., 2012). Similarly to conventional heating, MW with high temperatures and long irradiation time could lead to the formation of refractory compounds inhibiting the digestion (Marin et al., 2010; Shahriari et al., 2012).

- Autoclaving: it is a hydrothermal treatment where water is used as a reagent at increased temperature and pressure to hydrolyze and solubilize sugars, starch, proteins and hemicelluloses (Tampio et al., 2014). Autoclaving involves the high-pressure sterilization of waste by steam, which cooks the waste and destroys any bacteria in it (Ibrahim et al., 2011). The main factors influencing the process are temperature, pressure and time. Several studies investigated the effect of these process parameters by studying lighter and more aggressive treatment conditions. Time and temperature depend on the volume of waste feed into autoclave usually ranging between 120–160°C within 1 hour (Ibrahim et al., 2011). Marchesi et al. (2013) studied the biomethane potential of agro-industrial waste after autoclaving for 15-30 minutes at 2 bars and 134°C while Heerah et al. (2008) autoclaved grass clippings and acacia branches at 95°C and 1 bar for four consecutive cycles each lasting 45 minutes. Papadimitriou (2010) autoclaved commingled household waste for 1 hour at 200°C and 15.5 bars, Tampio et al. (2014) treated lignocellulosic biomass at 160°C and 6.2 bars and Wilson and Novak (2009) studied secondary sludge at 220°C and 28.7 bars for 2 hours. Most of the detected results showed an increase in COD (Heerah et al., 2008; Marchesi et al., 2013; Papadimitriou, 2010) and an increase in methane production (Heerah et al., 2008, Lissens et al., 2004). Bougrier et al. (2008) and Tampio et al. (2014) reported that more aggressive thermal and hydrothermal pre-treatments at higher temperatures (around 180°C) decrease biodegradability and biogas production. This is attributable to the

formation of complex and inhibitory Maillard compounds, produced by reactions between amino acids and carbohydrates. Another possible drawback of the treatment is the release of a high total ammonia nitrogen load due to protein solubilisation (Wilson and Novak, 2009) that could induce a methanogenic inhibition.

### 2.1.3 Chemical pretreatments

Chemical pretreatments are used to achieve the destruction of the organic compounds by means of strong acids, alkalis or oxidants. AD generally requires an adjustment of the pH by increasing alkalinity, thus alkali treatment is usually the preferred chemical method. Acidic pretreatments and oxidative methods such as ozonation are also used to enhance the biogas production and improve the hydrolysis rate.

- Alkali: during alkali pretreatment, the first reactions that occur are solvation and saphonication, which induce the swelling of solids. As a result, the specific surface area is increased and the substrates are easily accessible to bacteria. When substrates are pretreated with alkali methods, an important aspect is that the biomass itself consumes some of the alkali, thus higher alkali reagents might be required for obtaining the AD enhancement. Alkaline treatment often results in an increase of biogas production. Lopez-Torres and Llorens (2008) applied $Ca(OH)_2$ to the OFMSW. The optimal condition resulted the application of 62 mEq$Ca(OH)_2$ $L^{-1}$ for 360 min. The increase of soluble COD and methane production were approximately 11% and 172% respectively Similarly, Wang et al. (2009) obtained an increase of biogas production of about 50% adopting 4 gNaOH 100g$TS^{-1}$ for 60 min.
- Acid: it is more desirable than other chemical treatments not only because it is able to break down lignin, but also because the hydrolytic bacteria are able of acclimating to acidic conditions. The main reaction that occurs during acid pretreatments is the hydrolysis of hemicellulose into monosaccharides, while the lignin condensates and precipitates. Strong acidic treatments may result in the production of inhibitory by-products, such as carboxylic acids, furans and phenolic compounds. These undesirable by-products inhibit the fermentation process and can turn in less biogas production (Ma et al., 2011). Therefore, strong acidic treatments are commonly avoided. Other disadvantages associated with acid pretreatments include the loss of simple sugars due to the increased degradation of complex substrates, high operating costs and the additional cost for neutralizing the acidic conditions prior to the anaerobic process. Vavouraki et al. (2013) performed acid hydrolysis of kitchen

waste using HCl and $H_2SO_4$. Results showed that the amount of soluble sugars obtained could be increased up to the levels of total sugars. In fact, optimized results proved that chemical pretreatment, using either 1.12% HCl for 94 min or 1.17% HCl for 86 min, increased soluble sugars concentration by 120% compared to untreated samples. The increase of soluble sugars was mainly attributed to the production of monosaccharides as glucose and fructose.

- Ozonation: it does not increase salt concentration and no chemical residues remain as compared to other chemical treatment methods. Moreover, it also disinfects the pathogens. Ozone is a strong oxidant, which decomposes itself into radicals and reacts with organic substrates directly or indirectly. Cesaro and Belgiorno, 2013 performed ozonation (1.6 $gO^3$ $gTS^{-1}$) on OFMSW. No correlation was observed between increased solubilisation and biogas production: the application of ozone led to the formation of by-products less biodegradable than untreated substrate.

### 2.1.4 Biological pretreatments

Biological pretreatments include both the use of microorganisms with high ability in degrading a substrate and the addition of enzymes that support biological reactions. As such, biological treatments include the use of enzymes, aerobic bacteria and the two-stage technology.

- Enzymes: their use results in the increase of the microbial activity per unit of surface area. The use of fungi can be considered an enzymatic treatment since they secrete extracellular enzymes (laccases and II fungal peroxidases). By means of these enzymes, white rot fungi are able to successfully degrade lignin into cellulose. Moon and Song (2011) applied an enzyme mixture of carbohydrase, protease and lipase under the ratio 1:2:1 to FW for 10 h. The mixture dose corresponded to 0.2% w/w of FW. This concentration achieved the 95% of soluble COD removal efficiency. Kiran et al. (2015) performed FW treatment with *Aspergillums Awamori* at a substrate loading of 50% w/v. The methane yield from pretreated FW was found to be 2.3 times higher than control. After AD of pretreated FW, a volatile solids removal of 80.4% was achieved.
- Aeration: the efficiency of pre-composting is related to the aerobic pre-degradation of volatile solids. On the other hand, aeration is considered an energy intensive method. Therefore, the challenge is to optimize its rate, trying to avoid over-aeration.

Charles et al. (2009) found that a pre-aeration of 48 h generated enough biological heat to increase the temperature of OFMSW to 60°C. This self-heating was sufficient for the start-up of the following thermophilic AD. Pre-aeration reduced excess easily degradable organic compounds in OFMSW, which were the cause of acidification during the start-up of the anaerobic step. Finally, the combined pre-aeration and wet thermophilic AD was able to stabilise the OFMSW to soil amendment within a period of only 12 days. Lim et al. (2013) performed micro-aeration on FW. This consisted in the introduction of small amounts of oxygen (37.5 $mLO_2\ l_r^{-1}\ d^{-1}$) into an anaerobic tank. As such, aerobic and anaerobic biological activities occurred within a single bioreactor. The added oxygen was consumed fully by facultative bacteria and a reducing environment for organic matter degradation was maintained. This method achieved a high COD solubilisation and the conversion of short chain fatty acids to acetate. However, the improvement in methane yield (+21%) was observed after 25 d.

- Two-stage process: the two-stage technology is often considered a biological pretreatment since, in the first stage, fermentative bacteria degrades macro-polymers such as lipids, proteins and carbohydrates into VFAs readily available to methanogens in the second reactor. As such, the first tank acts as a real pretreatment of AD. This technology, object of the present research, is deeply presented in chapter 3.

**Table 2: Advantages and disadvantages of OFMSW pretreatments.**

| Mechanical pretreatments | |
|---|---|
| Advantages | Disadvantages |
| Easy implementation and moderate energy consumption. Energy consumption may increase due to the final particle size and the biomass characteristics. The strong structure of green waste makes its size reduction very energy intensive. The use of pressuring machines lays in the possibility to obtain a solid fraction optimal for aerobic process and a liquid fraction optimal for AD. Well-established technology due to a widespread application on full-scale. Sonication is characterized by high efficiency in improving organics solubilisation and methane production. | No pathogen removal, possibility of equipment clogging or scaling. Size reduction can lead to system instability where smaller particles are more readily degradable and accelerate the acidification process. As the methanogens are sensitive to acidic intermediates, excessive size reduction may result in a decreased AD process performance. Concerning Sonication limited application to wet digestion systems (TS < 10%) and energy consuming technology. |
| Thermal pretreatments | |
| Advantages | Disadvantages |
| High organic matter solubilisation. Thermal pretreatments lead to pathogen removal with improvement in dewatering performance viscosity of the digestate, with subsequent enhancement of digestate handling. | Formation of refractory compounds at high temperatures. Low increase of biogas production compared to organic matter solubilisation. The high-energy consumption is an obstacle in spreading this technology on a full-scale. |
| Chemical pretreatments | |
| Advantages | Disadvantages |
| Positive effect on substrates rich in lignin. Ozonation ensures short reaction times due to its strong oxidising power and no chemical residues remain after the treatment. Alkaline/acid: low capital cost. As for alkaline treatment methane production up to 100% higher than control. | Not suitable for easily degradable substrates (e.g. carbohydrates) due to their accelerated degradation and subsequent accumulation of VFA. Ozonation: high capital costs, possible formation of less biodegradable byproducts, limited application to wet digestion systems (TS<10%). Alkaline/Acid: high operating costs, possible formation of toxic compounds as carboxylic acids, furans and phenols). |
| Biological pretreatments | |
| Advantages | Disadvantages |
| No chemical addition, low capital and operating costs, reduced energy input, minimal production of inhibitors. | High reaction times, difficult application to very complex substrate. Enzymatic treatment of OFMSW may be difficult due to its complex and variable composition, which would require the use of a specific enzyme for each basic compound (carbohydrates, lipid and protein). |

### 2.1.5 Objective of the research

Many works have already investigated the effect of microwaving and autoclaving on the AD of several substrates. Autoclaving is generally applied to lignocellulosic agro-industrial waste (Heerah et al., 2008; Marchesi et al., 2013) since it is able to release the cellulosic materials enmeshed in lignin resulting in an increase of smaller molecules available for further processing (Papadimitrou et al., 2010). Conversely, microwaving is usually adopted for low biodegradable sludge (Appels et al., 2013; Kuglarz et al., 2013; Eskicioglu et al., 2007) and it is an optimal method to solubilize complex organics (Shahriari et al., 2013).

Nevertheless, it is still not clear whether these treatments are effective on a highly variable and lignocellulosic material such as it might be the OFMSW.

Under this perspective, the present work aims at evaluating MW and A on the AD of the OFMSW.

## 2.2 Materials and methods

The materials and methods section presents the substrates and the inocula used in the study, the experimental design, the experimental set-up (batch and semi-continuous tests and the tested treatments), the calculations performed for the energy balance and for the statistical analysis, the description of the analytical methods and the calibration of the laboratory equipment.

### 2.2.1 Substrates and inoculum

#### 2.2.1.1 Substrates

Two different samples of OFMSW with different lignocellulosic content were assessed. The two samples were achieved taking into account the main fractions of Italian OFMSW (Alibardi and Cossu, 2015) varying the different amounts in order to control proteins (meat), carbohydrates (pasta) and fibres content (fir sawdust and vegetables). Similar to Shahriari et al. (2013), M1 sample was characterized by (% w/w): fir sawdust (10%), grass (30%), carrot (10%), cabbage (10%), spinach (10%), cooked meat (7.5%), raw meat (7.5%) and cooked pasta (15%). M2 sample was composed by (% w/w): fir sawdust (25%), grass (20%), carrot (10%), cabbage (10%), spinach (10%), cooked meat (5%), raw meat (5%) and cooked pasta (15%). Pasta and meat were cooked for 10 minutes and then strained. In order to reduce the particle size to 3 mm diameter each fraction was treated in a food processor and sift with a strainer. Supplemental tap water was then added to the samples leading to two mashes to guarantee a dry matter content suitable for wet technology. Dilution ratios were determined 1.7 L $kg^{-1}$ for M1 and 2.5 L $kg^{-1}$ for M2. The mashes were then stored at 4°C until use. Samples characterization in terms of TS, TVS, pH, proteins, lipids, carbohydrates and fibres is presented in Table 3. The analytical method of each parameter is presented in Section 2.2.7. In particular, M1 presented a higher composition of macronutrients while M2 highlighted a higher fibre content.

**Figure 6: Fractions of M1 and M2 synthetic OFMSW.**

**Table 3: Characterization of M1 and M2 OFMSW in terms of total solids, total volatile solids, pH, proteins, lipids, carbohydrates and fibres. Values are expressed by averages and standard deviations (n = 3).**

| Parameters | M1 | M2 |
|---|---|---|
| TS (% w/w) | 9.2 ± 0.1 | 10.0 ± 0.1 |
| TVS $TS^{-1}$ (%w/w) | 96.5 ± 0.1 | 97.8 ± 0.1 |
| pH | 3.84 ± 0.01 | 4.22 ± 0.02 |
| Carbohydrates. (%w/w) | 12.1 | 12.0 |
| Proteins (%w/w) | 6.1 ± 0.3 | 4.8 ± 0.1 |
| Lipids (%w/w) | 4.0 ± 0.1 | 2.9 ± 0.3 |
| Fibers (% w/w) | 11.0 ± 1.2 | 25.1 ± 0.9 |

### 2.2.1.2 Inoculum

Digested sludge from an anaerobic reactor treating OFMSW was used as the mesophilic inoculum. It had a pH of 7.9 while TS and TVS contents were about 2.6 ± 0.1% (w/w) and 61.2 ± 4.6% on TS basis respectively.

### 2.2.2 Design Of the Experiment (DOE)

The experimental design was planned in order to study the influence of microwaving and autoclaving on the anaerobic performances varying pretreatments conditions (duration and amount of substrate) and composition of the OFMSW (Table 4). The organization of the tests followed a scale up strategy. Batch tests played the role of preliminary experiments where process parameters were varied in order to find the optimum condition to be tested on semi-continuous trials. Batch assays were performed in two series of analyses: Batch_exp1 and Batch_exp2. The former was carried out on M1 to find out the best treatment conditions for microwaving (Pecorini et al., 2015). These conditions were then applied to M2 in the latter set of experiments. In Batch-exp2, autoclaving was also tested on M1 and M2 aiming at assessing its feasibility depending on the lignocellulosic content (Pecorini et al., 2016). The conditions applied in Batch_exp2 were finally repeated in semi-continuous trials.

**Table 4: Design of the experiment for substrate pretreatments research.**

| Test | Treatment | Substrate | Substrate amount (kg) | Duration (min) | Acronym |
|---|---|---|---|---|---|
| Batch_exp1 | - | M1 | 0 | 0 | M1 |
| | MW | M1 | 0.50 | 4 | M1_MW_500/4 |
| | MW | M1 | 0.50 | 8 | M1_MW_500/8 |
| | MW | M1 | 0.50 | 16 | M1_MW_500/16 |
| | MW | M1 | 0.25 | 2 | M1_MW_500/2 |
| | MW | M1 | 0.25 | 4 | M1_MW_500/4 |
| | MW | M1 | 0.25 | 8 | M1_MW_500/8 |
| Batch_exp2 | - | M1 | 0 | 0 | M1 |
| | - | M2 | 0 | 0 | M2 |
| | MW | M1 | 0.50 | 4 | M1_MW_500/4 |
| | MW | M2 | 0.50 | 4 | M2_MW_500/4 |
| | A | M1 | 1.70 | 15+30 | M1_A |
| | A | M2 | 1.70 | 15+30 | M2_A |
| Semi-continuous trials | - | M1 | 0 | 0 | M1 |
| | - | M2 | 0 | 0 | M2 |
| | MW | M1 | 0.50 | 4 | M1_MW_500/4 |
| | MW | M2 | 0.50 | 4 | M2_MW_500/4 |
| | A | M1 | 1.70 | 15+30 | M1_A |
| | A | M2 | 1.70 | 15+30 | M2_A |

### 2.2.3 Batch assays

Assays were performed for 21 days in order to obtain the $BMP_{21}$ (Cossu and Raga, 2008). $BMP_{21}$ was determined as the cumulate methane production at normal conditions per kg of volatile matter of substrate added ($NLCH_4$ $kgTVS^{-1}$). The analysis were conducted using a modified method of Ponsà et al. (2008) and following the guidelines and advices included in Angelidaki et al. (2009).

Assays were performed in quadruplicate for each sample using 1 L stainless steel batch reactors (Figure 7). The vessels were incubated in a water bath at 37.0 ± 0.1 °C and tightly closed by a special cap provided with a ball valve to enable the gas sampling. Warm water was heated by a thermostatic bath (FA90, Falc Instruments s.r.l., Italy). Each reactor was initially filled with 500 mL of inoculum. The inoculum was then degassed for 5 days in order to deplete the residual biodegradable organic matter (Angelidaki et al., 2009) until the achievement of an endogenous metabolism phase. Successively, the vessels were loaded with different amounts of substrate, depending on the characteristics of the material, to achieve a concentration of substrate of about 2 gTVS 100 $mL^{-1}$. This concentration is a compromise of, one hand, the need to use a large sample to have a good representativeness and to get a high easy-to-measure gas production, and, on the other hand, to avoid too large and impractical volumes of reactors and gas production and keep the solution dilute to avoid inhibition from accumulation of VFA and ammonia (Hansen et al., 2004, Angelidaki et al., 2009). Furthermore, the inoculum to substrate ratio was kept under 10:1 weight ratio, according to Ponsà et al. (2008). In order to determine the background methane production a blank assay with only the inoculum was done in triplicate. The inoculum response toward a "standard" substrate (control vessels) was checked in duplicate with cellulose with a concentration of 2 gTVS 100 $mL^{-1}$ solution (Angelidaki et al., 2009). After set-up, the bottles were flushed with inert gas to ensure anaerobic conditions in the headspace of the batches.

Biogas production was estimated by measuring the pressure in the headspace of each reactor and then converting to volume by the application of the ideal gas law, Eq. (1). Pressure was measured using a membrane pressure gauge (Model HD2304.0, Delta Ohm S.r.L., Italy).

$$V_{biogas} = \frac{P_{measured} \cdot T_{NTP}}{P_{NTP} \cdot T_r} \cdot V_r \qquad (1)$$

where:

- $V_{biogas}$: volume of daily biogas production, expressed in Normal litre (NL);
- $P_{measured}$: headspace pressure before the gas sampling (atm);
- $T_r$ and $V_r$: temperature (K) and volume (L) of the reactor's headspace;
- $T_{NTP}$ and $P_{NTP}$: normal temperature and pressure, (273.15 K and 1 atm respectively).

The gas produced was routinely analysed in its methane content using an infrared gas analyser (ECOPROBE 5, RS Dynamics, Czech Republic). The bottles were daily shaken to guarantee homogeneous conditions in the vessels (Angelidaki et al. 2009).

According to Angelidaki et al. (2009), results from BMP tests were used to obtain further information on the studied substrates like the hydrolysis rate. Using the first part of the cumulated curve it is possible to define the first order hydrolysis constant $k_h$ ($d^{-1}$), which can be calculated thanks to the following equation, Eq. (2):

$$\ln \frac{B_\infty - B}{B_\infty} = -k_h t \qquad (2)$$

where:

- $B_\infty$: value of the ultimate methane production;
- B: methane produced at a given time t.

In this study, $B_\infty$ was approximated by $BMP_{21}$ due to a low substrates production (Pecorini et al., 2016). $k_h$ can then be determined as the slope of the obtained linear curve.

**Figure 7: Batch reactors and analytical instrumentation used for the study of pretreatments.**

### 2.2.4 Semi-continuous trials

A stainless steel 10-L reactor was used for the feeding of M1, M1_MW_4/500, M1_A, M2, M2_MW 4/500 and M2_A (Figure 8). The digester operated in a water bath at mesophilic conditions (37.0 ± 0.1 °C). Warm water was heated by a thermostatic bath (FA90, Falc Instruments s.r.l., Italy). The volume of the produced gas during the tests was measured by using volumetric counters connected to the upper side of the reactors through a 3-way valve. Each counter was composed of two concentric cylinders partially filled with water: when the gas flowed from the reactor to the external side of the counter, the water rose through the internal cylinder up to the level of an electrode. The electrode activated a 3-way valve, which connected the counter to a 10 L multilayer foil bag (SupelTM, Merck KGaA, Germany) that collected the gas. After bag filling, the water level in the counter dropped to a second electrode, which reconnected the counters to the reactors and the gas restarted to enter into them. Each impulse was related to a gas volume of 0.07 L. The collected gas was then daily measured in its methane using an infrared gas analyser (ECOPROBE 5, RS Dynamics, Czech Republic). The volume of biogas and methane daily produced was then converted to normal conditions. Mixing inside the reactor was manually provided a few times a day thanks to a block and tackle.

For the start-up, the reactor was filled with 5 L of inoculum degassed for one week in order to deplete the residual biodegradable organic matter until the achievement of an endogenous metabolism phase. Afterwards it was fed during 20 days with M1 in order to reach a steady phase. After this period the experiment was started. The study was conducted with the following chronology: M1, M1_MW_4/500, M1_A, M2, M2_MW_4/500 and M2_A. Each scenario was performed for one HRT. Only one digester was used in order to assure the same bacterial population inside the reactor for the whole period. In this way, problems due to the comparison of two digesters, as the birth of different process conditions inside the reactors, are avoided. The reactor was fed manually once a day with a 100-mL syringe with a consequent withdrawal of the same volume of effluent. OLR was established steady at 4.5 kgTVS $m_r^{-3}$ $d^{-1}$ (value in the range investigated by Tampio et al., 2014) while the HRT was determined to be 20 d (for M1), 20 d (for M1_MW), 24 d (for M1_A), 22 d (for M2), 19 d (for M2_MW) and 26 d (for M2_A).

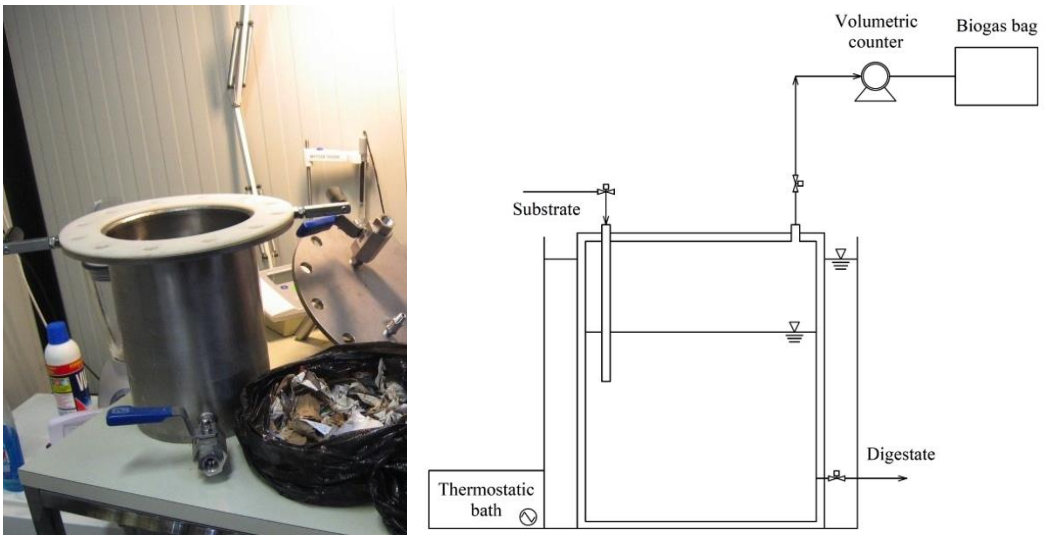


**Figure 8: Semi-continuous reactor used for the study of pretreatments.**

**Figure 9: Volumetric counters and pH and alkalinity measurements.**

Process stability was daily monitored on digestate by means of alkalinity, IA $PA^{-1}$ ratio and pH. Aiming at determining the volatile solids removal efficiency ($\eta_{TVS}$), digestate was daily controlled in its volatile solids content. Anaerobic performances were evaluated in terms of volatile solids removal, specific gas production (SGP) and methane content. $\eta_{TVS}$ was calculated based on the volatile solids content of the digestate ($TVS_{OUT}$) and the incoming substrate ($TVS_{IN}$) as reported in Eq. (3).

$$\eta_{TVS} = \frac{TVS_{IN} - TVS_{OUT}}{TVS_{IN}} \times 100 \quad (3)$$

### 2.2.5 Microwave and Autoclave pretreatments

A commercial domestic microwave oven (2,450 MHz frequency, 850 W) was used to irradiate the mashes. The microwave heating was performed in batch placing the substrate in a closed vessel to avoid losses caused by hot spot formation during the treatment (Appels et al., 2013; Rani et al., 2013).

According to Heerah et al. (2008) autoclaving was carried out using a batch system composed by a conventional pressure cooker operating at a maximum of 134°C and 2 bars (Marchesi et al., 2013) heated by a hot plate operating at 400 W. This configuration was assessed to avoid high pressure and temperature conditions, which result in an expensive treatment (Cesaro and Belgiorno, 2014) and could lead to the formation of Maillard compounds. The retention time consisted of 15 minutes to lead the mixtures from atmospheric conditions to 134°C and 2 bars followed by 30 minutes of heating at constant conditions.

Each sample was then stored at 4°C until use. The summary of microwaved and autoclaved substrates is provided in Table 5.

**Table 5: Summary of the treated samples.**

| Sample | Treatment | Substrate | Substrate am. (kg) | Duration (min) |
|---|---|---|---|---|
| M1_MW_500/4 | MW | M1 | 0.50 | 4 |
| M1_MW_500/8 | MW | M1 | 0.50 | 8 |
| M1_MW_500/16 | MW | M1 | 0.50 | 16 |
| M1_MW_500/2 | MW | M1 | 0.25 | 2 |
| M1_MW_500/4 | MW | M1 | 0.25 | 4 |
| M1_MW_500/8 | WW | M1 | 0.25 | 8 |
| M2_MW_500/4 | MW | M2 | 0.50 | 4 |
| M1_A | A | M1 | 1.70 | 15+30 |
| M2_A | A | M2 | 1.70 | 15+30 |

### 2.2.6 Energy profit

According to Kuglarz et al. (2013) and Pecorini et al. (2016), the specific energy profit of the pre-treatment $E_T$ (kJ $kgTVS^{-1}$) was calculated taking into account the energy produced in the form of biogas ($E_B$), the theoretical amount of energy produced in the form of heat ($E_Q$) and the energy demand of the pre-treatment ($E_D$), Eq. (4). The balance refers to the volatile mass of the substrate before the treatment.

$$E_T = E_B + E_Q - E_D \quad (4)$$

where:

- $E_B$: amount of energy produced in the form of biogas after subtracting the energy generated by raw substrates (kJ $kgTVS^{-1}$);
- $E_Q$: amount of energy produced in the form of heat (kJ $kgTVS^{-1}$);
- $E_D$: amount of energy used for samples pre-treatment performed in certain conditions (kJ $kgTVS^{-1}$).

$E_B$ was based on the lower heating value (LHV) of methane (35.8 kJ $NL^{-1}$, Siddiqui et al., 2011; Liu et al., 2013) and the surplus of methane production after pretreatment compared to untreated samples expressed as $NLCH_4$ $kgTVS^{-1}$. This calculation was performed taking into account $BMP_{21}$ results for batch tests, the specific methane production (SMP) for semi-continuous trials and an energy conversion factor (η) of 0.9 (Xiao et al., 2018). As for the semi-continuous trials, Eq. (5):

$$E_B = (SMP_{treated} - SMP_{untreated}) \cdot LHV_{CH4} \cdot \eta \quad (5)$$

The heat recovery from the pretreatment $E_Q$ was based on the heat of the organic mass after the treatment without considering the heat of biogas (Xiao et al., 2018):

$$E_Q = \frac{Q \cdot C_p \cdot (T_t - T_a) \cdot \phi}{Q \cdot VS} \quad (6)$$

where:

- Q: mass of treated substrate (kg);
- VS: volatile matter content of the substrate (kgTVS $kg^{-1}$);
- $C_p$: specific heat capacity of the treated organic mass (kJ $kg^{-1}$ $°C^{-1}$). $C_p$ was based on ratio of water and solids (TS). The values of $C_p$ used for calculations amounted to 4.18

and 1.95 kJ $kg^{-1}$ $°C^{-1}$ for water and solids respectively (Kim and Parker, 2008; Kuglarz et al., 2013);

- $T_T$: temperature of the treatment (°C);
- $T_a$: ambient temperature. It was assumed to be 20°C;
- ϕ: percentage of heat recovered, 80% (Xiao et al., 2018).

The specific energy demand ($E_D$) was calculated according to Kuglarz et al. (2013) taking into account the power of the microwave/autoclave heating system, the exposure time applied for each treatment and the mass of treated mash in kgTVS, Eq. (7):

$$E_D = \frac{P_D \cdot t_D}{Q \cdot VS} \quad (7)$$

where:

- $P_D$: power of microwave generator or hot plate (kW);
- $t_D$: exposure time (s);
- Q: mass of treated substrate (kg);
- VS: volatile matter content of the substrate (kgTVS $kg^{-1}$).

### 2.2.7 Analytical parameters

TS, TVS and pH were determined to characterize the inoculum, the substrates and the digestates of semi-continuous trials. Measurements were performed according to standard methods (APHA, 2006). The temperature reached by the substrates after the treatments was measured using a rigid tip digital thermometer (T1, Testo S.p.A., Italy).

The methane content of biogas was measured using an infrared gas analyser (ECOPROBE 5, RS Dynamics, Czech Republic).

With regard to Batch-exp2, in order to investigate the solubilisation effect of the pre-treatments, soluble COD (sCOD), soluble Carbohydrates (sCarb) and soluble Protein (sProt) were analysed before and after pre-treatments. sCOD was analysed to investigate the solubilisation of organic materials in the entire samples while sProt and sCarb were analysed to investigate the behaviour of two macromolecular organic components. The soluble part of each substrate was determined after centrifugation at 12,000 g for 30 min and subsequent filtration 0.45 μm microfiber filter paper (Marin et al., 2010; Rani et al., 2013). Proteins, lipids and fibres were obtained following the European Commission Regulation 2009/152/EC of 27 January 2009 (European Commission,

2009). Total carbohydrates were determined by subtracting the contents of humidity, ashes, proteins, lipids and fibres from the total amount.

With regard to semi-continuous trials, total alkalinity (TA), intermediate alkalinity (IA) and partial alkalinity (PA) were daily determined on the digestate according to Martín-González et al. (2013). The measurement consisted in a two-end point titration methodology to monitor VFAs/alkalinity ratio leading to obtain total alkalinity and partial alkalinity. The former included both VFA and bicarbonate alkalinity (pH = 4.3) while the latter was roughly related only to bicarbonate alkalinity (pH = 5.75). The difference, defined as intermediate alkalinity, was related only to VFA alkalinity. Several studies have included alkalinity ratios as monitoring parameters. For instance, the pilot scale digester was daily monitored through the ratios intermediate/partial alkalinity (IA $PA^{-1}$).

### 2.2.8 Statistical analysis

In order to assess the significance of batch tests results ($BMP_{21}$, sCOD, sProt, sCarb), the findings obtained by the different pretreaments were analysed using one-way ANOVA (Kuglarz et al., 2013). A confidence level of 95% was selected. All calculations were performed using XLStat2018 software packages.

### 2.2.9 Calibration and control of the experimental set-up

Analytical instruments and reactors were carefully calibrated and controlled before the start of the experiments.

The vessels used for batch tests and the reactor used for semi-continuous trials were subjected to leakage test in order to prevent biogas losses during the experiments. Both containers were realized to hold a 2 bar proof pressure.

The infrared gas analyser was periodically calibrated for methane and carbon dioxide measurements. The calibration curves were built using different synthetic gasses with known content methane and carbon dioxide.

The pH-meter was weekly calibrated using a 3-points calibration curve with proof buffer solutions of known pH (4, 7, 10).

The volume of gas displaced by a pulse of the volumetric counter was checked prior to the start of semi-continuous tests. Calibration was performed by flushing 5 different flow rates of

compressed air through the volumetric meters and recording the time interval between two pulses. Volumetric counters were also subjected to leakage test in order to prevent biogas losses during the experiments.

Each measurement of dry matter, volatile solids and pH was performed in triplicate while the quality of gas and the alkalinity were controlled in duplicate.

The measurements of proteins, lipids, carbohydrates, fibres, soluble COD, soluble proteins and soluble carbohydrates were performed by accredited external laboratories.

## 2.3 Results

### 2.3.1 Batch tests – experiment 1

Batch_exp_1 was performed on M1 aiming at defining the best MW condition for M1 OFMSW. Table 6 presents the temperature reached by the treatment and the effects of MW on the substrate in terms of TS, TVS and pH. As for the temperature, it was kept below 150°C in order to avoid the formation of refractory compounds that can inhibit the anaerobic process (Marin et al., 2010; Shahriari et al., 2012).

**Table 6: Batch_exp_1 - Temperature of microwaving and effects of the pretreatment on TS, TVS and pH. Values are expressed by averages and standard deviations (n = 3). Pecorini et al. (2015).**

| Samples | T (°C) | TS (%) | TVS $TS^{-1}$ (%) | pH |
|---|---|---|---|---|
| M1_MW_500/4 | 96.0 ± 0.5 | 8.6 ± 0.2 | 96.6 ± 0.1 | 3.65 ± 0.01 |
| M1_MW_500/8 | 99.3 ± 1.1 | 9.4 ± 0.1 | 96.7 ± 0.1 | 3.64 ± 0.01 |
| M1_MW_500/16 | 100.8 ± 1.3 | 12.9 ± 0.0 | 97.4 ± 0.1 | 3.28 ± 0.01 |
| M1_MW_250/2 | 85.5 ± 0.7 | 8.9 ± 0.1 | 96.6 ± 0.1 | 3.68 ± 0.01 |
| M1_MW_250/4 | 96.4 ± 0.9 | 9.5 ± 0.3 | 96.7 ± 0.1 | 3.65 ± 0.01 |
| M1_MW_250/8 | 98.7 ± 2.2 | 12.98 ± 0.29 | 96.6 ± 0.1 | 3.49 ± 0.01 |

#### 2.3.1.1 BMP tests

BMP assays showed the typical trend of the test without the occurrence of any acidification process (Figure 10). Control test showed an average methane production of 126.2 $NLCH_4$ $kgTVS^{-1}$, which underlines a good quality response of the inoculum to a standard substrate as cellulose. The average methane content ranged between 62.4% and 65.4% for all the tested samples, in the optimal range of the anaerobic treatment (APAT, 2005). All treatments noticed an increase in methane production compared to the blank substrate (M1). This is in agreement with what reported in previous batch studies (Eskicioglu et al., 2007; Sólyom et al., 2011; Kuglarz et al., 2013). In particular, the enhancement in methane production occurred at increasing $E_D$, with the highest result observed for M1_MW_250/8 (+32.7%, Table 7). Analysis of variance performed on $BMP_{21}$ results indicated the finding of statistically different results ($p < 0.05$) supporting the importance of the treatment on methane production. Moreover, treatments performed on the smaller amount (0.25 kg) determined slightly higher methane productions compared to treatments performed on 0.50 kg of substrate. These behaviours are probably attributable to a better application of the treatment to the mass of substrate. In this way, a stronger solubilisation effect is produced leading

to a sample composition more suitable for anaerobic bacteria. The solubilisation effect of the pretreatments was confirmed by the lower pH found after all treatments reported in Table 6, that could be associated to a release of organic acids during the process (Pecorini et al., 2016).

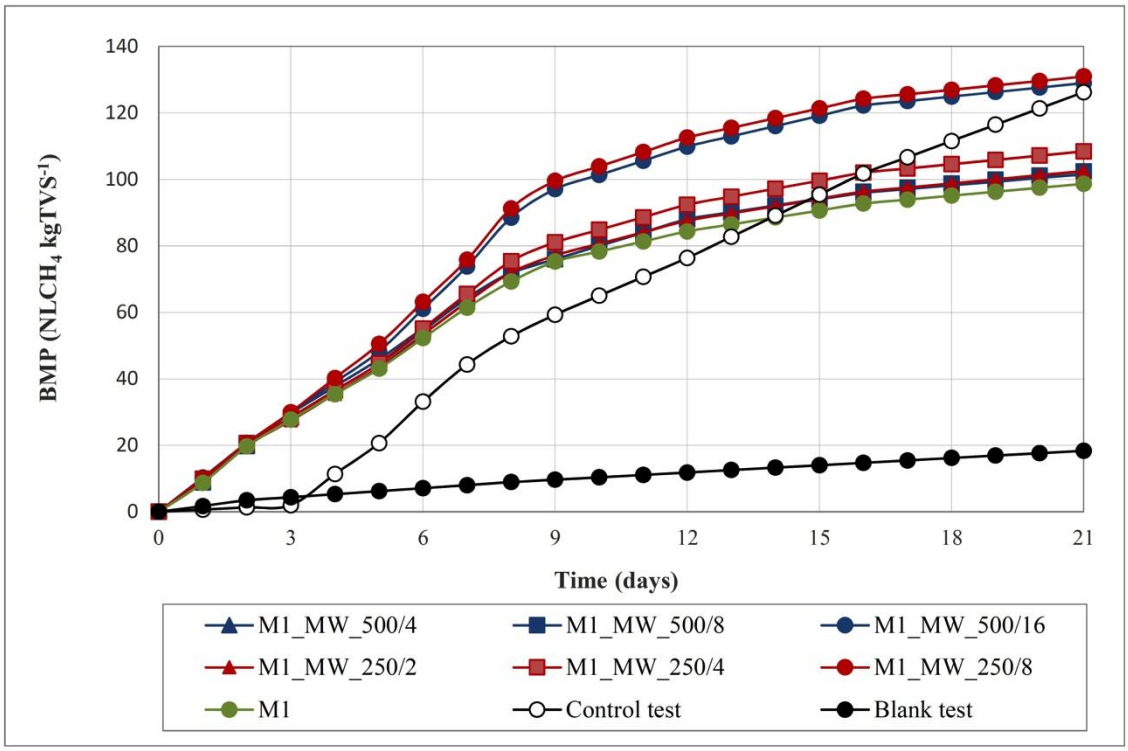


**Figure 10: Batch_exp_1 - Mean BMP test curves with control and blank assays. Pecorini et al. (2015).**

**Table 7: Batch-exp_1 – Results of anaerobic biodegradability assays in terms of methane content and $BMP_2$. Values are expressed by averages and standard deviations (n = 3). Pecorini et al. (2015).**

| Parameter | M1 | M1_MW_500/4 | M1_MW_500/8 | M1_MW_500/16 |
|---|---|---|---|---|
| $CH_4$ (%) | 63.8 ± 1.8 | 62.4 ± 2.3 | 64.4 ± 2.3 | 65.4 ± 3.5 |
| $BMP_{21}$ ($NLCH_4$ $kgTVS^{-1}$) | 98.7 ± 2.3 | 101.5 ± 0.1 | 102.4 ± 1.3 | 129.0 ± 8.4 |
| Parameter | M1_MW_250/2 | M1_MW_250/4 | M1_MW_250/8 | Control test |
| $CH_4$ (%) | 64.1 ± 2.1 | 63.6 ± 2.4 | 64.6 ± 2.4 | 49.1 ± 0.4 |
| $BMP_{21}$ ($NLCH_4$ $kgTVS^{-1}$) | 102.5 ± 7.9 | 108.4 ± 10.8 | 130.9 ± 12.9 | 126.2 ± 2.6 |

### 2.3.1.2 Energy profit

Energy efficiency is a crucial factor influencing the economic feasibility of substrate pretreatments (Kuglarz et al., 2013). The calculated $E_D$, $E_B$, $E_Q$ and $E_T$ for the different MW conditions are presented in Table 8. Analysing the specific energy balance, no energy profit was registered for all treatments. This was mainly due to the low increase in biogas production compared to raw substrate digestion and to laboratory scale conditions. The amount of $E_B$ and $E_Q$

was not enough to balance $E_D$. A negative energy balance was also reported by previous studies performing low-energy thermal treatments: Houtmeyers et al., 2014 and Appels et al., 2013 carried out MW by applying 96 kJ $kg^{-1}$ and 336 kJ $kg^{-1}$ respectively. Under these conditions, energy balances proved that MW was not energetically feasible. Conversely, Kuglarz et al. (2013) obtained an increase in total energy by applying severe treatment conditions ($E_D$ 8,094 kJ $kgTVS^{-1}$), which resulted in high $E_B$ and $E_Q$, not found in the present work.

Even if with negative results, lighter treatments showed better energetic response than more aggressive one due to a lower $E_D$. For this reason, M1_MW_500/4 was selected as the best condition to be tested on M2 in new batch tests (section 2.3.2) and continuous experiments (section 2.3.3).

**Table 8: Batch_exp_1 - Energy balance.**

| Sample | $E_B$ (kJ $kgTVS^{-1}$) | $E_Q$ (kJ $kgTVS^{-1}$) | $E_D$ (kJ $kgTVS^{-1}$) | $E_T$ (kJ $kgTVS^{-1}$) |
|---|---|---|---|---|
| M1_MW_500/4 | 90 | 2,731 | - 4,596 | - 1,774 |
| M1_WW_500/8 | 119 | 2,837 | - 9,191 | - 6,235 |
| M1_MW_500/16 | 976 | 2,834 | - 18,383 | - 14,572 |
| M1_MW_250/2 | 122 | 2,350 | - 4,596 | - 2,123 |
| M1_MW_250/4 | 313 | 2,732 | - 9,191 | - 6,147 |
| M1_MW_250/8 | 1,037 | 2,759 | - 18,383 | - 14,686 |

## 2.3.2 Batch tests – experiment 2

Focusing on the lignocellulosic fraction of biowaste, the study was conducted by varying the lignocellulosic content of OFMSW while the treatment conditions were not changed. As such, it has been selected a single condition for A and MW characterized by low treatment energy with the intention of limiting the energy expenses and prevent the formation of refractory compounds. As for MW, treatment conditions were chosen based on the best conditions highlighted in Batch_exp_1 (4 minutes and 0.5 kg of substrate). Changes in the soluble fractions of the organic matter (measured by soluble COD, carbohydrates and proteins), the first order hydrolysis constant $k_h$ and the cumulated methane production were used to evaluate the efficiency of microwaving and autoclaving on substrates solubilisation and AD process.

Table 9 shows the temperature reached and the effects on the treatment in terms of TS, TVS and pH. Also in this case, MW and A temperatures were kept below 150°C.

**Table 9: Batch_exp_2 - Temperatures and effects of MW and A on TS, TVS and pH. Values are expressed by averages and standard deviations (n = 3). Pecorini et al. (2016).**

| Samples | T (°C) | TS (%) | TVS $TS^{-1}$ (%) | pH |
|---|---|---|---|---|
| M1_MW_500/4 | 96.0 ± 0.5 | 9.1 ± 0.1 | 96.6 ± 0.1 | 3.51 ± 0.02 |
| M1_A | 134.0 ± 1.3 | 11.1 ± 0.0 | 96.9 ± 0.1 | 3.41 ± 0.02 |
| M2_MW_500/4 | 95.3 ± 0.3 | 8.6 ± 0.4 | 97.6 ± 0.0 | 3.69 ± 0.02 |
| M2_A | 134.0 ± 0.5 | 11.9 ± 0.1 | 97.8 ± 0.1 | 3.46 ± 0.01 |

#### 2.3.2.1 Substrate solubilisation

MW and A treatments led to the solubilisation of the organic material of both the OFMSW samples. sCOD, sCarb and sProt were found higher for M1 substrates than M2 substrates (Table 10). This feature is concurring with the OFMSWs initial composition (Table 3), which shows a higher content of proteins, carbohydrates and lipids for M1 mash.

An increase of sCOD was found for both treatments and both OFMSW tested samples. This trend was found particularly relevant for MW with an increase of about 219.8% for M1_MW_500/4 and 142.4% for M2_MW_500/4. The increase of sCOD for MW agreed with Coelho et al. (2011), Elagroudy and El-Gohary (2013), Kuglarz et al. (2013), Marin et al. (2010) and Toreci et al. (2008). The solubilisation effect on carbohydrates and proteins was registered for both treatments (in agreement with Marin et al., 2010; Rani et al., 2013) but it was mainly relevant for autoclaving. Compared to the abovementioned studies, results showed a lower solubilisation effect of carbohydrates and proteins. This was due to the application of treatments characterized by low temperatures and short duration times that translates into the application of little energy per treatment. The higher increase of sProt and sCarb found for autoclaving is attributable to the higher temperature reached in A compared to MW. Indeed, as reported by previous studies (Appels et al., 2010; Wilson and Novak, 2009), the increase in temperature is associated to a major release of soluble proteins and carbohydrates. In particular, the thermal effect acts on both decomposition of extracellular polymer substances and cell lysis (Appels et al., 2010; Eskicioglu et al., 2007). The analysis of variance performed on sCOD, sCarb and sProt results indicated the finding of statistically different results ($p < 0.05$) supporting the relevance of the treatment in the solubilisation of the macromolecules.

Furthermore, also in this case, the lower pH found after all treatments could be associated to a release of organic acids during the process (Table 6, Heerah et al., 2008; Papadimitriou, 2010).

**Table 10: Bacth_exp_2 - Substrates solubilisation in terms of soluble COD, carbohydrates, proteins and sCarb $sProt^{-1}$ ratio. Values are expressed by averages and standard deviations (n = 3). Pecorini et al. (2016).**

| Parameter | M1 | M1_MW_500/4 | M1_A |
|---|---|---|---|
| sCOD (mg $L^{-1}$ $O_2$) | 19,700 ± 4,400 | 63,000 ± 14,000 | 25,500 ± 5,600 |
| sCarb (%TS) | 49.9 ± 4.2 | 54.0 ± 5.1 | 64.1 ± 6.3 |
| sProt (%TS) | 19.8 ± 5.5 | 19.4 ± 5.8[a] | 25.7 ± 6.3 |
| sCarb $sProt^{-1}$ | - | 2.78 | 2.49 |
| Parameter | M2_MW | M2_A_500/4 | M2 |
| sCOD (mg $L^{-1}$ $O_2$) | 41,700 ± 9,200 | 32,200 ± 7,100 | 17,200 ± 3,800 |
| sCarb (%TS) | 30.8 ± 5.9 | 35.4 ± 6.8 | 26.2 ± 5.1 |
| sProt (%TS) | 15.2 ± 3.7 | 18.1 ± 4.3 | 14.8 ± 3.4 |
| sCarb $sProt^{-1}$ | 2.03 | 1.95 | - |

### 2.3.2.2 BMP tests

Similar to Batch_exp_1, the assays showed the typical trend of the test without the occurrence of any acidification process (Figure 11). In this case, the control test showed an average methane production of 154.6 $NLCH_4$ $kgTVS^{-1}$, which underlines a good quality response of the inoculum to a standard substrate as cellulose. Results showed a higher methane yield for all M1 substrates compared to M2 substrates, which was attributable to the sample composition, more suitable for anaerobic bacteria (M2 was characterized by a higher fibre and lignocellulosic content). Similarly, also the methane content was registered higher for M1 (ranging between 59.9% and 61.6%) than M2 substrates (between 56.2% and 58.1%). MW led to a $BMP_{21}$ increase of 8.5% for both the tested OFMSW while A had an increase of about 1.0% for M1 and 4.4% for M2. Therefore, MW was found to be an efficient treatment for both OFMSW while A was found to be more suitable for a more lignocellulosic substrate (M2). This statement is concurring with Lissens et al. (2004) and Marchesi et al. (2013) who determined a higher increase in BMP for lignocellulosic substrates after wet oxidation and autoclaving, respectively. Also in this case, the analysis of variance performed on $BMP_{21}$ results indicated the finding of statistically different results ($p < 0.05$) supporting the relevance of the treatment on methane production.

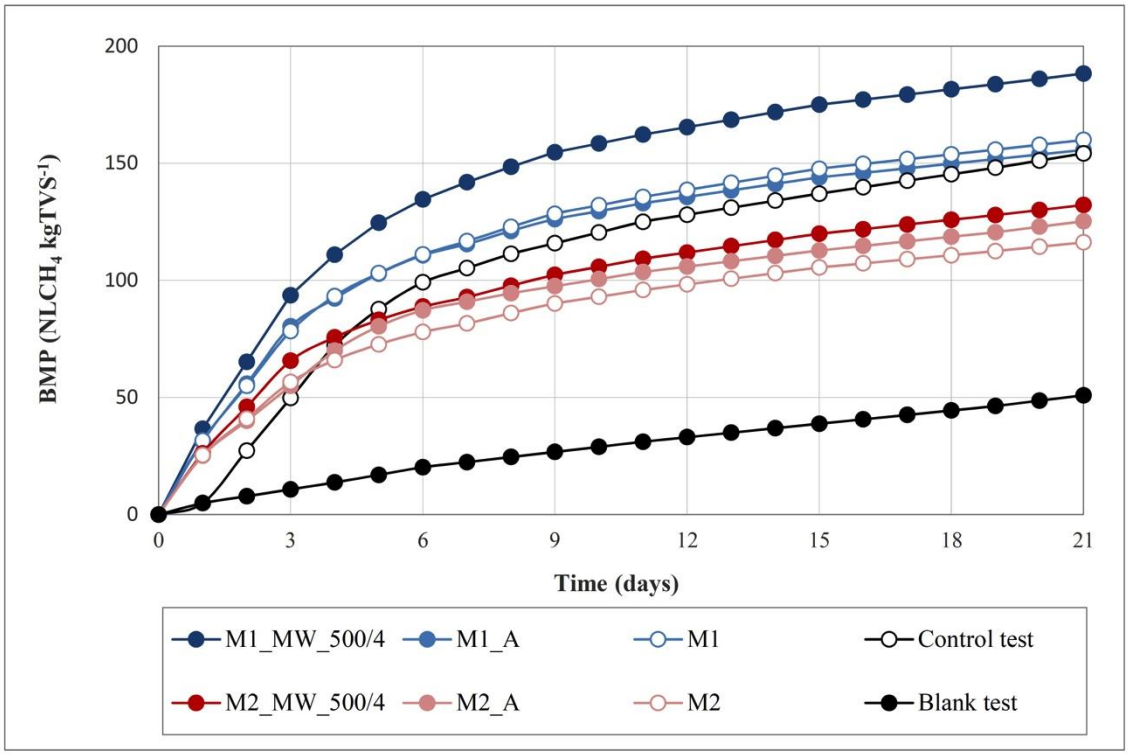


**Figure 11: Batch_exp_2 - Mean BMP test curves with control and blank assays. Pecorini et al. (2016).**

**Table 11: Batch_exp_2 - Results of anaerobic biodegradability assays expressed in terms of methane content, $k_h$ and $BMP_{21}$. Values are expressed by averages and standard deviations (n = 3). Pecorini et al. (2016).**

| Parameter | M1 | M1_MW_500/4 | M1_A |
|---|---|---|---|
| $CH_4$ (%) | 61.6 ± 0.2 | 59.9 ± 0.8 | 60.0 ± 0.9 |
| $BMP_{21}$ ($NLCH_4$ $gTVS^{-1}$) | 172.1 ± 9.8 | 186.7 ± 6.5 | 173.8 ± 16.6 |
| $k_h$ ($d^{-1}$) | 0.221 ± 0.003 | 0.218 ± 0.005 | 0.202 ± 0.008 |
| Parameter | M2 | M2_MW_500/4 | M2_A |
| $CH_4$ (%) | 58.1 ± 0.7 | 57.2 ± 0.1 | 56.2 ± 0.5 |
| $BMP_{21}$ ($NLCH_4$ $gTVS^{-1}$) | 119.9 ± 4.7 | 130.2 ± 0.7 | 125.2 ± 4.2 |
| $k_h$ ($d^{-1}$) | 0.210 ± 0.006 | 0.196 ± 0.003 | 0.200 ± 0.011 |

The increase in methane production was found directly related to sCOD release with the highest increase of methane production found together with the highest release of sCOD (Figure 12). The coefficient of determination ($R^2$) was calculated for both mashes and found above 0.98, guarantying a good approximation of the linear correlation. This finding concurred with Beszédes et al., (2008) and Elagroudy and El-Gohary, (2013) which reported an increase of methane production together with an increase in sCOD. Nevertheless, the significant increase in sCOD did not correspond with a similar increase in methane production (e.g. for M1_MW_500/4: +219.8% for sCOD and +8.5% for $BMP_{21}$). This is in agreement with Ariubaatar et al. (2014a) who stated

that thermal treatments generally result in a slight increase of biogas production compared to organic matter solubilisation (Table 2). Furthermore, this outcome suggested that most of the released sCOD was not biodegradable and it was not converted into methane. Indeed, 1 gram of biodegradable COD produces around 400 mL of $CH_4$ (Field et al., 1988) and, according to the increase of sCOD, the increase in methane production did not reflect this relation supporting the case that the sCOD produced from MW and A was mainly composed of non-biodegradable substances. In this regard, the non-biodegradable fraction can be associated to recalcitrant compounds such lipid hydrolysis products (Alibardi and Cossu, 2016; Chen et al., 2008).

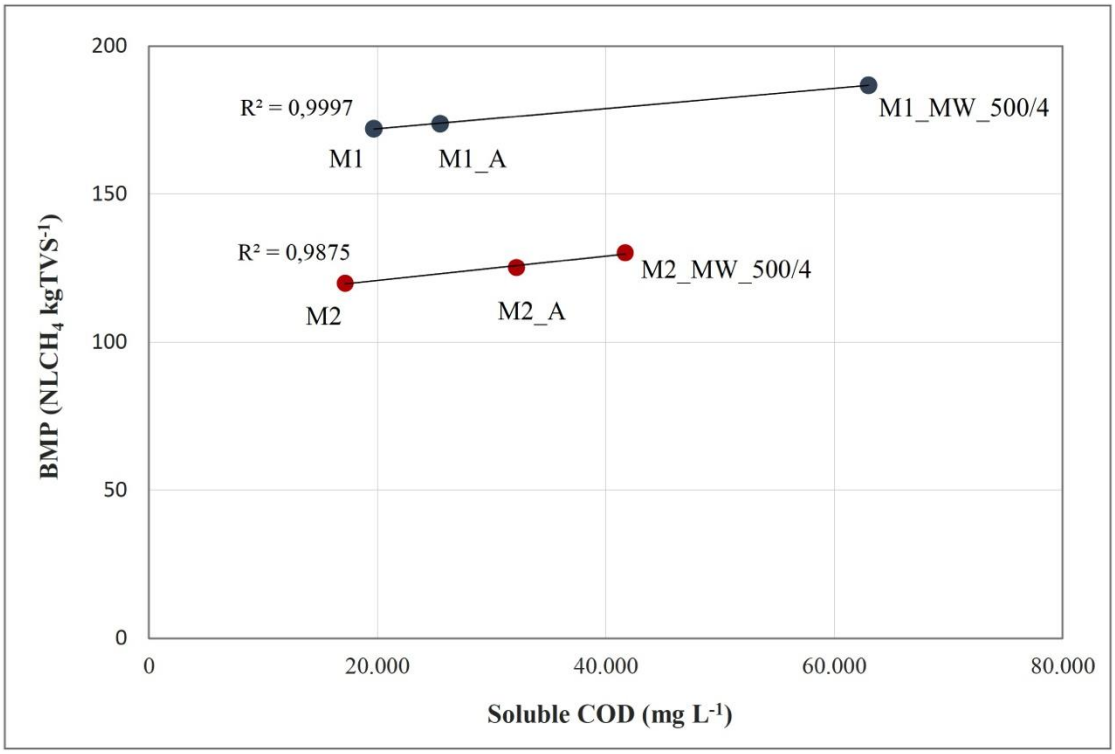


**Figure 12: Batch_exp_2 - Correlation between the release of soluble COD and $BMP_{21}$. $R_2$ represents the coefficient of determination. Pecorini et al. (2016).**

The higher increase in biogas production and sCOD recorded for MW compared to A is attributable to the different action mechanism of the pretreatments, and, in particular, to the athermal effect of MW. Indeed, while autoclaving normally increases the ionized products of water which are able to hydrolyse the macromolecules at elevated temperature and pressure (Yin et al., 2014), microwaving can improve the rupturing of the cell wall in two different ways: thermal and athermal effect (Cesaro and Belgiorno, 2014; Houtmeyers et al., 2014; Sólyom et al., 2011). The former corresponds to degradation caused by temperature increase. The latter occurs when the alternating electric field of microwaves is able to force the polarized side chains of the cell wall macromolecules to break their hydrogen bonds, and thus alter their structure. As reported by

previous studies (Eskicioglu et al., 2007; Pino-Jelcic et al., 2006), the athermal effect of MW is manifested through a difference in sCOD and/or increased rates of biogas production compared to other treatments.

Furthermore, autoclaving was probably affected by a release of total ammonia nitrogen due to the solubilisation of proteins that partially inhibited the anaerobic process (Wilson and Novak, 2009). This statement is supported by the inverse correlation found between the increase of methane production and the increase of protein solubilisation. Indeed, the more is the protein solubilisation, the less is the increase in methane production. Analysing the ratio sCarb $sProt^{-1}$ calculated for both treatments and presented in

Table 10, this parameter was directly related to methane. Even in the presence of a relevant increase in sCarb, the increase in sProt reduces the ratio and simultaneously the methane production.

Results on the first order hydrolysis underlines what previously reported. $k_h$ was found higher for M1 samples than M2 ones; furthermore, for M1 $k_h$ was registered superior for MW while for M2 $k_h$ was determined slightly superior for A confirming the efficiency on the hydrolysis phase of MW on a meagre lignocellulosic substrate and A on a rich lignocellulosic substrate.

#### 2.3.2.3 Energy profit

Similar to Batch_exp1, the energy balance performed for Batch_exp_2 proved that using batch tests, pretreatments were not energetically feasible (Table 12). Comparing the two treatments, even if with negative results, MW showed better energetic responses than A, due to the lower energy demand. Among the two forms of produced energy, $E_Q$ was the main one. As for MW, $E_Q$ accounted for the 85-88% of the total energy produced while for A, this percentage increased up to 96-98%. This result was mainly due to the high temperature of the media after the treatment and to the low increase in methane generation. The same approach was then used for semi-continuous trials in order to verify the relevance of the scale conditions in the balance.

**Table 12: Batch_exp_2 – Energy balance.**

| | $E_B$ (kJ $kgTVS^{-1}$) | $E_Q$ (kJ $kgTVS^{-1}$) | $E_D$ (kJ $kgTVS^{-1}$) | $E_T$ (kJ $kgTVS^{-1}$) |
|---|---|---|---|---|
| M1_MW_500/4 | 470 | 2,724 | - 4,596 | - 1,383 |
| M1_A | 55 | 4,040 | - 7,156 | - 3,061 |
| M2_MW_500/4 | 332 | 2,457 | - 4,172 | - 1,402 |
| M2_A | 171 | 3,650 | - 6,496 | - 2,575 |

### 2.3.3 Semi-continuous trials

Results are firstly presented by analysing process stability through pH and alkalinity data. The effect of MW and A was then analysed in the anaerobic performance through biogas production, biogas quality and volatile solids removal efficiency ($\eta_{TVS}$). Finally, similar to batch tests, the energy balance was also provided. The effect of the pretreatments on TS, TVS and pH was the same of Bartch_exp_2 (Table 9).

#### 2.3.3.1 Process stability

Process stability can be represented by pH and alkalinity measurements. These parameters indicate the degree of inhibition inside the digester. When their values exceed the proper ranges, inhibition happens and the digester get unstable. Figure 13 depicts TA and and IA $PA^{-1}$ ratio trends over time while Table 13 reports their averages and standard deviations. pH, IA and PA trends over time are reported in the Appendix as additional data.

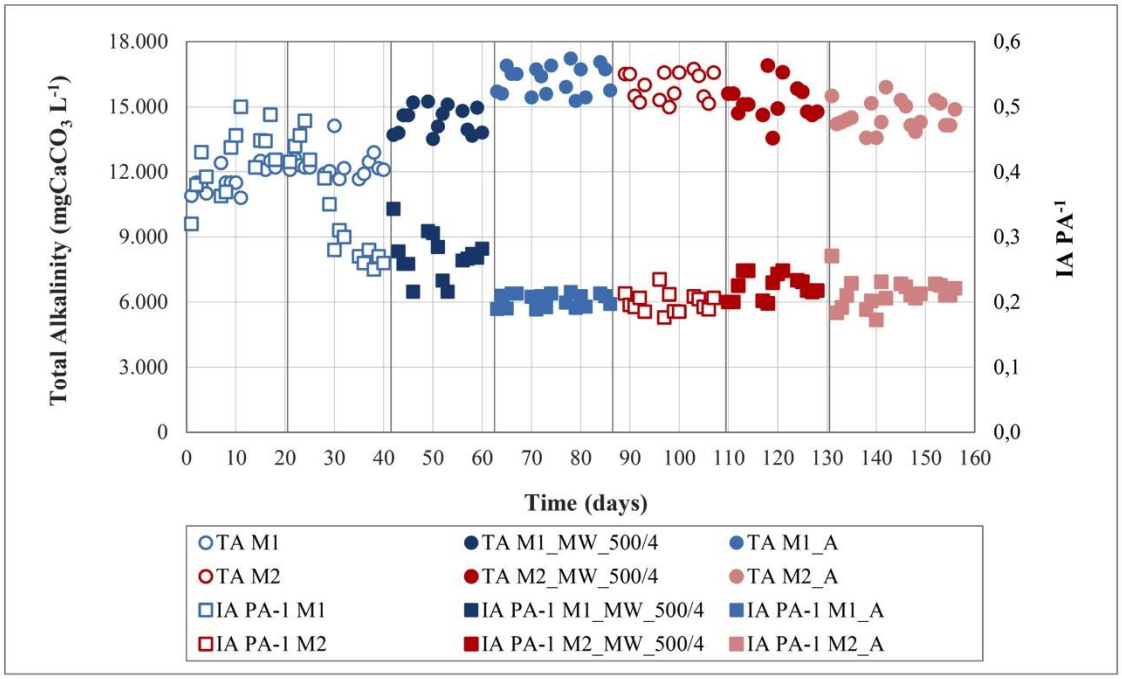


**Figure 13: Semi-continuous trials on substrate pretreatments - Process stability indicators over time: total alkalinity and IA $PA^{-1}$ ratio.**

pH assumed optimal values during the whole period ranging between 7.16 and 7.99 and no acidification process occurred (the process is considered steady when pH is approximately in the neutral range, between 6.5 and 8, APAT, 2005). The trend was found steadier after the acclimatization phase. The IA $PA^{-1}$ ratio ranged between 0.17-0.50. According to Martín-González et al. (2013) an IA $PA^{-1}$ ratio of below 0.3 is a useful indicator of stable performances of a digester

treating OFMSW with total VFAs between 2.5–3.5 mg $L^{-1}$ in a range of TA between 13,000 and 15,000 mg$CaCO_3$ $L^{-1}$. This statement was reached after 30 days leading to the conclusion that 20 days of acclimatization were not enough. This result suggests the use of longer period of time to test different scenarios.

**Table 13: Semi-continuous trials on substrate pretreatments - Process stability indicators. Values are expressed by averages and standard deviations (n = 3).**

| Parameter | M1 | M1_MW | M1_A |
|---|---|---|---|
| pH | 7.42 ± 0.36 | 7.60 ± 0.15 | 7.72 ± 0.11 |
| Total Alkalinity (mg$CaCO_3$ $L^{-1}$) | 12,280 ± 583 | 14,380 ± 615 | 16,240 ± 645 |
| IA $PA^{-1}$ | 0.34 ± 0.03 | 0.27 ± 0.03 | 0.20 ± 0.01 |
| Parameter | M2 | M2_MW | M2_A |
| pH | 7.73 ± 0.09 | 7.64 ± 0.08 | 7.68 ± 0.09 |
| Total Alkalinity (mg$CaCO_3$ $L^{-1}$) | 15,940 ± 640 | 15,220 ± 841 | 14,580 ± 657 |
| IA $PA^{-1}$ | 0.20 ± 0.01 | 0.22 ± 0.02 | 0.22 ± 0.02 |

### 2.3.3.2 Anaerobic performances

The average results of SGP, methane content, specific methane production (SMP) and $\eta_{TVS}$ obtained are reported in Table 14.

**Table 14: Semi-continuous trials on substrate pretreatments - Yields of the process. Values are expressed by averages and standard deviations (n = 3).**

| Parameter | M1 | M1_MW | M1_A |
|---|---|---|---|
| SGP (NL $kgTVS^{-1}$ $d^{-1}$) | 389.5 ± 50.1 | 463.6 ± 60.7 | 430.2 ± 80.2 |
| $CH_4$ (%) | 72.2 ± 3.2 | 72.0 ±3.2 | 70.9 ± 2.6 |
| SMP ($NLCH_4$ kgTVS $d^{-1}$) | 262.8 ± 74.0 | 310.4 ± 91.5 | 288.4 ± 78.5 |
| $\eta_{TVS}$ (%) | 65.6 ± 14.3 | 72.5 ± 10.2 | 80.3 ± 5.1 |
| Parameter | M2 | M2_MW | M2_A |
| SGP (NL $kgTVS^{-1}$ $d^{-1}$) | 222.4 ± 25.9 | 222.1 ± 21.5 | 242.3 ± 32.3 |
| $CH_4$ (%) | 67.6 ± 3.0 | 66.9 ± 3.9 | 67.7 ± 4.0 |
| SMP ($NLCH_4$ kgTVS $d^{-1}$) | 141.2 ± 41.9 | 138.7 ± 42.6 | 157.4 ± 41.2 |
| $\eta_{TVS}$ (%) | 56.2 ± 8.3 | 50.2 ± 14.8 | 82.4 ± 4.2 |

Similar to Batch_exp_2, results showed a better anaerobic performance for all M1 substrates compared to M2 substrates, which is attributable to the sample composition, more suitable for anaerobic bacteria characterized by a low content of fir sawdust (Table 3). As for SMP, it is related

to the specific gas production and its methane content, as it is the product between the two variables. Figure 14 and Figure 15 show the trends of SGP and methane content over time.

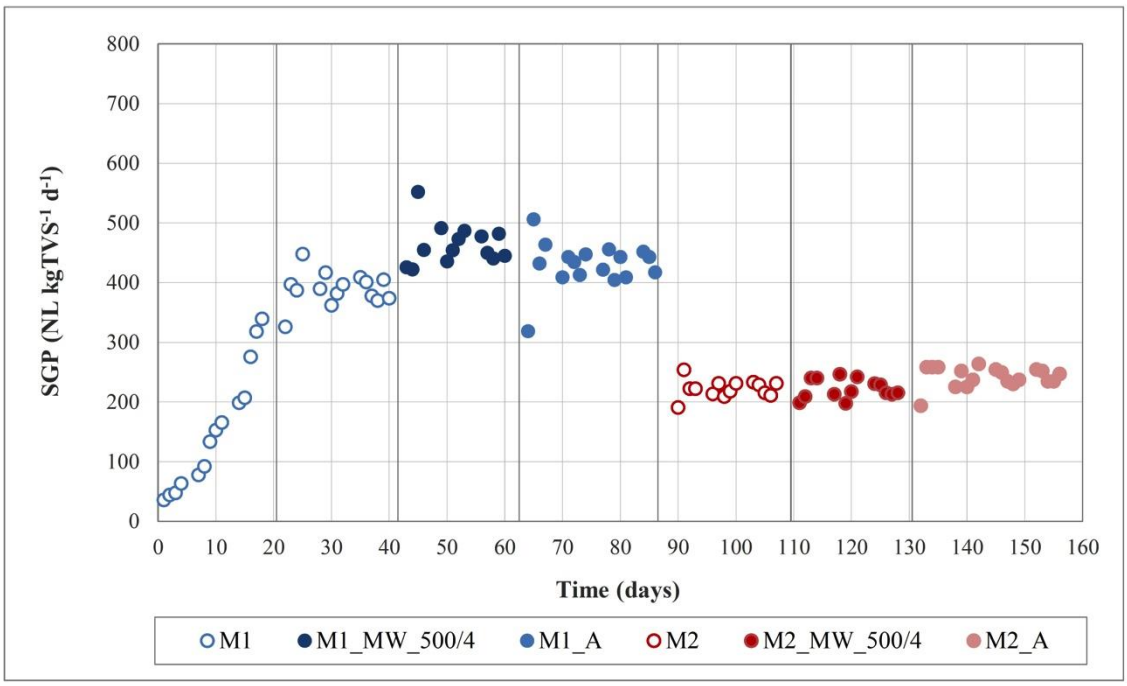


**Figure 14: Semi-continuous trials on substrate pretreatments – Specific gas production over time.**

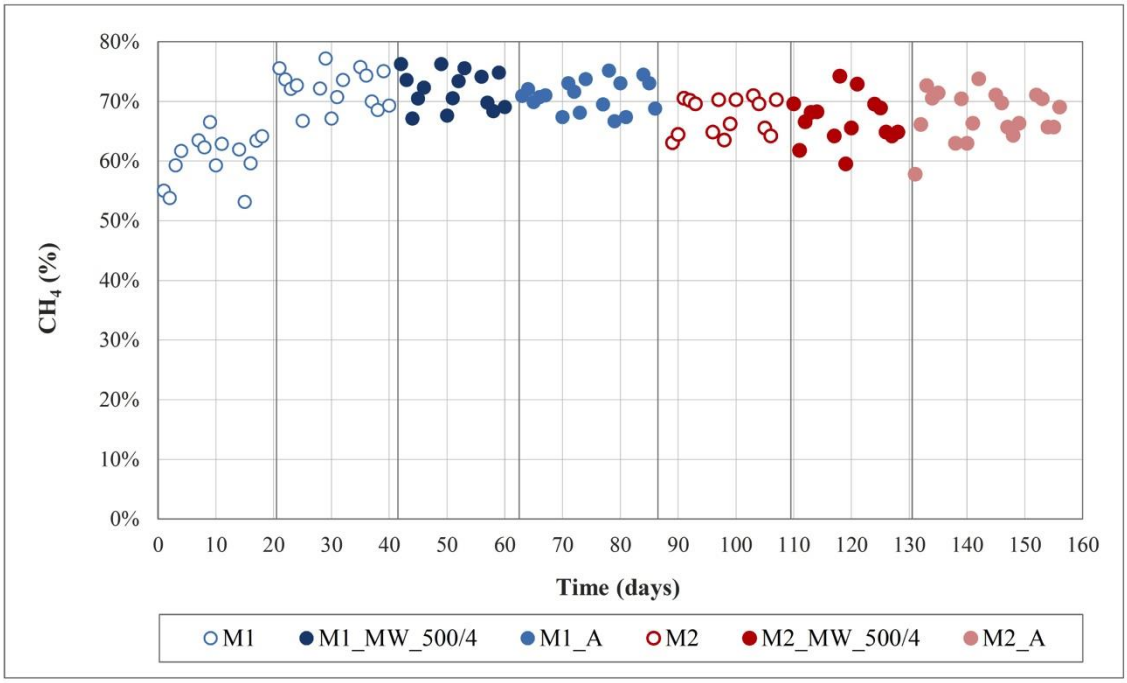


**Figure 15: Semi-continuous trials on substrate pretreatments – Methane content over time.**

SMP outcomes confirmed the pretreatments efficiency on methane production previously found with BMP assays, underlining that MW was more suitable for a meagre lignocellulosic substrate (M1) while A was more suitable for a richer lignocellulosic substrate (M2). Concerning MW,

while BMP tests showed a substantial equivalence of the application of the treatment on the two OFMSW (+ 8.5 for both samples), the adoption of semi-continuous trials confirmed the adequacy of MW on M1 (+18.1), while highlighted the unsuitableness for the substrate most rich in lignocellulosic content (M2, - 1.8%). As for autoclaving, the increases of methane production obtained in batch assays (+1.0% for M1 and +4.4% for M2) were amplified by performing semi-continuous trials, which observed SMP increases of approximately 9.8% and 11.5%, respectively for M1 and M2. This could be attributable to the optimal pH and alkalinity conditions observed inside the digester. The buffer capacity developed into the semi-continuous reactor was able to minimize acidification problems and total ammonia nitrogen loading. This result highlights the limitation of batch assays, which are rapid and low cost tools to assess the feasibility of the anaerobic process but they are not able to counteract problems that can occur during the experiment, thus often leading to underestimation of methane production.

As for the methane content, results observed a substantial equivalence of the average values between treated and untreated samples (Table 14). As such, the increase of SMP was totally ascribable to the increase in biogas production (SGP) rather in methane content, which is in agreement with previous works (Appels et al., 2013; Kuglarz et al., 2013; Rani et al., 2013). The percentage was typical of a healthy anaerobic process with values in the range of 57.8% and 77.2% during the steady state (APAT, 2005). The average values were found always higher than what reported for batch tests (Table 11), confirming the better response of semi-continuous trials than batch experiments.

Concerning the volatile solids removal efficiency, results reflected the outcomes obtained for SMP (Table 14). Microwaving highlighted an increase of the degradation of the volatile matter of approximately 10.5% for M1 while it resulted in a decrease of degradation for M2 (-10.6%). As for autoclaving, the increase of degradation was more evident for M2 (+11.1%) than for M1 (+7.2%). Figure 16 depicts the trend of $\eta_{TVS}$ over time.

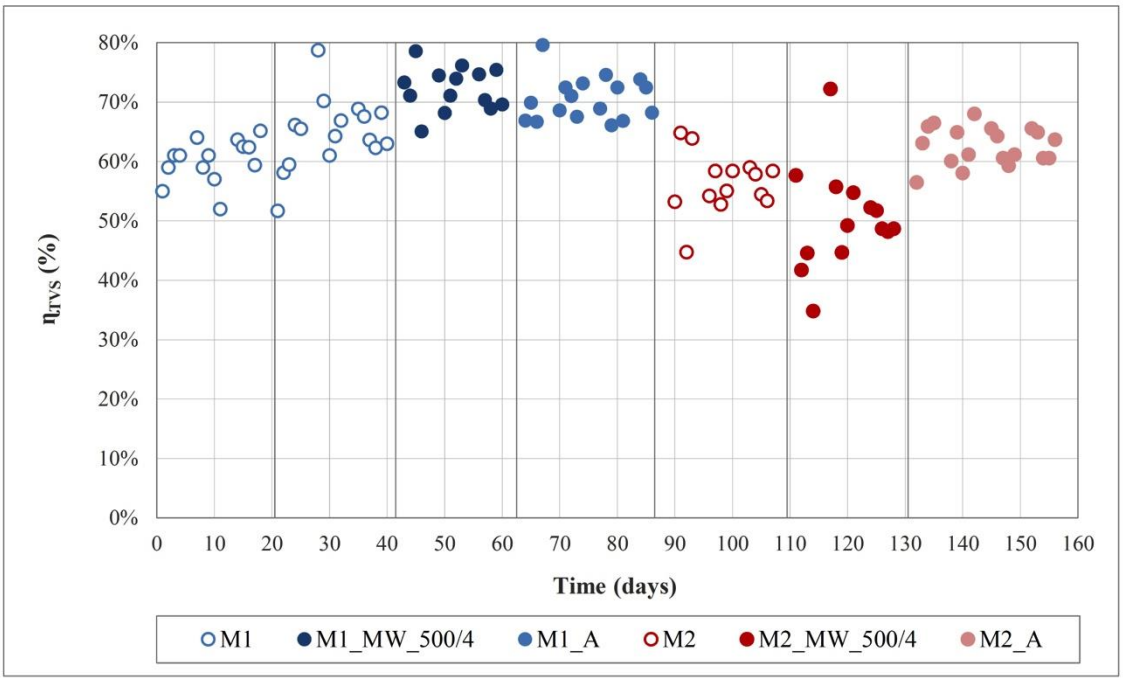


**Figure 16: Semi-continuous trials on substrate pretreatments – Volatile solids removal efficiency over time.**

### 2.3.3.3 Energy profit

Similar to batch tests, the energy balance was performed for semi-continuous trials. Table 15 highlights energy balance results and the comparison with the outcomes of Batch_exp_2. Likewise batch tests, the calculation proved that pretreatments were not energetically feasible. Nevertheless, with the exception of M2_MW_500/4, the higher increase of methane production determined a relevant improvement of the energy profit for semi-continuous trials. For instance, the best response was obtained for MW on M1 with a total energy of -338 kJ $kgTVS^{-1}$. The same calculation conducted with batch tests highlighted a result of -1,383 kJ $kgTVS^{-1}$. Despite the higher increase of methane production, the main source of energy remained $E_Q$. Therefore, the heat of the organic mass after the treatment is a relevant item to take advantage. By comparing the two treatments, it can be highlighted that MW resulted in a better energetic response than A mainly due to the lower energy demand, which is approximately the 30% less than A.

**Table 15: Semi-continuous trials on substrate pretreatments – Energy balance.**

| | $E_B$ (kJ $kgTVS^{-1}$) | $E_Q$ (kJ $kgTVS^{-1}$) | $E_D$ (kJ $kgTVS^{-1}$) | $E_T$ (kJ $kgTVS^{-1}$) | $E_T$ – Batch (kJ $kgTVS^{-1}$) |
|---|---|---|---|---|---|
| M1_MW_500/4 | 1,534 | 2,724 | - 4,596 | - 338 | - 1,383 |
| M1_A | 825 | 4,040 | - 7,156 | - 2,323 | - 3,061 |
| M2_MW_500/4 | - 81 | 2,457 | - 4,172 | - 1,796 | - 1,402 |
| M2_A | 522 | 3,650 | - 6,496 | - 2,291 | - 2,575 |

## 2.4 Conclusions

The first line of research investigated the application of autoclaving and microwaving on lignocellulosic OFMSW aiming at verifying their effect on the anaerobic process. Batch tests were carried out as preliminary tools to obtain the best treatment conditions to be successively applied in semi-continuous mode. Therefore, microwaving was studied by varying the duration and the amount of substrate subject to the treatment and the lignocellulosic content of the substrate whereas autoclaving was investigated only in this latter feature. Finally, the feasibility of treatments on the AD was assessed through biogas production, biogas quality (methane content), volatile matter degradation and energy profit (Table 16).

The first set of batch experiments (Batch_exp_1) performed with microwaving observed that, even if the most severe treatment ($E_D$ = 20,404 kJ $kgTVS^{-1}$) obtained the best results with a methane production improvement of approximately 32.7%, the best energetic response was found for the lighter treatment ($E_D$ = 5,101 kJ $kgTVS^{-1}$: 4 minutes on a substrate sample of 0.5 kg). Therefore, this condition was further applied on the second set of batch experiments and on semi-continuous trials.

The second set of batch experiments (Batch_exp_2) tested the application of microwaving and autoclaving on two samples of OFMSW with different lignocellulosic content. This experimental campaign highlighted a better response in terms of methane production, macromolecules solubilisation and energy balance of microwaving on a substrate with low lignocellulosic content (M1) and autoclaving on a substrate rich in fibres (M2). The increase in methane production was found directly related to the release of soluble COD after the treatment. Nevertheless, the significant increase in soluble COD did not correspond with an equivalent increase in methane production leading to the conclusion that a relevant part of the soluble COD was not readily biodegradable. As for the energy balance, no energy profit was registered. This was mainly due to the low increase in biogas production compared to raw substrate digestion and to laboratory scale conditions. The amount of energy recovered in the form of biogas ($E_B$) and heat ($E_Q$) was not enough to overcome the energy demand of the treatment ($E_D$).

The same conditions tested in Batch_exp_2 were replied in semi-continuous trials. Table 16 summarises the improvement of the anaerobic performances obtained for microwaving with M1 and autoclaving with M2. The adoption of semi-continuous trials amplified the methane and biogas production increases found in batch tests. Therefore, SMP observed an increase of 18.1% adopting MW on M1, and 11.5% adopting A on M2. This could be attributable to the optimal pH and

alkalinity conditions observed inside the digester. The buffer capacity developed into the semi-continuous reactor was able to minimize acidification problems and total ammonia nitrogen loadings. This result highlights the limitation of batch assays, which are rapid and low cost tools to assess the feasibility of the anaerobic process but they are not able to counteract problems that can occur during the experiment, thus often leading to underestimation of methane production. Concerning the volatile solids removal efficiency, results reflected the outcomes obtained for the SMP. Microwaving highlighted an increase of the degradation of the volatile matter of approximately 10.5% for M1. Similarly, an increase of about 11.1% was obtained for autoclaving on M2. As for the energy balance, thanks to the higher increase of methane production, the energy profit observed a relevant improvement for semi-continuous trials compared to batch tests. Nevertheless, the balance was found negative. The improvement of the anaerobic performances did not counteract the energy expended for the treatments. Among the forms of produced energy, $E_Q$ was the prevalent. The heat of the organic mass after the treatment is a relevant item to take advantage. By comparing the two treatments, it can be highlighted that MW resulted in a better energetic response than A mainly due to the lower energy demand, which was approximately 30% less than A. Having regard the improvement observed by passing from batch tests to semi-continuous trials, a scale factor can play a determinant role also in the transfer at industrial scale. Therefore, further tests are required on pilot scale with larger reactor volumes in order to assess a realer energy feasibility.

The main outcome of this study is the identification of the better application of microwaving (using light conditions: 4 min, 0.5 kg) on a OFMSW with low lignocellulosic content and autoclaving on a substrate rich in fibres.

**Table 16: Summary of the best results obtained employing microwaving and autoclaving on OFMSW.**

| Parameter | Microwaving | Autoclaving |
|---|---|---|
| Duration (min) | 4 | 15+30 |
| Amount of substrate (kg) | 0.5 | 1.7 |
| Optimal substrate (% fibres w/w) | M1 (11% fibres) | M2 (25% fibres) |
| Biogas production increase – SGP (%) | +19.0% | +8.9% |
| Methane production increase - SMP (%) | +18.1% | +11.5% |
| Volatile matter degradation increase - $\eta_{TVS}$ (%) | +10.5% | +11.1% |
| Energy balance (kJ $kgTVS^{-1}$) | - 338 | - 2,291 |

# CHAPTER 3 - TWO-STAGE ANAEROBIC DIGESTION

## 3.1 Introduction

With the aim of improving AD, the scientific community has recently increased its interest in the study of biological hydrogen production during the acidogenic phase, commonly known as Dark Fermentation (DF). Hydrogen is considered the fuel of the future owing to its high-energy content and environmentally friendly production (Ghimire et al., 2015). As a matter of fact, hydrogen is characterized by the highest energy content per unit mass (122 kJ $g^{-1}$) and no greenhouse gases emissions when combusted (Khan et al., 2016). Such potential benefits are further enhanced if hydrogen is produced from biodegradable wastes (Cappai et al., 2014). Among them, FW appears to be a promising feedstock due to its relevant carbohydrates content, as well as wide availability (De Gioannis et al., 2013; Cappai et al., 2014).

Despite the full-blown benefits, an efficient hydrogen production is closely dependent on a complex microbial system influenced by several parameters such as substrate type, substrate loading, hydraulic retention time, pH, temperature, inoculum pretreatment and inoculum type, to mention just the major ones.

The following introductive paragraph provides an insight on the factors that influence the biochemical process of hydrogen production.

### 3.1.1 Biochemical pathways

In DF, carbohydrate-rich substrates are broken down anaerobically by hydrogen-producing bacteria (HPB). Figure 17 provides a schematic representation of the different steps and biochemical pathways involved.

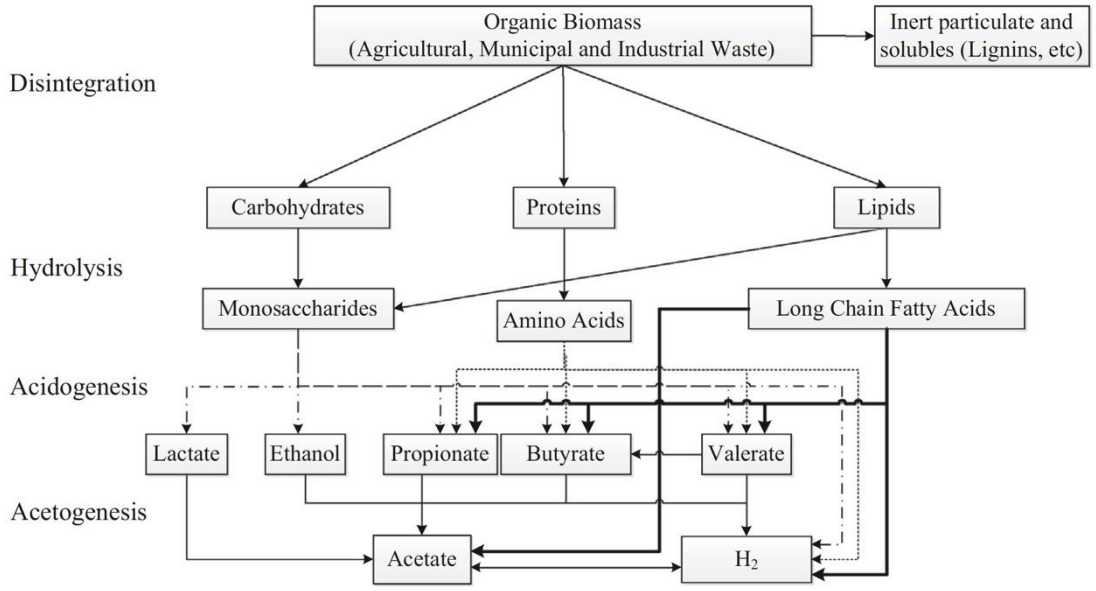


**Figure 17: Biodegradation pathway involved in fermentative hydrogen production (Ghimire et al., 2015).**

As shown in Figure 17, the DF of the organic biomass is a complex process, which results in a wide range of intermediates and by-products. Referring to a model substrate as an hexose (e.g.: glucose), the biochemical reactions undertaken by fermentative bacteria are mainly two (Ghimire et al., 2015). When the metabolic pathway is such that it favours the production of acetic acid, the stoichiometric yield of $H_2$ is 4 mol for a mole of glucose (Reaction 1), whereas the yield of $H_2$ is 2 mol for a mole of glucose when the final product is butyric acid (Reaction 2):

Reaction 1: $C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$

Reaction 2: $C_6H_{12}O_6 \rightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$

Nevertheless, the actual hydrogen yield is lower than the theoretical yield as part of the substrate is used for biomass production and substrate degradation might follow other biochemical pathways without hydrogen production (De Gioannis et al., 2013). For instance, the metabolic pathways can lead to ethanol and acetate production, lowering the stoichiometric hydrogen yield to 2 mol of $H_2$ for a mole of glucose (Reaction 3). Similarly, other fermentation pathways involve the direct formation of propionate (Reaction 4), ethanol (Reaction 5) and lactate (Reaction 6), which are hydrogen-consuming ways:

Reaction 3: $C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2OH + CH_3COOH + 2CO_2 + 2H_2$

Reaction 4: $C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$

Reaction 5: $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$

Reaction 6: $C_6H_{12}O_6 + \rightarrow CH_3CHOHCOOH + 2CO_2$

### 3.1.2 Type of substrate

The substrate plays an important role in the $H_2$ yield, $H_2$ production rate and the overall economy of the process. These are mainly dependent on the substrate's composition, bioavailability and biodegradation rate (Alibardi and Cossu, 2016; De Gioannis et al., 2013). Concerning the composition, Alibardi and Cossu (2016) found a linear correlation between hydrogen production and the carbohydrate content of organic waste while proteins and lipids did not produce significant contributions. Conversely, low hydrogen yields resulting from the fermentation of lignocellulosic substrates are related to partial and incomplete hydrolysis and digestibility of cellulose (Hendriks and Zeeman, 2009). Carbohydrate-rich substrates have been extensively used in DF studies, in particular pure glucose, sucrose and starch mixtures (Chen et al., 2006; Lin and Lay, 2004; Dellosso Penteado, 2013). Nevertheless, with the integration of DF within a biorefinery concept, the substrate has to come from renewable resources (Cappai et al., 2014). Second generation biomass sources, such as organic waste and sludge, are abundant and can thus support the supply of renewable substrates for DF.

The OFMSW is generally composed by a significant amount of FW, which is a carbohydrate-rich substrate making it an optimal substrate for DF and leading to its adoption in a wide number of works (Lee et al., 2010; Luo et al., 2011; Voelklein et al., 2016; De Gioannis et al., 2017; Lee and Chung, 2010; Chu et al., 2010; Cavinato et al., 2012; Chinellato et al., 2013; Micolucci et al., 2014; Yeshanew et al., 2016).

Co-digestion of sludge and FW is a valuable solution to improve the digestion efficiency and increase the energy output using the spare digestion capacity at WWTPs (Iacovidou et al., 2012; Appels et al., 2011). Both substrates can provide a positive contribution to the anaerobic process. FW provides essential carbon necessary for the improvement of digestion performance (Iacovidou et al., 2012). Conversely, sludge are protein-rich substrates whose anaerobic degradation releases hydroxide and ammonia ions (Jung et al., 2011). Optimal levels of ammonia ions (up to 200 mg $L^{-1}$) ensure adequate supply of nitrogen as nutrient substance for anaerobic biomass and together with hydroxide ions increase system's buffer capacity, counteracting

acidification lead by VFA production and thus helping to guaranteeing the stability of the process (Polizzi et al., 2018).

On this basis, the present research focuses on the study of hydrogen production from the co-digestion of AS and FW.

### 3.1.3 Type of inoculum

The hydrogen producing seed inoculum is a crucial element for the start-up and the proper operation of the DF process. Regarding the microorganism's species, Clostridia have been identified as the dominant HPB in DF. Fang et al. (2002) identified that 64.6% of all the microorganisms were affiliated with Clostridium species, 18.8% with Enterobacter and 3.1% with Streptococcus. In addition, the presence of Escherichia Coli can aid in increasing the $H_2$ yield by diverting the metabolic pathways to the acetate and butyrate hydrogen producing pathways (Reaction 1 and Reaction 2). With regard to Clostridia, several works found *Clostridium Perfringens sp*. to be the most dominant hydrogen producing specie (Huang et al., 2010; Wong et al., 2018; Sivagurunathan et al., 2014; Jame et al., 2016) while others adopted them as inoculum when evaluating hydrogen production from a pure culture (Wang et al., 2011; Zhi and Wang, 2014). On the other hand, other undesirable bacteria reduce the total $H_2$ yield either by consuming the $H_2$ produced or by altering the biochemical pathways of the $H_2$ synthesis (Fang, 2007). The main hydrogen-consuming bacteria (HCB) include methanogens, homoacetogenic bacteria and sulfate reducing bacteria.

On this basis, several studies use pure hydrogen producing cultures to avoid the presence of HCB (Huang et al., 2010; Wang et al., 2011; Wong et al., 2018, Sivagurunathan et al., 2014; Jame et al., 2016).

Nevertheless, since in full scale digesters bacteria coexist in a wide range of species and the fermentation of substrates into biohydrogen involves a wide consortium of microorganisms (Park et al., 2005), the use of mixed cultures belonging to operative facilities is mainly preferred by most authors (Alibardi and Cossu, 2015; Alibardi and Cossu, 2016; Chinellato et al., 2013; Favaro et al., 2013; Lavagnolo et al., 2015; Pan et al., 2008; Akhlagi et al., 2017; De Gioannis et al., 2017; Cappai et al., 2014). Moreover, using mixed-microorganism media is more functional and economically advantageous than using unique microbial populations. In this context, BHP tests reported in literature are currently being performed with different types of mixed cultures. Several works use anaerobic sludge from anaerobic digesters (Alibardi and Cossu, 2015; Alibardi and Cossu, 2016; Chinellato et al., 2013; Favaro et al., 2013; Lavagnolo et al., 2015; Pan et al.,

2008) while others use activated sludge (Akhlagi et al., 2017; De Gioannis et al., 2017; Cappai et al., 2014).

### 3.1.4 Treatment of inoculum

The activity of the HCB when using mixed cultures can be controlled by inoculum pretreatment methods or bioreactor operating conditions (Alibardi and Cossu, 2015; Wang and Wan, 2009).

Inoculum pretreatment is important in batch tests or at process start-up. This method relies on the spore forming characteristics of $H_2$ producers such as Clostridia. These organisms have a better chance to survive the harsh conditions during the pretreatment of the inoculum than the non-spore forming bacteria such as methanogens, as the spores can germinate again under favourable conditions (Li and Fang, 2007). Heat treatment of mixed cultures for the enrichment of HPB is a simple and effective method (Li and Fang, 2007). The sludge is treated at 80-105 °C (Alibardi and Cossu, 2016; Akhlagi et al., 2017) for a period of time ranging from 15 min. (Alibardi and Cossu, 2016) to 4 h (Alibardi and Cossu, 2015). Similarly, acid treatment is based on the notion that the activity of methanogens drops sharply at a pH below 6.5 or above 8 (Li and Fang, 2007), while the activity of HPB is not affected by an acidic pH (below pH 6.5). Other pretreatment methods are chemical pretreatment and aeration. Therefore, oxygen can inhibit methanogens activity during aeration (Wang and Wan, 2008) aerated the inoculum sludge with air for 24 h to inhibit the activity of methanogens. Likewise, chemical like sodium 2-bromoethasulfonic acid, iodopropane, chloroform and acetylene are used to inhibit methanogens (Fang, 2007; Ghimire et al., 2015).

Unlike inoculum pretreatments, proper bioreactor operating conditions (mainly pH, HRT, OLR) are required to select a stable fermentative microflora when reactors are already working. Fermentative pH, HRT and OLR are lately discussed in sections 3.1.6and 3.1.7, respectively.

### 3.1.5 Temperature

The $H_2$ yields depend on temperature as it affects the hydrolysis rate (Shin et al., 2004; Valdez-vazquez et al., 2005). Similar to AD, temperature can affect the metabolic pathways of DF, thus shifting the composition of the by-products. Mesophilic and thermophilic temperatures have been studied to determine their effect on the biohydrogen production. Valdez-vazquez et al. (2005) reported higher $H_2$ yields for thermophilic fermentation than in the mesophilic temperature range

using organic waste as substrate. In addition, acetic acid was a dominant by-product in thermophilic digestion, whereas butyrate was formed in a higher proportion during mesophilic digestion. Similarly, results of the extreme thermophilic (70 °C) DF of household organic waste also showed acetic acid as the dominant by-product in DF tests conducted at pH 7 (Liu et al., 2008). In contrast, Shin et al. (2004) showed acetate as major end-product at mesophilic culture while butyrate levels and hydrogen production were higher by the thermophilic culture, obtained in DF of OFMSW carried at pH 5.5. In another study, Wang and Wan (2011) found the maximum substrate degradation efficiency, maximum $H_2$ yield and production rate at 37.8 °C in DF of glucose. In conclusion, the difference between the optimum operational temperatures is due to the difference in the fraction of easily biodegradable compounds present in the substrate. Complex materials rich in lignocellulosic compounds are better degraded using high temperatures whereas easily biodegradable substrates such as FW or monomers are well degraded even under mesophilic conditions.

### 3.1.6 pH

pH is recognized as being a crucial parameter for the fermentation process as it affects the degree of substrate hydrolysis, the activity of hydrogenase, the efficiency of energy utilization by the microbial cells as well as the metabolic pathways (Kim et al., 2011). Hydrogen production is maximised at operating pH values from 5 up to 6.5 (Cappai et al., 2014; De Gioannis et al., 2014; Moon et al., 2015). Under these conditions the acetate and butyrate pathways are predominant (Reaction 1 and Reaction 2). Conversely, high acidity or basicity negatively affect the activity of hydrogen-producing bacteria, since under such conditions ATP is used to ensure cell neutrality rather than to produce hydrogen (Nazlina et al., 2011). At values below 5, hydrogenase activity is inhibited and non-hydrogen producing pathways, including solventogenesis and lactate production, take over (Micolucci et al., 2014; Nazlina et al., 2011).

As a result, maintaining pH within the suitable range for hydrogen production is crucial. Several studies have focused on the effect of the initial pH of the feeding mixture (Argun et al., 2008; Bao et al., 2013; Giordano et al., 2011; Ramos et al., 2012; Xiao et al., 2013; Zhou et al., 2013). In most cases, pH is adjusted using NaOH or HCl with no further control during the test (Giordano et al., 2011; Ramos et al., 2012; Zhou et al., 2013). The major constraint is the pH decrease caused by the acidogenic reactions which may lead to the inhibition of the hydrogenase activity (Bao et al., 2013; Giordano et al., 2011; Ramos et al., 2012; Xiao et al., 2013; Zhou et al., 2013). Bao et al. (2013) and Xiao et al. (2013), who adopted initial pHs of 7 and 8, observed

an inhibition of hydrogen production due to an excessive acidification of the system. Similar constraints were observed by Argun et al. (2008), who adopted a pH adjustment strategy through intermittent addition of NaOH to counteract the pH drop observed in the early stages of the experiment. With the same purpose, recent studies adopted pH control methods based on the addition of buffers or alkaline solutions, including 2-(N-morpholino)ethanesulfonic acid (MES; Alibardi and Cossu, 2015; Alibardi and Cossu, 2016; Favaro et al., 2013; Lavagnolo et al., 2018), phosphate (Favaro et al., 2013) or carbonate (Lavagnolo et al., 2018) solutions to maintain acid, neutral and basic conditions, respectively. Other investigators (Cappai et al., 2014; De Gioannis et al., 2017; Akhlaghi et al., 2017) adopted a continuous pH control strategy through automatic addition of a NaOH solution. The pH control method adopted is expected to affect the hydrogen production yield, as even relatively small pH fluctuations during the process are recognized to influence the activity of the hydrogenogenic biomass. In the case of two-stage system with a fermentative reactor followed by a methanogenic one, besides the use of a chemical control of pH, in recent years a less expensive alternative has been developed. It consists in applying a recirculation to the head to the process from the stage of methanogenesis in order to exploit the residual buffer capacity (ammonia and bicarbonate) of the methanogenic digestate (Chinellato et al., 2013; Micolucci et al., 2014). Moreover, the application of a recirculation flow allows balancing the nutrients intake and helps dilute the feedstock (Lee et al., 2010). Therefore, also from an economical point of view, it is convenient to develop a pH control system, which allows managing the process in a sustainable approach, because neither chemical addition nor high costs devices would have to be used to reach the target. Nevertheless, considering a long-term management of the process, the continuous recirculation of the methanogenic digestate can lead to an accumulation of ammonia, which is strongly inhibitive above 1,500 mg $L^{-1}$ for both the stages (APAT, 2005; Cavinato et al., 2012; Salerno et al., 2006).

### 3.1.7 HRT and OLR

HRT and OLR are closely related to each other and defining a specific value for either one actually depends on both. In fermentative hydrogen production, the HRT and the OLR, affect substrate hydrolysis and thus the production of intermediates and products, affecting fermentative $H_2$ production. The influence of HRT and OLR on hydrogen yield is controversial in the literature.

It is generally acknowledged that long HRTs favour the build-up of HCB, such as methanogens. The different growth rates of HPB and HCB make it possible to use the HRT as a

controlling parameter to inhibit the activity of HCB. More specifically, it has been reported that low HRTs favour hydrogen production as the methanogens are washed out, and hydrogen production increases as the HRT decreases (Kim et al., 2006; Liu et al., 2008). On the other hand, too low HRTs may reduce the substrate utilization efficiency, in particular in the case of complex substrates, which need an adequate hydrolysis period, and cause the washout of the active biomass, in turn impairing the conversion yield.

Concerning the OLR, a microbial culture can shift from substrate-limited to substrate-sufficient growth depending on the relative availability of substrate and biomass, thus affecting the production of hydrogen. Differently, the operation at high loads of substrate involves an accumulation of VFAs that can lead to the inhibition of the hydrogenase activity (Micolucci et al., 2014).

In this context, the optimum HRT for biohydrogen production in DF closely depends on the type of substrate used as the hydrolysis rate depends on the biodegradability of the substrate (Ghimire et al., 2015). Concerning FW, most studies that used stirred reactors with continuous or semi-continuous operation adopted HRT values between 21 h and 4 d. The related OLR values fall within the ranges 8–38 kgTVS $m_r^{-3}$ $d^{-1}$ (Lee and Chung, 2010; Hong and Haiyun, 2010; Chu et al., 2008; Lee et al., 2010; Chinellato et al., 2013; Cavinato et al., 2012; Yeshanew et al., 2016).

### 3.1.8 Use of by-products: the two-stage technology

The low process yield and the incomplete conversion of organic biomass are two major bottlenecks for commercial dark fermentative biohydrogen production (Ghimire et al., 2015). DF residues mainly contain VFAs, major by-products of the DF process, which need to be further used to achieve a complete conversion of the organic biomass.

Under this perspective, AD can be considered the final stage to stabilize the residues of DF. The two-stage technology considers the separation of the traditional one-stage AD into a two-stage process equipped with a fermentative reactor and a methanogenic reactor. While the first stage produces $H_2$ and $CO_2$ as gaseous products and releases VFAs into the liquid solution, the second one converts VFAs and the residual organic biodegradable matter into $CH_4$ and $CO_2$ (De Gioannis et al., 2013; Ghimire et al., 2015, Figure 18). Therefore, in this context, the role of the fermentative reactor is twofold: producing a hydrogen-rich biogas and acting as a pretreatment for the methanogenic reactor. Indeed, by degrading the macro-polymers, fermentative bacteria make the substrate more easily accessible to the methanogens, thus improving methane production in the second reactor (Lee et al., 2010; Luo et al., 2011; Voelklein et al., 2106; De

Gioannis et al., 2017). The multiple advantages of this technology include a better degradation of the organic matter and an overall increase in the energy output by generating two gases with high combustion power (Liu et al., 2013).

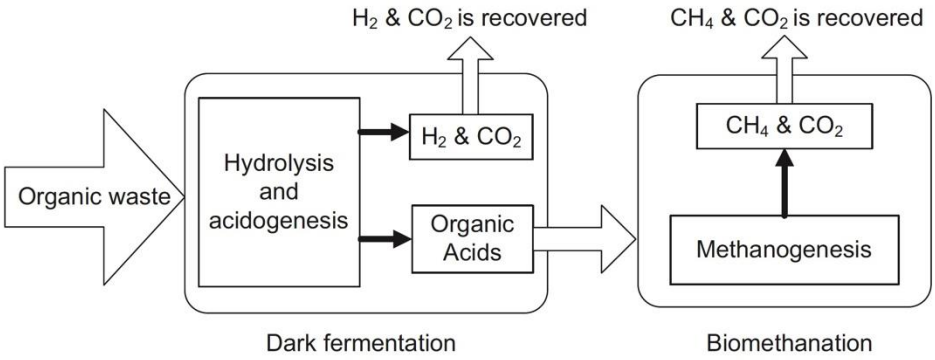


Figure 18: Two-stage technology (Ghimire et al., 2015).

Table 17 summarizes the optimal conditions for DF hydrogen production, previously discussed.

**Table 17: Optimal condition of the DF stage.**

| Parameters | Optimal conditions |
|---|---|
| Type of substrate | Carbohydrate-rich |
| Type of inoculum | Clostridia-rich |
| Treatment of inoculum | Heating: 80-105°C, 15-240 min. |
| Temperature (°C) | 37 – 55 |
| pH | 5 – 6.5 |
| HRT (d) | 1 - 4 |
| OLR (kgTVS $m_r^{-3}$ $d^{-1}$) | 8 - 38 |

## 3.1.9 Objective of the research

Owing to the heterogeneity of the literature data, the present research aims at shedding light on some of these parameters (inoculum type, pH, substrate type and substrate loading) in order to determine the best conditions for the fermentation process. The study was firstly conducted using FW of the OFMSW. Subsequently, hydrogen production was assessed on the co-digestion of FW and AS, which to the author's knowledge has never been tested before.

## 3.2 Materials and methods

The materials and methods section presents the substrates and the inocula used in the study, the experimental design, the experimental set-up (batch and semi-continuous tests), the calculations performed for the energy balance and for the statistical analysis, the description of the analytical methods and the calibration of the laboratory equipment.

### 3.2.1 Substrates and inocula

#### 3.2.1.1 Substrates

Two different samples of FW were used during the experimentation due to the long duration of the research (2 years) and the biodegradability of the substrates. Concerning the co-digestion trials, 200 L of activated sludge (AS) were collected from the aerobic unit of the municipal WWTP of Viareggio (Italy). The sample of AS was stored in plastic tanks and kept under refrigeration at 4°C until use. Each sample of FW was manually sorted from source-separated OFMSW collected in Viareggio (Italy) by means of a kerbside collection system. As for the batch tests, the sample was immediately treated aiming at obtaining mashes with a dry matter content of 5% by weight. More specifically, it was shredded in a food processor (Problend 6, Philips, Netherlands) and diluted with tap water. Finally, the obtained mash (FW1) was stored at -20 °C until use. As for semi-continuous trials, FW was initially treated with the same procedure of FW1 in order to obtain a homogenised slurry with a dry matter content of approximately 20% by weight that can be easily stored in plastic vessels at -20 °C (FW2). A sample of approximately 200 mL of FW2 was daily removed from storage conditions and mixed with additional tap water or AS for the digestion (Semi-continuous_exp1) and the co-digestion trials (Semi-continuous_exp2), respectively. The mixtures were finally treated to obtain mashes with a dry matter content of 5% by weight. The ratio FW2:AS was approximately 1:5 by weight. A detailed description of the substrates is presented in Table 19.

#### 3.2.1.2 Inocula

Four different types of inocula were adopted to perform fermentative tests:

- IN1: activated sludge collected from the aerobic unit of a municipal WWTP;
- IN2: digested sludge collected from an anaerobic reactor treating the organic fraction of municipal solid waste (OFMSW) and cattle manure;

- IN3: digested sludge collected from an anaerobic reactor treating agro-industrial residues;
- IN4 digested sludge collected from an anaerobic reactor of a municipal WWTP.

Fermentative inocula were heat treated at 80°C for 30 minutes prior to the tests with the aim of selecting only HPB while inhibiting hydrogenotrophic methanogens (Alibardi and Cossu, 2016; Cappai et al., 2014). The treatment was performed in 250 mL beakers placed in a static oven (UM200, Memmert GmbH, Germany). The temperature of the medium was continuously measured with a digital tip thermometer (T1, Testo S.p.A., Italy). Treatment time started when the temperature of the medium reached 80°C. After 30 minutes, the inocula were removed from the oven and cooled down to ambient air temperature. Tests were carried out when the inoculum temperature reached mesophilic conditions.

IN2 was also used as inoculum for the methanogenic reactor.

A detailed description of the substrates is presented in Table 19.

**Figure 19: Samples of active sludge and FW1.**

**Figure 20: Samples of inoculum after heating.**

### 3.2.2 Design Of the Experiment (DOE)

The experimental design was planned in order to assess the performance of the two-stage technology in comparison with the traditional one-stage AD. Also for this line of research, the organization of the tests followed a scale up strategy. Batch tests played the role of preliminary experiments where process parameters were varied in order to find the optimum condition to be tested on semi-continuous trials (Table 18).

Batch assays were performed in two series of analyses: Batch_exp_1 and Batch_exp_2. The former was carried out aiming at identifying the best type of inoculum for the start-up of fermentative assays (Pecorini et al., 2019). Batch_exp_2 studied the influence of pH and Food-to-Microorganism ratio (F/M) (Baldi et al., 2019a). Both investigations considered the study of the best strategy to control pH: by using of an initial buffer solution (BHP1 assays) or by the automatic addition of an alkaline solution (BHP2 assays). The results of batch experiments were useful to perform proper semi-continuous trials (Baldi et al., 2019b). Therefore, the digestion of FW and the co-digestion of FW and AS were lately carried out in semi-continuous trials. The former tested a unique value of fermentative HRT (3 d) while the latter studied two HRT (3 d and 1.5 d).

The first set of batch tests was carried out with a pH of 5.5 and a F/M of 1/1 w/w according to previous studies (Alibardi and Cossu, 2015; Chinellato et al., 2013; Favaro et al., 2013). Nevertheless, other works obtained good results testing different values of pH and substrate loadings (Akhlagi et al., 2017; Lavagnolo et al., 2018; Ramos et al., 2012), thus suggesting the necessity of a further analysis (Batch_exp_2). In Batch_exp_1, hydrogen production was also evaluated from sucrose since it is an easy biodegradable feed adopted in several researches as standard substrate (Chen et al., 2006; Lin and Lay, 2004; Dellosso Penteado, 2013).

In addition, an analytical characterization of the inocula and the substrates was performed to provide a detailed description of the media and to assess the extent of potential inhibitory compounds.

**Table 18: Design of the experiment for the two-stage technology research.**

| Test | Set-up | Inoculum | Substrate | pH | F/M (w/w – gTVS gTVS$^{-1}$) |
|---|---|---|---|---|---|
| Batch_exp_1 | BHP1 | IN1 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN2 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN3 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN4 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN1 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN2 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN3 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN4 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN1 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN2 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN3 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN4 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN1 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN2 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN3 | Sucrose | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN4 | Sucrose | 5.5 | 1/1 – 4.0 |
| Batch_exp_2 | BHP1 | IN1 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP1 | IN1 | FW1 | 5.5 | 1/3 – 1.3 |
| | BHP1 | IN1 | FW1 | 6.5 | 1/1 – 4.0 |
| | BHP1 | IN1 | FW1 | 6.5 | 1/3 – 1.3 |
| | BHP2 | IN1 | FW1 | 5.5 | 1/1 – 4.0 |
| | BHP2 | IN1 | FW1 | 5.5 | 1/3 – 1.3 |
| | BHP2 | IN1 | FW1 | 6.5 | 1/1 – 4.0 |
| | BHP2 | IN1 | FW1 | 6.5 | 1/3 – 1.3 |
| Test | Configuration | | Substrate | HRT (d) | OLR (kgTVS $m_r^{-3}d^{-1}$) |
| Semi-continuous trials | S1: one-stage (AD) | | FW2 | 17** | 2.5** |
| | S2: two-stages (DF+AD) | | FW2 | 3* – 12.8** | 14.2* - 2.5** |
| | S1: one-stage (AD) | | FW2+AS | 17** | 2.5** |
| | S2: two-stages (DF+AD) | | FW2+AS | 3* – 11.9** | 14.6* - 2.5** |
| | S3: two-stages (DF+AD) | | FW2+AS | 1.5* – 11.9** | 27.6* - 2.5** |

*: it refers to the fermentative reactor (DF)
**: it refers to the methanogenic reactor (AD)

### 3.2.3 Batch assays

BHP tests were performed with two different methods. Assays were carried out without (BHP1) and with (BHP2) automatic pH control. Each test lasted 48 h. Temperature was constantly kept at mesophilic conditions (37.0 ± 0.1 °C). The SHP was determined as the cumulate hydrogen production at normal conditions per kg of volatile matter of substrate added ($NLH_2$ $kgTVS^{-1}$).

#### 3.2.3.1 BHP1

Tests were based upon the methodology reported by previous studies (Alibardi and Cossu, 2015; Alibardi and Cossu, 2016; Favaro et al., 2013; Lavagnolo et al., 2018) that involves the use of an initial buffer solution to control pH during the test. Assays were carried out in triplicate using 1 L stainless steel bottles (Pecorini et al., 2016, Figure 21).

**Figure 21: BHP1 – Pressure sampling and biogas collection.**

The reactors were placed on a hot plate magnetic stirrer and incubated in a water bath. The working volume of the reactor was roughly 0.5 L comprising substrate, inoculum, MES (2-N-Morpholino-EthaneSulfonic acid, VWR, Italy) buffer solution and HCl 2.5M to set the initial pH at the desired value. Before the start of the experiment, the reactors were flushed with nitrogen for a few minutes to guarantee anaerobic conditions. Biogas generation was regularly appraised by measuring the pressure in the headspace of each bottle by means of a membrane pressure gauge (HD2304.0, Delta Ohm S.r.L., Italy). Pressure data were then converted to volume using the ideal gas law (Eq. 1). The produced gas was then sampled in a 0.6 L multilayer foil bag (Supel

TM, Merck KGaA, Germany) and measured in its hydrogen content by gas-chromatography (section 3.2.6).

#### 3.2.3.2 BHP2

Tests were based upon the methodology reported by previous studies (Akhlagi et al., 2017; Cappai et al., 2014; De Gioannis et al., 2017) that involves the continuous pH control through the automatic addition of an alkaline solution. Assays were carried out using stainless steel (AISI 316) reactors of 6 L (working volume of 3 L, Figure 22).

**Figure 22: BHP2 – Reactors.**

Continuous mixing inside the reactors was ensured by a mixing blade connected to an electric gear motor (COAX MR 615 30Q 1/256, Unitec s.r.l., Italy). Temperature was constantly kept at mesophilic conditions (by a jacket where warm water heated up by a thermostat (FA90, Falc Instruments s.r.l., Italy) was continuously recycled. pH was continuously measured by pH probes (InPro4260i, Mettler Toledo, Italy). The volume of the produced gas during the tests was measured by using volumetric counters connected to the upper side of the reactors through a 3-way valve (Figure 9). Each counter was composed of two concentric cylinders partially filled with water: when the gas flowed from the reactor to the external side of the counter, the water rose through the internal cylinder up to the level of an electrode. The electrode activated a 3-way valve, which connected the counter to a 10 L multilayer foil bag (SupelTM, Merck KGaA, Germany) that collected the gas. After bag filling, the water level in the counter dropped to a second electrode, which reconnected the counters to the reactors and the gas restarted to enter

into them. Each impulse was related to a gas volume of 0.07 L. In order to convert gas volume data at normal conditions, a pressure transducer (HD 9908T Baro, Delta Ohm S.r.l., Italy) and a T-type thermocouple (PT100, Delta Ohm S.r.l., Italy) measured ambient pressure and temperature respectively. All signals coming from the reactors were acquired by a cRIO 9030 controller (National Instruments, USA) and were processed by a software specifically developed in Labview® environment. Data were recorded every 5 minutes. The acquisition system and the software were used also to control a peristaltic pump (Reglo ICC, Ismatec, Germany) dedicated to the dosage of an alkaline solution for pH control. The communication between the acquisition device and the pump occurred via a serial RS-232 connection. More specifically, 3 mL of NaOH 2M solution were automatically added when the pH decreased under the set value in order to constantly keep the pH in the range of ± 0.1 all through the tests. HCl 2.5M solution was used to set the initial pH at the desired value. After filling, the reactors were flushed with nitrogen for a few minutes to ensure anaerobic conditions. The hydrogen content in the collected biogas was measured by gas-chromatography (section 3.2.6).

#### 3.2.3.3 Kinetic model

The mean cumulative hydrogen production curves were obtained over the course of the batch experiments and analysed using the modified Gompertz equation (Van Ginkel et al., 2005). Eq. 8 is used to describe the kinetic of hydrogen production from batch fermentation assays (Pan et al., 2008).

$$H(t) = H_{max} \exp\left\{-\exp\left[\frac{r \cdot e}{H_{max}}(\lambda - t) + 1\right]\right\} \quad (8)$$

where:

- H(t): specific hydrogen production at time t ($NLH_2$ $kgTVS^{-1}$);
- $H_{max}$: total amount of hydrogen produced ($NLH_2$ $kgTVS^{-1}$);
- r: maximum hydrogen production rate ($NLH_2$ $kgTVS^{-1}$ $h^{-1}$);
- λ: length of the lag phase (h).

The time needed to attain 95% of the maximum hydrogen yield ($t_{95}$), was obtained from the Gompertz equation as follows (Cappai et al., 2014):

$$t_{95} = \frac{H_{max}}{R \cdot e}(1 - \ln(-\ln 0.95)) + \lambda \quad (9)$$

Constants were estimated by minimizing the sum square of errors between the experimental data and model results. The estimations were carried out by using the solver function of Microsoft Excel version 2016.

#### 3.2.3.4 Theoretical hydrogen production

As for Batch_exp_1, the theoretical maximum hydrogen production was obtained with the hypothetical assumption of Van Ginkel et al. (2005) and Logan et al. (2002) that sucrose, fructose, glucose and cellulose were the sole carbohydrates and the primary product was acetate. Under this assumption, calculations were performed taking into account a theoretical yield of 4 mol of $H_2$ $mol^{-1}$ of glucose, fructose and cellulose and 8 mol of $H_2$ $mol^{-1}$ of sucrose (Logan et al., 2002). Based on the considerations above, the conversion efficiency was calculated on a mass basis using the equation of De Gioannis et al. (2013), Eq. (10):

$$E_m = \frac{\text{mol } H_2 \text{ produced/mass of substrate}}{\text{Theoretical mol } H_2 \text{ produced/mass of substrate}} \times 100 \quad (10)$$

#### 3.2.3.5 Volatile solids removal efficiency

As for Batch_exp_1, the volatile solids removal efficiency ($\eta_{TVS}$) achieved during the test was calculated using Eq. (3).

### 3.2.4 Semi-continuous trials

The reactor used for BHP2 was also employed as semi-continuously stirred tank reactor (CSTR) to perform the fermentative stage. The methanogenic stage was performed in a similar stainless steel (AISI 316) reactor of 20 L (12 L working volume). The experimental set-up and the acquisition system was the same reported for BHP2.

Substrates were daily fed to the reactors by means of a syringe. The digestion of FW and the co-digestion of FW and AS were characterized by two scenarios. In the first scenario (S1), the methanogenic reactor was run alone aiming at evaluating the traditional one-stage AD (Figure 23). Simultaneously, the fermentative reactor was also fed in order to reach steady state conditions. In the second scenario (S2), the two digesters were connected in series aiming at evaluating the two-stage process (Figure 24). Each scenario was performed for three HRTs of the methanogenic reactor: 51 days S1 and 36 days S2. As for the methanogenic reactors, the first 34 and 24 days of S1 and S2 respectively were considered as the acclimatization phase (equal to

two HRT), while the last HRT of each scenario was considered as the steady state and its data were used for comparison. As for the fermentative reactors, the whole S1 was considered as a trial stage, while S2 was entirely considered as steady. Both scenarios were characterized by an OLR of the methanogenic reactor of 2.5 kgTVS $m^{-3}d^{-1}$. This value was selected as the optimum value for wet digestion technologies and mesophilic conditions (APAT, 2005) and in the range of previous studies (Voelklein et al., 2016). Consequently, similarly to other works (Cavinato et al., 2012; Chinellato et al., 2013, Micolucci et al., 2014; Zhu et al., 2011) the HRT was approximately 17 d for S1 and 12 d for S2. As for the fermentative reactor, the HRT was set to 3.0 d based on previous studies (Chinellato et al., 2013, Micolucci et al., 2014). The related OLR was then calculated to be approximately 14 kgTVS $m^{-3}d^{-1}$. As for the co-digestion study, an additional scenario was investigated (S3). The OLR and the HRT of the methanogenic reactor were the same of S2: 2.5 kgTVS $m^{-3}d^{-1}$ and 12 d, respectively. Regarding the fermentative reactor, the HRT was reduced to 1.5 d leading to an increase of OLR to approximately 28 kgTVS $m^{-3}d^{-1}$. Similar to S2, the scenario was performed for 36 days.

Table 18 summarizes the operational conditions applied to the reactors during the tests.

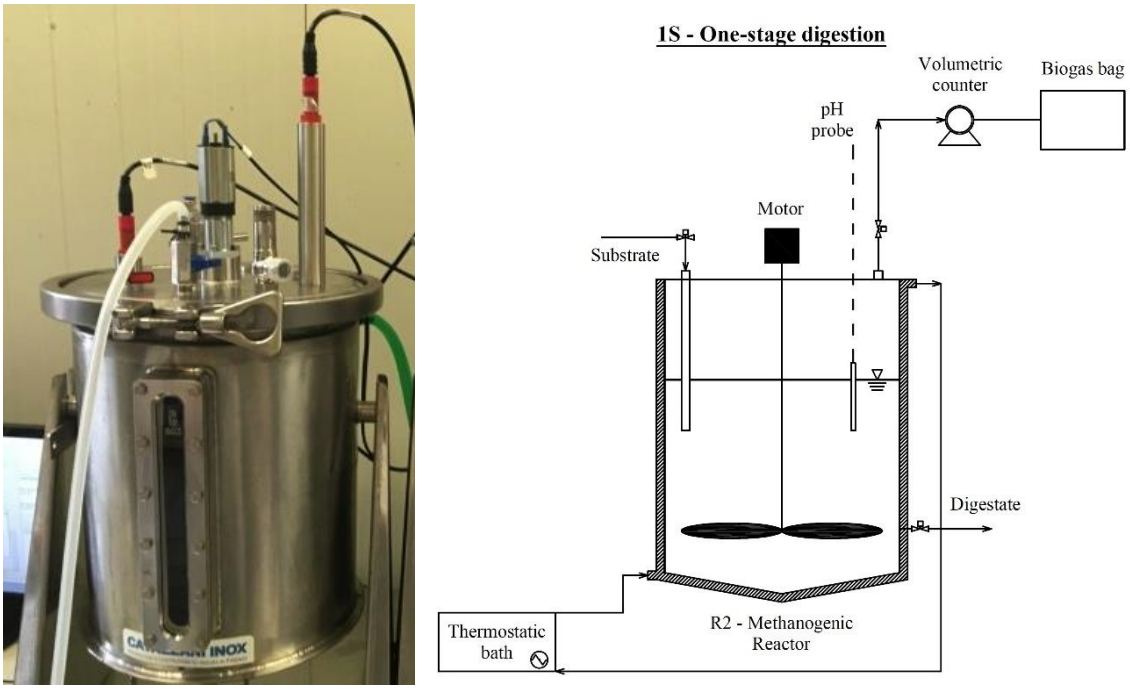


**Figure 23: Semi-continuous reactor used for the study of the one-stage technology. Baldi et al. (2019b).**

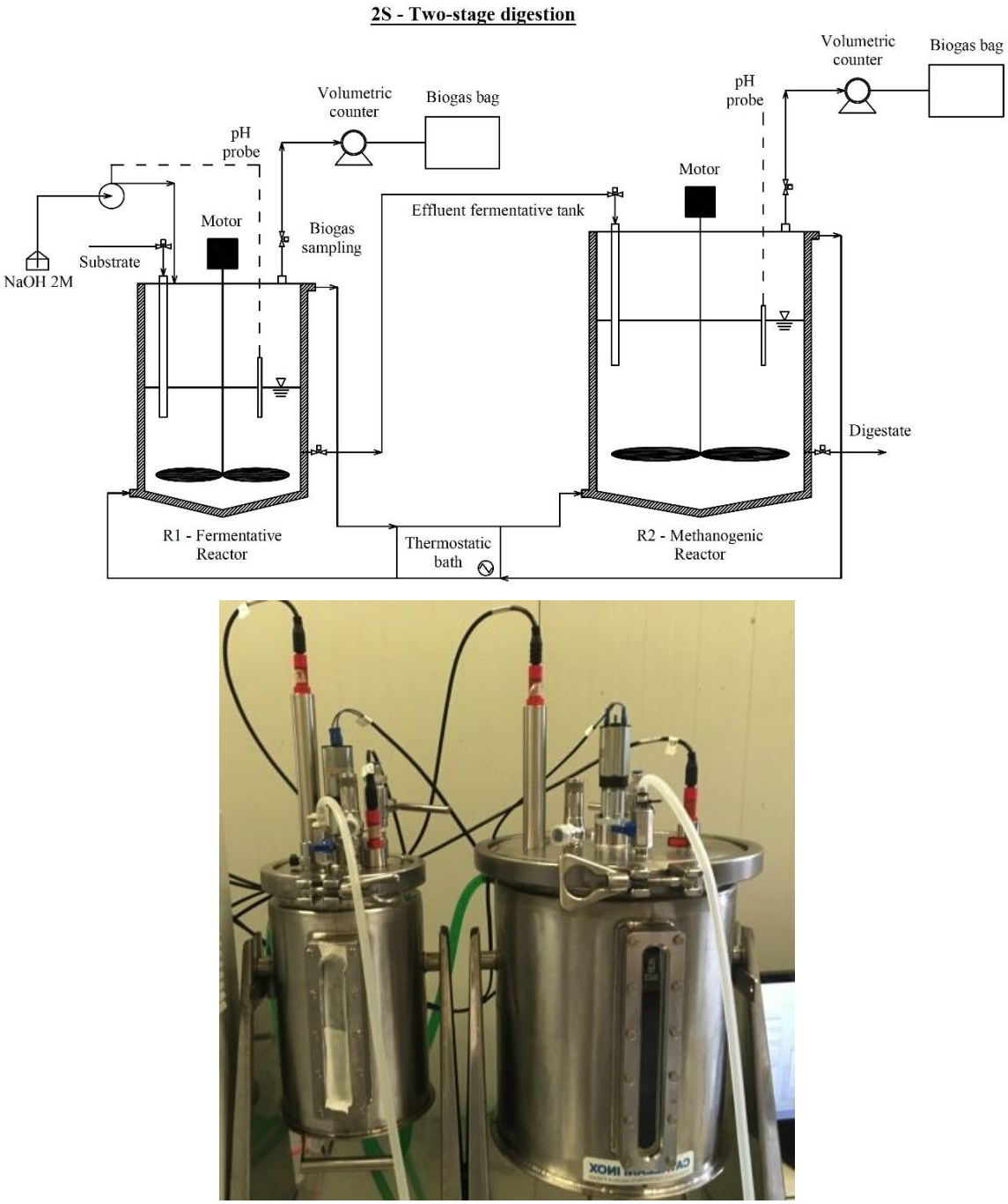


**Figure 24: Semi-continuous reactors used for the study of the two-stage technology. Baldi et al. (2019b).**

Process stability was daily monitored on digestate by means of alkalinity, IA $PA^{-1}$ ratio, pH and VFA. Aiming at determining the volatile solids removal efficiency ($\eta_{TVS}$, Eq. (3)), digestate was daily controlled in its volatile solids content, Anaerobic performances were evaluated in terms of $\eta_{TVS}$, specific gas production (SGP) and methane and hydrogen content in biogas.

### 3.2.5 Energy profit

Similar to the study of pretreatments (Eq. (4)), the specific energy balance, expressed as kJ $kgTVS^{-1}$, was calculated for semi-continuous trials taking into account the energy produced in the form of biogas ($E_B$), the theoretical amount of energy produced in the form of heat ($E_Q$) and the energy demand of a new fermentative reactor to be included in the treatment chain ($E_D$). The balance referred to the volatile mass of the substrate entering the digester daily.

$E_B$ was determined as the energy surplus produced by the two-stage technology compared to the traditional one-stage AD (Eq. 11). The calculation was performed taking into account the specific productions of hydrogen and methane (SHP in $NLH_2$ $kgTVS^{-1}$, SMP in $NLCH_4$ $kgTVS^{-1}$), the LHV of the two gases (10.8 kJ $NLH_2^{-1}$ and 35.8 kJ $NLCH_4^{-1}$, Siddiqui et al. 2011; Liu et al. 2013; Xiao et al., 2018) and an energy conversion factor (η) of 0.9 (Xiao et al., 2018).

$$E_B = (SHP_{S2} \cdot LHV_{H2} \cdot \eta + SMP_{S2} \cdot LHV_{CH4} \cdot \eta) - (SMP_{S1} \cdot LHV_{CH4} \cdot \eta) \quad (11)$$

The heat recovery $E_Q$ estimated as Eq. (12) based on the heat of the effluent without considering the heat of biogas (Xiao et al., 2018):

$$E_Q = \frac{Q \cdot C_p \cdot (T_d - T_a) \cdot \phi}{Q \cdot VS} \quad (12)$$

where:

- Q: effluent and influent flow rate (kg $d^{-1}$);
- VS: volatile matter content in the influent (kgTVS $kg^{-1}$);
- $C_p$: specific heat capacity of the effluent (kJ $kg^{-1}$ $°C^{-1}$). $C_p$ was based on ratio of water and solids (TS). The values of $C_p$ used for calculations amounted to 4.18 and 1.95 kJ $kg^{-1}$ $°C^{-1}$ for water and solids respectively (Kim and Parker, 2008; Kuglarz et al., 2013);
- $T_d$: anaerobic digester temperature (37°C);
- $T_a$: ambient temperature. It was assumed to be 20°C;
- ϕ: percentage of heat recovered, 80% (Xiao et al., 2018).

The energy demand ($E_D$) was calculated taking into account the energy consumption for mixing ($E_m$), the energy to heat the digester ($E_h$) and the energy of the peristaltic pump to dose the alkaline solution.

$$E_D = E_m + E_h + E_p \quad (13)$$

$E_m$ was estimated as follows, Eq. (14):

$$E_m = \frac{P_m \cdot t_m \cdot \xi}{Q \cdot VS} \quad (14)$$

where:

- Q: influent flow rate (kg $d^{-1}$);
- VS: volatile matter content in the influent (kgTVS $kg^{-1}$);
- $P_m$: power of the electric gear motor (0.024 kW);
- $t_m$: daily mixing time (52,800 s $d^{-1}$);
- $\xi$: mixing rate, 40%.

$E_h$ was estimated as follows, Eq. (15):

$$E_h = \frac{Q \cdot C_p \cdot (T_{H2O} - T_i)}{Q \cdot VS} \quad (15)$$

where:

- Q: influent flow rate (kg $d^{-1}$);
- VS: volatile matter content in the influent (kgTVS $kg^{-1}$);
- $C_p$: specific heat capacity of water (4.18 kJ $kg^{-1}$ $°C^{-1}$);
- $T_{H2O}$: temperature of the water bath. It was set at 37.8 °C to have 37 °C inside the reactor, therefore considering also the heat losses;
- $T_i$: ambient temperature. It was assumed to be 20 °C.

$E_p$ was calculated with Eq. (16):

$$E_p = \frac{(P_p/n) \cdot t_p}{Q \cdot VS} \quad (16)$$

where:

- $P_p$: power of the peristaltic pump (0.075 kW);
- n: number of channels, 3;
- $t_p$: average daily working time (720 s $d^{-1}$ for S2 of the digestion test, 600 s $d^{-1}$ and 1,800 s $d^{-1}$ for S2 and S3 of the co-digestion trial);
- Q: influent flow rate (kg $d^{-1}$);
- VS: volatile matter content in the influent (kgTVS $kg^{-1}$).

### 3.2.6 Analytical parameters

Substrates and inocula were characterized in terms of TS, TVS, pH, proteins, lipids, carbohydrates, Total Organic Carbon (TOC), Total Kjeldahl Nitrogen (TKN), ammonia, VFAs, C, H, N, O, S, Ca, Mg and Na. As for the inocula the concentration of *Clostridium Perfringens sp.* spore was also measured while FW1 was also characterized in terms of sucrose, fructose and glucose contents. The effluent of semi-continuous reactors was monitored daily in terms of TS, TVS, pH, alkalinity and VFAs. Hydrogen and methane contents of the biogas produced in batch tests and semi-continuous trials were measured by gas-cromatography.

TS, TVS and pH were measured following standard procedures (APHA, 2006).

Proteins, lipids and cellulose were obtained following the European Commission Regulation 2009/152/EC of 27 January 2009. Total carbohydrates were determined by subtracting the contents of humidity, ashes, proteins, lipids and fibres from the total amount. Lignin was obtained as reported by Martillotti et al. (1987).

C, H, N were measured in accordance with EN 15407 (2011) while S, P, Ca, Mg and Na were obtained following EPA 6010 D (2014) and EN 13657 (2004). The oxygen content was calculated by subtracting the contents of C, H, N, S and P from the total.

TOC was determined as stated in EN ISO 13127 (2002) while ammonia was obtained following APHA (2012). TKN, sum of organic nitrogen and ammonia, was obtained in accordance with the European Commission Regulation 2009/152/EC of 27 January 2009.

Sucrose, fructose and glucose content of FW were determined according to official methods (AOAC, 2003).

The concentration of *Clostridium Perfringens sp.* spores was investigated as bacterial indicator for the inocula. Spores were measured because *Clostridium sp.* and other HPB are able to sporulate and survive the extreme pretreatment conditions unlike nonsporulating bacteria which are suppressed (Zumar Bundhoo and Mohee, 2016). *Clostridum Perfringens spores* were measured using a tryptose sulphite cycloserine agar method (Wang et al., 2011; Araujo et al., 2004; Junqueira et al., 2012). 50 mg of inoculum were heated at 80°C for 30 minutes and then filtered through 47 mm Whatman WCN 0.45 μm pore size membrane filters. Samples were placed onto agar plates (Merck KGaA, Germany) and incubated at 44 ± 1 °C for 21 ± 3 h under anaerobic conditions. All black colonies were then counted as *Clostridium Perfringens* (Wang et al., 2011; Araujo et al., 2004).

Alkalinity was measured according to the methodology of Martín-González et. (2013), presented in section 2.2.7.

VFAs, including acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids were measured using a gas chromatograph (7890B, Agilent Technology, US) with hydrogen as gas carrier, equipped with a CPFFAP column (0.25 mm / 0.5 μm / 30 m) and with a flame ionization detector (250°C). The temperature during the analysis started from 60°C and reached 250°C with a rate of 20 °C/min. Samples were centrifuged (30 minutes, 13,500 rpm) and filtrated on a 0.45 μm membrane. 500 μL of filtrate were mixed with isoamyl alcohol (1.00179, Merck KGaA, Germany) in a volumetric ratio of 1:1, 200 μL of phosphate buffer solution (pH 2.1), sodium chloride and 10 μL of hexanoic-D11 acid solution (10.000 ppm) used as internal standard. The blend was mixed with a Mortexer™ Multi-Head vortexer (Z755613-1EA, Merck KGaA, Germany) for 10 minutes. The liquid suspension of the sample was then inserted in the gas chromatograph by means of an auto-sampler.

Concerning gas quality, hydrogen, methane, carbon dioxide, nitrogen, oxygen and hydrogen sulphide contents in biogas were analysed using a gas chromatograph (3000 Micro GC, INFICON, Switzerland) equipped with a thermal conductivity detector. Carbon dioxide and hydrogen sulphide passed through a PLOTQ column (10μm/320μm/8m) using helium as gas carrier at temperature of 55°C. The other gas passed through a Molsieve column (30μm/320μm/10m) using argon as gas carrier at a temperature of 50°C.

### 3.2.7 Statistical analysis

Statistical analysis was performed for batch tests results.

With the aim of evaluating the differences obtained from the adoption of two different experimental settings, simple linear regression was used to compare the results of BHP1 and BHP2.

In order to assess the significance of the results, the findings obtained by the different experimental conditions (type of inoculum, pH, F/M ratio) were analysed using one-way ANOVA (Alibardi and Cossu, 2016).

As for Batch_exp_1, in order to identify linear relationships between the variables of concern, two Pearson matrices were obtained, one for each substrate (FW1 and sucrose). The analyses was performed taking into account experimental findings (SHP, $\eta_{TVS}$, $E_m$), Gompertz model

results ($H_{max}$, $\lambda$, r and $t_{95}$) and *Clostridium perfringens* sp. spore concentrations in the four inoculum.

A confidence level of 95% was selected for all statistical comparisons. All data analysis calculations were performed using XLStat2018 software packages.

### 3.2.8 Calibration and control of the experimental set-up

Analytical instruments and reactors were carefully calibrated and controlled before the start of the experiments.

The vessels used for batch tests and the reactor used for semi-continuous trials were subjected to leakage test in order to prevent biogas losses during the experiments. Both containers were realized to hold a 2-bar proof pressure.

The micro-gas-chromatograph adopted for gas measurements was periodically calibrated to assess the content of $CH_4$, $CO_2$, $N_2$, $H_2S$, $O_2$ and $H_2$. The calibration curves were built using different synthetic gasses with known content.

The laboratory pH-meter and the pH-probes of the reactors were weekly calibrated using a 3-points calibration curve with proof buffer solutions of known pH (4, 7, 10).

The volume of gas displaced by a pulse of the volumetric counter was checked prior to the start of semi-continuous tests. Calibration was performed by flushing 5 different flow rates of compressed air through the volumetric meters and recording the time interval between two pulses. Volumetric counters were also subjected to leakage test in order to prevent biogas losses during the experiments.

The gas-chromatograph adopted for VFA measurements was periodically calibrated to assess the content of acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids. The calibration curves were built using different samples with known content of the different VFA (100, 200, 500, 1,000, 2,000, 5,000, 10,000 mg $L^{-1}$). The calibration curves attached as annex in the appendix.

Each measurement of dry matter, volatile solids and pH was performed in triplicate while the quality of gas, VFAs and alkalinity were controlled in duplicate.

The measurements of proteins, lipids, carbohydrates, fibres, lignin, TOC, TKN, ammonia, elemental composition, Ca, Mg, Na, *Clostridium Perfringens sp.* Spore, sucrose, fructose and glucose were performed by accredited external laboratories.

## 3.3 Results

### 3.3.1 Analytical characterization of the media

Analytical characterization of inocula and substrates was performed in order to provide a detailed description of the media and to assess the extent of potential inhibitory compounds for the fermentative process. Results expressed with averages and standard deviations are presented in Table 19.

**Table 19: Substrates and inocula characterization. Values are expressed by averages and standard deviations (n = 3).**

| Parameters | IN1 (AS) | IN2 | IN3 | IN4 | FW1 | FW2 |
|---|---|---|---|---|---|---|
| TS (% w/w) | 2.1 ± 0.2 | 2.9 ± 0.1 | 3.0 ± 0.2 | 2.1 ± 0.0 | 5.6 ± 0.1 | 19.9 ± 0.6 |
| TVS $TS^{-1}$ (% w/w) | 74.2 ± 0.4 | 61.5 ± 1.3 | 61.0 ± 0.5 | 79.3 ± 0.3 | 91.6 ± 0.3 | 80.6 ± 0.9 |
| pH | 7.1 ± 0.0 | 8.2 ± 0.1 | 7.8 ± 0.1 | 6.7 ± 0.1 | 3.8 ± 0.1 | 3.8 ± 0.1 |
| Carboh. (% w/w) | < 0.1 | < 0.1 | 0.1 | 0.1 | 2.0 | 7.4 |
| Proteins (% w/w) | 0.9 ± 0.1 | 0.6 ± 0.1 | 1.2 ± 0.1 | 0.8 ± 0.1 | 1.0 ± 0.1 | 3.9 ± 0.2 |
| Lipids (% w/w) | < 0.3 | < 0.3 | < 0.3 | < 0.3 | 0.3 ± 0.1 | 3.9 ± 0.2 |
| Fibres (% w/w) | 0.4 ± 0.0 | 0.5 ± 0.0 | 0.4 ± 0.0 | < 0.3 | 1.8 ± 0.2 | 7.9 ± 1.1 |
| Cellulose (% w/w) | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | < 0.1 | 1.5 ± 0.1 | 3.0 ± 0.4 |
| Lignin (% w/w) | 0.3 ± 0.0 | 0.3 ± 0.1 | 0.3 ± 0.0 | < 0.3 | 0.3 ± 0.1 | 4.9 ± 0.7 |
| TOC (%C w/w) | 1.2 ± 0.2 | 1.0 ± 0.1 | 1.2 ± 0.2 | 0.6 ± 0.1 | 1.9 ± 0.2 | 10.0 ± 1.8 |
| TKN (%N w/w) | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.2 ± 0.0 | 0.1 ± 0.0 | 0.2 ± 0.0 | 0.6 ± 0.0 |
| $NH_4^+$ (mgN $L^{-1}$) | 341 ± 47 | 2,025±304 | 1,040 ± 82 | 980 ± 79 | 191 ± 5 | 969 ± 78 |
| Acetate (mg $L^{-1}$) | 830 ± 120 | < 25 | < 20 | < 20 | 958 ± 30 | 2,320±470 |
| Propionate (mg $L^{-1}$) | 390 ± 71 | < 25 | < 40 | < 40 | < 40 | < 40 |
| C (%TS) | 58.9 ± 4.3 | 34.6 ± 5.2 | 50.8 ± 3.7 | 52.1 ± 3.8 | 36.0 ± 1.9 | 44.4 ± 3.2 |
| H (%TS) | 6.4 ± 0.5 | 5.7 ± 0.8 | 3.9 ± 0.3 | 5.2 ± 0.4 | 5.8 ± 0.2 | 7.5 ± 0.6 |
| N (%TS) | 7.5 ± 0.9 | 8.9 ± 1.4 | 8.0 ± 0.9 | 10.5 ± 1.2 | 2.9 ± 0.3 | 3.4 ± 0.5 |
| S (%TS) | 0.9 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.1 | 1.2 ± 0.2 | 0.2 ± 0.0 | 0.1 ± 0.0 |
| P (%TS) | 0.4 ± 0.1 | 0.9 ± 0.1 | 0.4 ± 0.1 | 1.7 ± 0.2 | 0.4 ± 0.1 | 0.2 ± 0.0 |
| O (%TS) | 27.9 | 49.2 | 36.3 | 29.3 | 54.6 | 44.4 |
| Ca (mg $L^{-1}$) | 703 ± 85 | 840 ± 74 | 1,240 ±140 | 617 ± 56 | 1,120 ± 13 | 4,200±490 |
| Mg (mg $L^{-1}$) | 109 ± 25 | 154 ± 14 | 220 ± 44 | 103 ± 24 | 166 ± 34 | 590 ±120 |
| Na (mg $L^{-1}$) | 121 ± 27 | 769 ± 66 | 710 ± 140 | 173 ± 36 | 550 ± 110 | 1,340±260 |
| *Clos.* (CFU $mg^{-1}$) | 150 | 13 | 50 | 110 | - | - |

Butyric, isobutyric valeric and isovaleric acid contents were not shown since their content was found to be below the limit of detection (LOD = 40 mg $L^{-1}$). Acetic and propionic acids were also undetected for IN2, IN3, IN4, while acetic acid was the prevalent VFA for IN1 and FW.

With regard to C:N ratio, FW1 and FW2 showed values of 12.4 and 13.1, which was slightly below other FW findings. Zhang et al. (2007) reported an average value of 14.8, while Pan et al. (2008) and Han and Shin (2004) obtained C:N ratios of 17.1 and 14.7 respectively. Concerning sludges, their C:N ratios were found to be lower than FW, being between 3.9 (IN2) and 7.9 (IN1). This result was comparable with the range of 6-9 defined by Iacovidou et al. (2012) for sewage sludge and it is explained by the high N contents and the high ammonia concentration. Previous studies found that a proper C:N ratio enhances biohydrogen production by shifting the microorganism metabolic pathway (Lin and Lay, 2004). Argun et al. (2008) studied the effects of the C:N ratio on the hydrogen yield and specific $H_2$ production rate in DF of wheat powder solution by supplementing nitrogen. The results of the study showed that the highest $H_2$ production was obtained at a C:N ratio of 200. Lin and Lay (2004) achieved a 500% and 80% increased hydrogen yield and hydrogen production rate at a C:N ratio of 47 compared with the blank while Kayhanian (1999) reported an optimum C:N ratio in the range of 27-32. Although the optimum ratio covers a wide range of values, the C:N ratio of the four inocula was found to be significantly lower than each of them.

The problem that might occur with the use of these sludges is the failure of the process due to toxicity ammonia inhibition (Iacovidou et al., 2012). Under this perspective, Ghimire et al. (2015) advised against the use of swine, poultry and dairy manure due to their low C:N ratio and high levels of ammonia (cattle slurry: 1,040–1,900 mg $L^{-1}$ and chicken manure: 7,000–12,800 mg $L^{-1}$). As reported for methane enzymes (Kayhanian, 1999), the accumulation of ammonia inside the cell membrane may be toxic and inhibit HPB activities. Several studies have made an effort to find the inhibitory threshold of ammonia on hydrogen production. Sterling et al. (2001) investigated hydrogen production from dairy cattle manure under different ammonia concentrations (600, 1,500 and 3,000 mgN $L^{-1}$). In the first two set-ups, an increment in hydrogen generation was observed after 24 h while in the 3,000 mgN $L^{-1}$ set-up, the fermentative process was arrested. Salerno et al. (2006) studied ammonia inhibition of biohydrogen production in batch and continuous reactors with glucose as substrate. In batch tests, an ammonia threshold concentration of 7,000 mgN $L^{-1}$ was found under a pH of 5.2, while in continuous flow conditions the suppression of biohydrogen production occurred when ammonia concentrations in the feed exceeded 800 mgN $L^{-1}$. As can be highlighted, even if the probability of having an inhibitory effect and ammonia concentration seems to follow a linear correlation, a unique threshold limit

is still not defined. Ammonia content in the four inocula ranged between 341 mgN $L^{-1}$ (IN1) and 2,025 mgN $L^{-1}$ (IN2), while FW highlighted a weaker concentration (191 mgN $L^{-1}$). All the inocula showed concentrations lower than the threshold limit for batch tests of 3,000 and 7,000 mgN $L^{-1}$ set respectively by Sterling et al. (2001) and Salerno et al., (2006).

Among the macromolecules, carbohydrates were the main component for FW while inocula highlighted a predominance of proteins (Wilson and Novak, 2008). FW proteins and carbohydrates were found to be slightly below previous works due to the dilution employed in the present study. Indeed, protein and carbohydrate contents in FW generally range between 3.1-4.9 %w/w and 6.2-13.4% w/w respectively (Yeshanew et al., 2016; Chu et al., 2008).

The content of Clostridium Perfringens sp. spores was studied since previous works found Clostridium Perfringens sp. to be the most dominant hydrogen producing culture (Huang et al., 2010; Wang et al., 2011; Wong et al., 2018; Sivagurunathan et al., 2014; Jame et al., 2016). The concentration was observed to vary by a factor of up to 12, ranging from a maximum of 150 CFU $mg^{-1}$ found for activated sludge sample (IN1) to a minimum of 13 CFU $mg^{-1}$ (IN2).

Light metals, such as magnesium, sodium and calcium, are crucial important requirements of DF as they assist in microbial metabolism, cell growth, hydrogen and enzyme generation. Nonetheless, significant contents of metal ions in inocula or substrates may result in an inhibitory behaviour and hinder hydrogen generation (Zumar Bundhoo and Mohee, 2016).

Magnesium constitutes bacterial cell walls and it is needed for cellular protein synthesis. Therefore, a minimum content of magnesium ions is needed for an efficient fermentative process and it has been highlighted to improve hydrogen generation (Zumar Bundhoo and Mohee, 2016). Concerning the inhibitory threshold, Srikanth and Mohan (2012) defined a magnesium concentration of 600 mg $L^{-1}$ as the limit value.

Sodium is an element necessary for microbial growth (Hao et al., 2006). Nevertheless, a significant sodium content may hinder hydrogen generation because of a variation of the metabolic route. In this case, alternative metabolites such as lactate are produced instead of hydrogen (Zumar Bundhoo and Mohee, 2016; Kim et al., 2009). In particular, Cao and Zhao (2009) found an inhibitory threshold level of 14,410 mg $L^{-1}$.

Concerning Calcium, it is a constituent of extracellular polysaccharides, which plays a key role in the formation of biofilms, and thereby improves cell granulation and settleability (Elbeshbishy et al., 2017). Lee et al. (2004) reported that addition of a small amount of $Ca^{2+}$ (5.4 mg $L^{-1}$) spurred a significant improvement in hydrogen production. Yuan et al. (2010) reported

enhancing bacterial growth and mechanical strength. Chang and Lin (2006) showed that $Ca^{2+}$ in the range of 0–150 mg $L^{-1}$ is non-inhibitory as reflected by yields of 3.6 mol $mol^{-1}$.

In conclusion, the tested compounds were always below the inhibitory threshold. The co-digestion trials were performed with a mixture of IN1 (AS) and FW2, which therefore resulted suitable for the fermentative process.

### 3.3.2 Batch test – experiment 1

The results of specific cumulative hydrogen productions, volatile solids reduction and conversion efficiency obtained from the four inocula (IN1, IN2, IN3, IN4), the two substrates (FW and sucrose) and the two experimental set-ups are reported in Table 20. SHP curves are expressed in terms of $NLH_2$ $kgTVS^{-1}$ and depicted in Figure 25, Figure 26, Figure 27 and Figure 28. In these figures, the experimental data are shown as single points while solid lines represent Gompertz model curves. In order to avoid visual misunderstandings, BHP2 experimental data were represented every 2 hours.

**Table 20: Average experimental results of fermentative tests in terms of SHP (in $NLH_2$ $kgTVS^{-1}$), $\eta_{TVS}$ (in %) and $E_m$ (in %) (± SD, n = 3). Results of the simple regression analysis are expressed by R and p-value. Pecorini et al. (2019).**

| | | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 |
|---|---|---|---|---|---|---|---|
| Inoculum | Substrate | SHP | SHP | $\eta_{TVS}$ | $\eta_{TVS}$ | $E_m$ | $E_m$ |
| IN1 | FW1 | 90.2 ± 3.2 | 101.6 ± 2.5 | 24.1 ± 0.7 | 48.2 ± 1.2 | 27.0 ± 1.0 | 30.1 ± 0.7 |
| IN2 | FW1 | 29.3 ± 0.7 | 56.0 ± 2.5 | 14.3 ± 0.5 | 31.2 ± 1.5 | 14.9 ± 0.4 | 19.5 ± 0.9 |
| IN3 | FW1 | 50.2 ± 1.4 | 65.5 ± 2 | 14.8 ± 0.5 | 43.0 ± 1.7 | 7.2 ± 0.2 | 16.3 ± 0.5 |
| IN4 | FW1 | 74.0 ± 4.1 | 79.2 ± 2.6 | 23.5 ± 0.6 | 37.8 ± 0.8 | 20.2 ± 0.5 | 23.2 ± 0.8 |
| | R | 0.97 | | 0.52 | | 0.98 | |
| | *p-value* | 0.03 | | > 0.05 | | 0.02 | |
| IN1 | Sucrose | 153.1 ±2.1 | 155.3 ± 1.8 | 46.9 ± 1.0 | 58.2 ± 2.0 | 29.2 ± 0.4 | 29.6 ± 0.3 |
| IN2 | Sucrose | 74.6 ± 1.6 | 95.1 ± 2.1 | 34.6 ± 1.5 | 31.7 ± 1.4 | 20.9 ± 0.4 | 21.0 ± 0.5 |
| IN3 | Sucrose | 109.4 ± 1.0 | 110.2 ± 2.3 | 41.1 ± 1.1 | 46.3 ± 1.7 | 11.4 ± 0.1 | 17.7 ± 0.4 |
| IN4 | Sucrose | 149.9 ± 1.7 | 151.0 ± 2.5 | 44.8 ± 1.2 | 46.5 ± 1.5 | 28.6 ± 0.3 | 28.0 ± 0.5 |
| | R | 0.98 | | 0.95 | | 0.97 | |
| | *p-value* | 0.02 | | 0.04 | | 0.03 | |

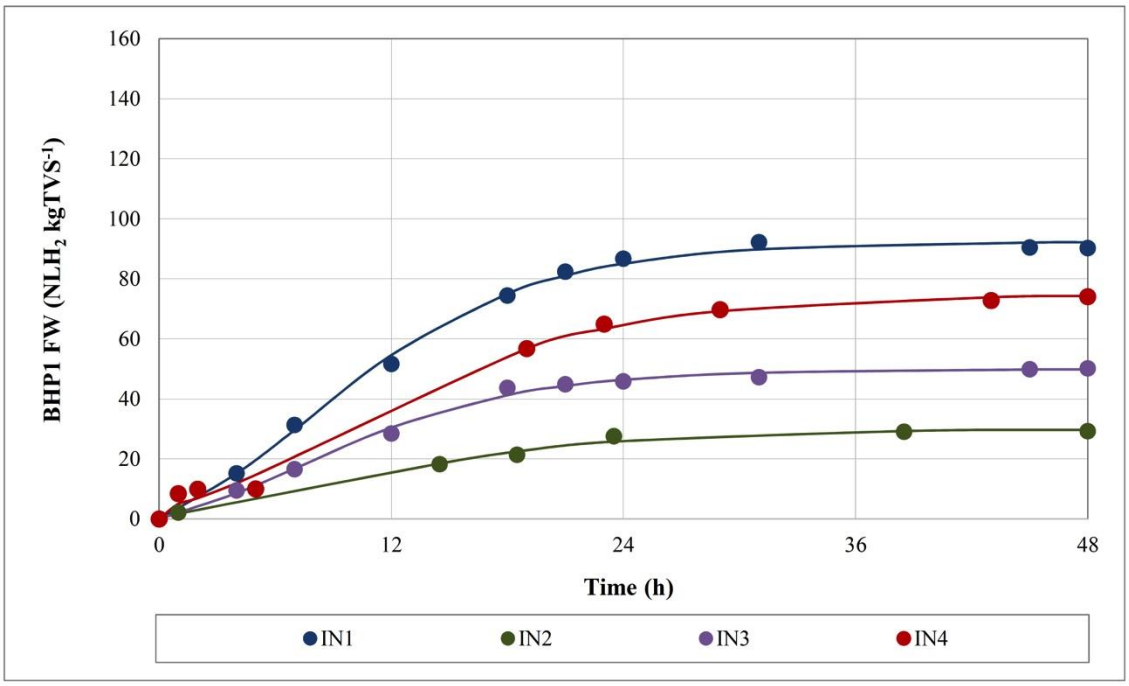


**Figure 25: Batch_exp_1 - Mean SHP curves obtained for the tests without automatic pH control (BHP1) and FW as substrate. Points indicate experimental results, solid lines Gompertz model trends. Pecorini et al. (2019).**

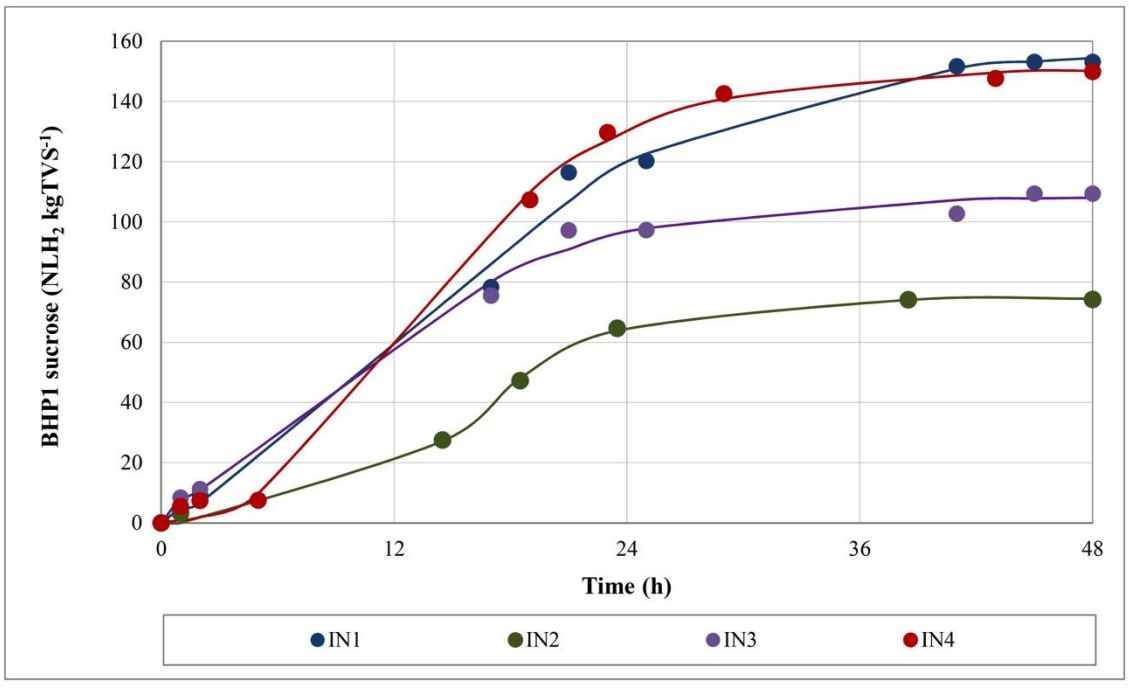


**Figure 26: Batch_exp_1 - Mean SHP curves obtained for the tests without automatic pH control (BHP1) and sucrose as substrate. Points indicate experimental results, solid lines Gompertz model trends. Pecorini et al. (2019).**

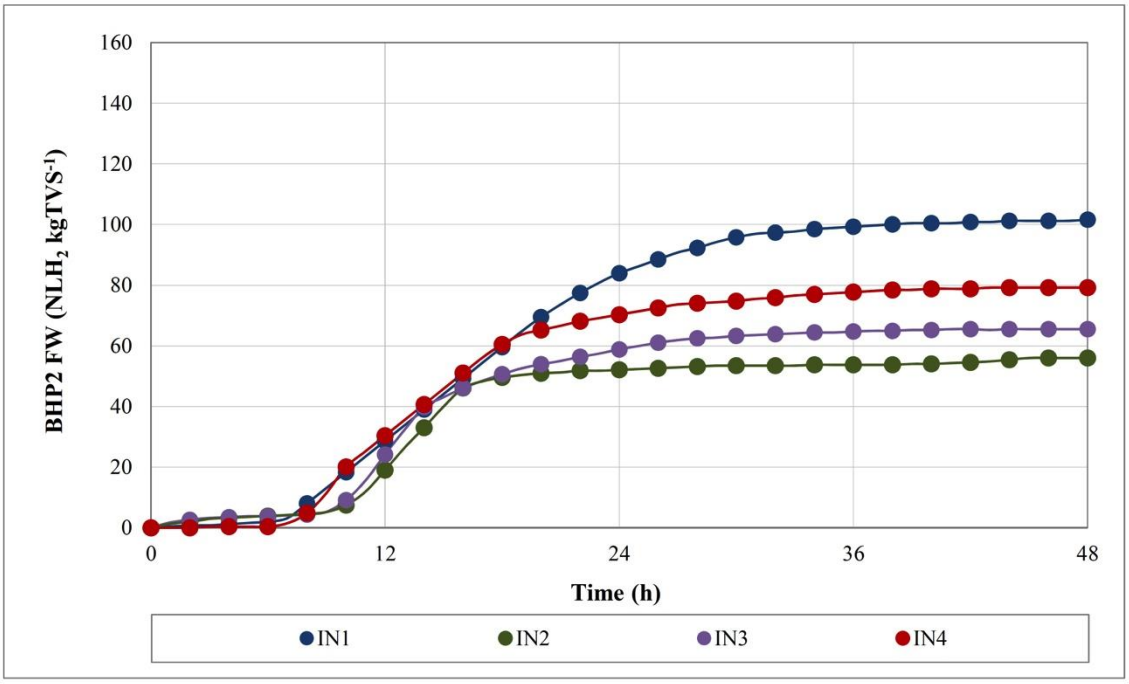


**Figure 27: Batch_exp_1 - Mean SHP curves obtained for the tests with automatic pH control (BHP2) and FW as substrate. Points indicate experimental results, solid lines Gompertz model trends. Pecorini et al. (2019).**

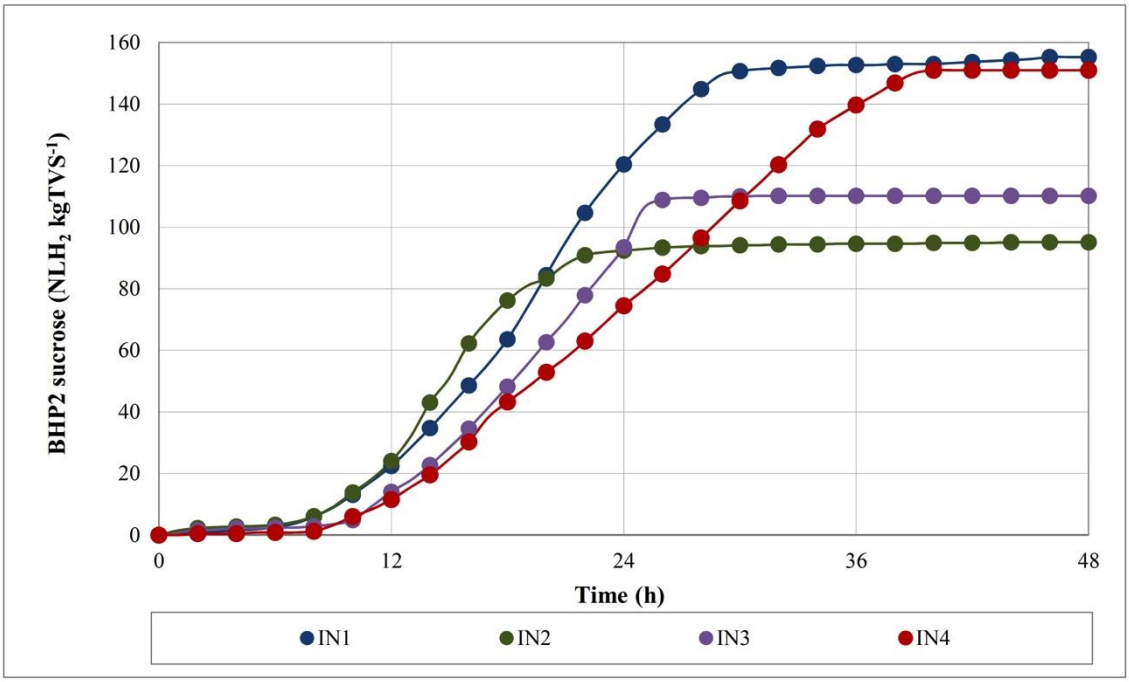


**Figure 28: Batch_exp_1 - Mean SHP curves obtained for the tests with automatic pH control (BHP2) and sucrose as substrate. Points indicate experimental results, solid lines Gompertz model trends. Pecorini et al. (2019).**

During all fermentation assays no methane was detected. Thermal pretreatment of the inoculum was therefore efficient in the inhibition of hydrogenotrophic methanogens. SHPs from FW1 (29.3 – 90.2 $NLH_2$ $kgTVS^{-1}$ for BHP1 and 65.4 – 101.6 $NLH_2$ $kgTVS^{-1}$) were comparable to findings of previous works. Specifically, Alibardi and Cossu (2015) highlighted results in the

range of 25 - 85 $NLH_2$ $kgTVS^{-1}$, De Gioannis et al. (2017) and Pecorini et al. (2017) reported final SHPs from FW of 58.6 and 55.0 $NLH_2$ $kgTVS^{-1}$ respectively while Alibardi and Cossu (2016) found 78 - 135 $NLH_2$ $kgTVS^{-1}$ for different organic waste mixtures. As for sucrose, the conversion of hydrogen productions to $molH_2$ $mol_{sucrose}^{-1}$ ranged between 0.9 - 2.3 $molH_2$ $mol_{sucrose}^{-1}$ for BHP1 and 1.4 - 2.4 $molH_2$ $mol_{sucrose}^{-1}$ for BHP2. These data were concurrent with Dellosso Penteado et al. (2013). Hydrogen yields from sucrose were in the range of 0.7 – 2.0 $molH_2$ $mol_{sucrose}^{-1}$ using different seed sludge sources in up-flow anaerobic fixed-bed reactors.

TVS removal efficiencies were found to be in the ranges of 14.3 – 48.3% for FW and 31.7 – 58.2% for sucrose. Similarly to hydrogen production results, IN1 showed the highest removal efficiencies followed by IN4, IN3 and IN2.

Aiming at calculating the conversion efficiency ($E_m$), the contents of sucrose (0.4 ± 0.2 % w/w), glucose (1.1 ± 0.4 % w/w) and fructose (0.2 ± 0.1 % w/w) were measured in FW1. $E_m$ were below 30% with the highest conversion efficiencies found for the tests performed with activated sludge (IN1). Previous literature findings reported conversion efficiencies of 23% (Logan et al., 2002) and 58% (Van Ginkel et al., 2001) for sucrose and 19.3% for FW (Han and Shin, 2004). These results were obtained since, in real practice, fermentation processes do not generate only acetate as product, differently from what is assumed by the theoretical calculation. Several other reduced products are formed to sustain microbial cell synthesis, including ethanol, butanol, butyrate and lactate (De Gioannis et al., 2013). Consequently, the real yield is lower than 4 $molH_2$ $mol_{glucose}^{-1}$ and 8 $molH_2$ $mol_{sucrose}^{-1}$ (Alibardi and Cossu, 2016; Zumar Bundhoo and Mohee, 2016).

Observing the results, it was evident that the hydrogenic process was dependent on the type of inoculum. Analysis of variance performed separately for each substrate and each test configuration on SHP, $\eta_{TVS}$ and $E_m$ data indicated the finding of statistically different results ($p < 0.01$). This confirms that results were due to the different inocula used in the tests. In particular, the highest SHP and efficiencies were obtained for activated sludge (IN1). SHP obtained using IN1 were almost double compared to IN2 results in all test configurations and 4.5 – 5.8% and 23.4 - 31.4% higher than IN4, the second most performing inoculum, respectively for FW and sucrose tests. In order to compare the results of the two experimental set-ups, simple linear regression was carried out between SHP, $\eta_{TVS}$ and $E_m$ data obtained for BHP1 and BHP2. Results were statistically significant ($p < 0.05$) and Pearson correlation coefficients (R) were always found to be above 0.95 with the exception of $\eta_{TVS}$ calculated for FW assays.

The kinetic parameters derived from fitting of the experimental data with the Gompertz equation (Eq. (9)) are shown in Table 21. The Gompertz model fitted the experimental data well, with high coefficients of determination for all the assays ($R^2(G) > 0.977$).

**Table 21: Kinetic parameters obtained from the application of the Gompertz model: total amount of hydrogen produced ($H_{max}$ in $NLH_2$ $kgTVS^{-1}$), maximum hydrogen production rate (r in $NLH_2$ $kgTVS^{-1}$ $h^{-1}$), length of the lag phase (λ in h). The time needed to attain 95% of the maximum hydrogen yield ($t_{95}$ in h) and the linear correlation with the experimental data ($R^2(G)$) are also presented. Results of the simple regression analysis are expressed by R and p-value. Pecorini et al. (2019).**

| | | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| In. | Sub. | $H_{max}$ | $H_{max}$ | r | r | λ | λ | $t_{95}$ | $t_{95}$ | $R^2(G)$ | $R^2(G)$ |
| IN1 | FW | 92.4 | 102.4 | 5.2 | 5.9 | 1.4 | 7.4 | 27.1 | 22.9 | 0.99 | 0.99 |
| IN2 | FW | 29.9 | 54.1 | 1.4 | 7.3 | 1.3 | 9.2 | 31.6 | 20.0 | 0.99 | 0.99 |
| IN3 | FW | 49.9 | 64.5 | 2.9 | 5.7 | 1.2 | 7.8 | 26.3 | 24.3 | 0.98 | 0.99 |
| IN4 | FW | 74.9 | 78.0 | 3.5 | 6.0 | 0.9 | 7.2 | 32.3 | 26.0 | 0.99 | 0.99 |
| | R | 0.97 | | - 0.73 | | 0.41 | | - 0.07 | | | |
| | *p-value* | 0.03 | | > 0.05 | | > 0.05 | | > 0.05 | | | |
| IN1 | Sucrose | 157.5 | 158.8 | 6.4 | 10.1 | 3.4 | 10.9 | 39.4 | 34.0 | 0.99 | 0.99 |
| IN2 | Sucrose | 74.6 | 95.4 | 5.7 | 9.7 | 9.7 | 9.3 | 28.8 | 23.6 | 0.99 | 0.99 |
| IN3 | Sucrose | 108.5 | 113.1 | 5.4 | 8.8 | 0.7 | 12.0 | 30.2 | 30.7 | 0.99 | 0.99 |
| IN4 | Sucrose | 150.8 | 150.0 | 8.5 | 6.4 | 5.0 | 11.9 | 30.9 | 26.2 | 0.99 | 0.99 |
| | R | 0.99 | | - 0.81 | | - 0.85 | | 0.82 | | | |
| | *p-value* | 0.01 | | > 0.05 | | > 0.05 | | > 0.05 | | | |

The maximum hydrogen production rate for FW1 was in the ranges of 1.4 - 5.2 $NLH_2$ $kgTVS^{-1}$ $h^{-1}$ and 5.9 – 7.3 $NLH_2$ $kgTVS^{-1}$ $h^{-1}$ for BHP1 and BHP2 respectively. These findings were in the range of previous literature data. Cappai et al. (2014) reported maximum hydrogen production rates in the range of 2.4 – 16.6 $NLH_2$ $kgTVS^{-1}$ $h^{-1}$ depending on pH and inoculum pretreatment, while De Gioannis et al. (2017) found a maximum hydrogen production rate of 3.9 $NLH_2$ $kgTVS^{-1}$ $h^{-1}$. Concerning sucrose, maximum hydrogen production rates were higher than those found for FW1, due to the higher biodegradability of the substrate: 5.4 - 8.4 $NLH_2$ $kgTVS^{-1}$ $h^{-1}$ and 6.4 – 10.1 $NLH_2$ $kgTVS^{-1}$ $h^{-1}$ for BHP1 and BHP2 respectively.

In the matter of $t_{95}$, the time required to attain 95% of the maximum hydrogen yield was found to be slightly higher for sucrose (28.8 – 39.4 h for BHP1 and 23.6 – 34.0 h for BHP2) than for FW1 (26.3 – 32.3 h for BHP1 and 20.0 - 26.0 h for BHP2). These results were concurrent with previous works (Cappai et al. (2014): $t_{95}$ = 9.1 – 32.2 h; De Gioannis et al. (2017): $t_{95}$ = 26.4 h).

Comparing BHP1 and BHP2 data, the continuous adjustment of pH (BHP2) appeared to accelerate the kinetics, with $t_{95}$ values always below 26 hours for FW1 and 34 h for sucrose.

In analogy with SHP results, $H_{max}$ data highlighted a good correlation between BHP1 and BHP2 findings ($R > 0.97$, $p < 0.05$). Nevertheless, the linear correlation between BHP1 and BHP2 was not evident for the other kinetic parameters probably due to differences in data acquisition methods. Indeed, while for BHP2 assays, volumetric gas production was recorded every 5 minutes, for BHP1 tests the time between two pressure measurements was in the range of a few hours. As such, the cumulative curves depicted different trends, thus leading to the calculation of different kinetic parameters.

In order to identify linear relationships between the variables of concern two Pearson matrices showing the correlation coefficients (R) were carried out for FW1 and sucrose separately. Given the correlation between BHP1 and BHP2 results, the data from the two experimental set-ups were treated together. What is relevant from Table 22 is the high degree of correlation between the experimental variables (SHP, $\eta_{TVS}$ and $E_m$) and *Clostridium perfringens sp.* spore concentration, which is particularly related to the production of hydrogen ($R > 0.92$). This confirms the analogy between the experimental results obtained from two different set-ups and the relevance of the *Clostridium perfringens sp.* in the production of hydrogen. On the other hand, no correlation was found between the kinetic variables of the Gompertz model, especially between r, $\lambda$ and $t_{95}$.

In conclusion, from the first set of batch experiments, it was evident that the hydrogenic process was highly dependent on the type of inoculum. In particular, assays performed with an aerobic inoculum such as activated sludge (IN1) highlighted higher specific hydrogen productions (SHP), TVS removal efficiencies ($\eta_{TVS}$) and conversion efficiencies ($E_m$). The presence of high concentration of Clostridia in this media seemed to play a key role in the development of an optimal fermentative process.

**Table 22: Pearson correlation matrices. Data in bold are are significantly correlated ($p < 0.05$). *Clos.* means *Clostridium Perfringes sp.* spore concentration. Pecorini et al. (2019).**

| FW1 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Parameters | SHP | $\eta_{TVS}$ | *Clos.* | $E_m$ | $H_{max}$ | r | λ | $t_{95}$ |
| SHP | **1.00** | **0.67** | **0.92** | **0.83** | **1.00** | 0.58 | 0.26 | 0.20 |
| $\eta_{TVS}$ | **0.67** | **1.00** | 0.39 | **0.61** | **0.64** | **0.77** | **0.82** | -0.10 |
| *Clos.* | **0.92** | 0.39 | **1.00** | **0.76** | **0.94** | 0.25 | -0.10 | 0.46 |
| $E_m$ | **0.83** | **0.61** | **0.76** | **1.00** | **0.84** | 0.55 | 0.32 | 0.26 |
| $H_{max}$ | **1.00** | **0.64** | **0.94** | **0.84** | **1.00** | 0.55 | 0.22 | 0.24 |
| r | 0.58 | **0.77** | 0.25 | 0.55 | 0.55 | **1.00** | **0.84** | -0.58 |
| λ | 0.26 | **0.82** | -0.10 | 0.32 | 0.22 | **0.84** | **1.00** | -0.52 |
| $t_{95}$ | 0.20 | -0.10 | 0.46 | 0.26 | 0.24 | -0.58 | -0.52 | **1.00** |
| Sucrose | | | | | | | | |
| Parameters | SHP | $\eta_{TVS}$ | *Clos.* | $E_m$ | $H_{max}$ | r | λ | $t_{95}$ |
| SHP | **1.00** | **0.80** | **0.96** | **0.73** | **1.00** | 0.20 | -0.09 | **0.70** |
| $\eta_{TVS}$ | 0.80 | **1.00** | **0.85** | 0.51 | **0.82** | 0.29 | 0.14 | 0.56 |
| *Clos.* | **0.96** | **0.85** | **1.00** | **0.76** | **0.97** | 0.13 | -0.11 | **0.69** |
| $E_m$ | **0.73** | 0.51 | **0.76** | **1.00** | **0.74** | 0.31 | 0.26 | 0.53 |
| $H_{max}$ | **1.00** | **0.82** | **0.97** | **0.74** | **1.00** | 0.22 | -0.08 | **0.69** |
| r | 0.20 | 0.29 | 0.13 | 0.31 | 0.22 | **1.00** | 0.46 | -0.34 |
| λ | -0.09 | 0.14 | -0.11 | 0.26 | -0.08 | 0.46 | **1.00** | 0.11 |
| $t_{95}$ | **0.70** | 0.56 | **0.69** | 0.53 | **0.69** | -0.34 | 0.11 | **1.00** |

### 3.3.3 Batch test – experiment 2

After studying the fermentation process in the inoculum type, the second set of experiments concerned the study of pH and substrate loading. Taking stock of Batch_exp_1 findings, all experiments were performed using activated sludge as inoculum. SHP curves are expressed in terms of $NLH_2$ $kgTVS^{-1}$ and are depicted in Figure 29 and Figure 30. In these figures, the experimental data are shown as single points while solid lines represent Gompertz model curves. In order to avoid visual misunderstandings, BHP2 experimental data were represented every 2 hours.

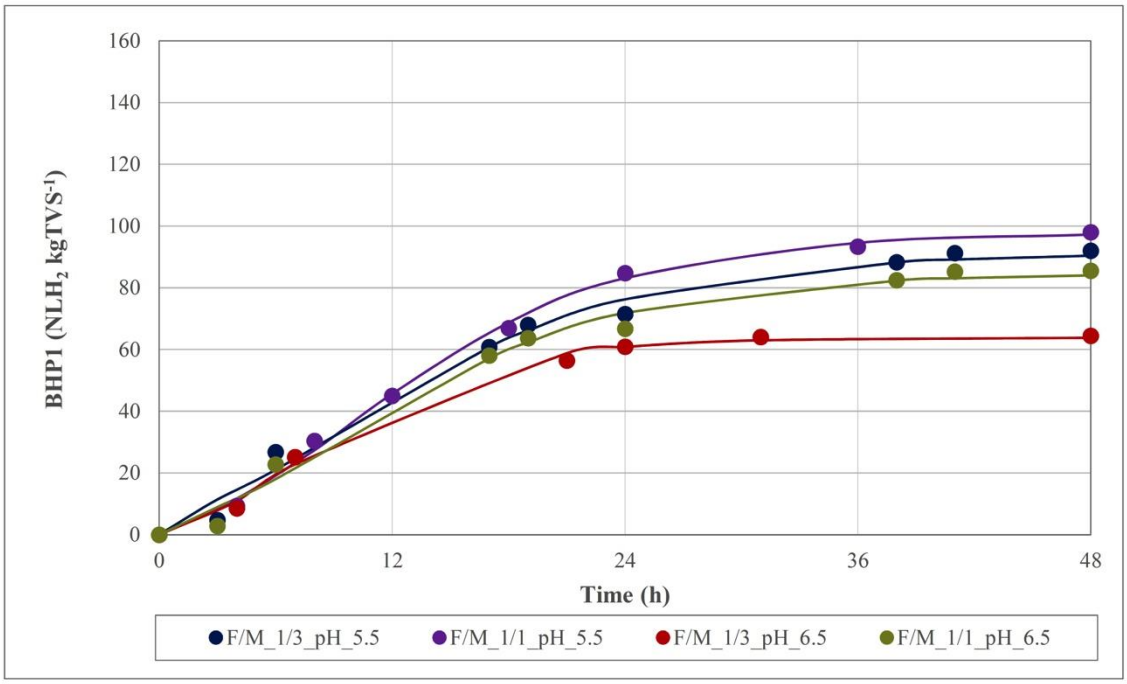


**Figure 29: Batch_exp_2 - Mean SHP curves obtained for the tests without automatic pH control (BHP1). Points indicate experimental results, solid lines Gompertz model trends.**

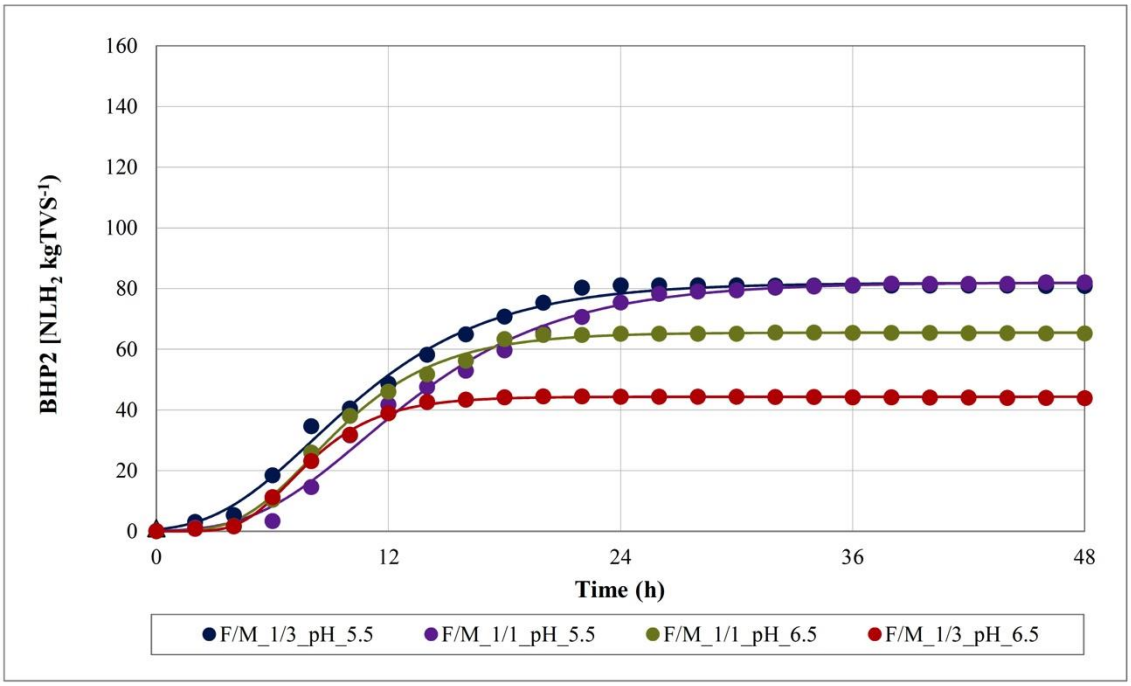


**Figure 30: Batch_exp_2 - Mean SHP curves obtained for the tests with automatic pH control (BHP2). Points indicate experimental results, solid lines Gompertz model trends.**

Also in this case, the heating of the inoculum prior to the fermentative process was proved effective since no methane was detected in the biogas over the entire duration of the experiments and the major components were only hydrogen and carbon dioxide. The final experimental SHPs were for BHP1 and BHP2, respectively: 82.3 ± 3.1 and 91.0 ± 6.4 $NLH_2$ $kgTVS^{-1}$ (pH 5.5, F/M

1/3), 82.1 ± 2.7 and 92.0 ± 0.0 $NLH_2$ $kgTVS^{-1}$ (pH 5.5, F/M 1/1), 43.9 ± 5.6 and 64.4 ± 10.5 $NLH_2$ $kgTVS^{-1}$ (pH 6.5, F/M 1/3), 65.2 ± 4.6 and 85.6 ± 18.6 $NLH_2$ $kgTVS^{-1}$ (pH 6.5, F/M 1/1). Analysis of variance performed separately for each test configuration on SHPs indicated the finding of statistically different results ($p < 0.05$). This result highlighted that the final hydrogen production was dependent on the experimental conditions, with the highest yields observed when pH was set to 5.5 and F/M to 1/1. Moreover, it concurred with previous works (Alibardi and Cossu, 2015; Chinellato et al., 2013; Favaro et al., 2013) and underlined the proper adoption of such conditions in Batch_exp_1.

As for the pH control, for this set of experiment the final pH of the digestate was also recorded at the end of BHP1. When the automatic addition of the NaOH solution was employed (BHP2), pH was rather stable all over the tests, with fluctuations within ± 0.1 units. Conversely, the initial addition of the buffer solution (BHP1) was not suitable for an adequate pH control, so that the final pH was significantly lower than the desired set value. Nevertheless, pH was in all cases found to lie above the commonly recognised threshold for potential inhibition of the hydrogenase activity (Micolucci et al., 2014). The pH decrease was found to be slightly larger for the tests performed at higher F/M ratios. More specifically, the final pH was found to be 5.3 ± 0.1 and 5.0 ± 0.0 for F/M_1/3_pH_5.5 and F/M_1/1_pH_5.5, while 5.9 ± 0.1 and 5.7 ± 0.0 for F/M_1/3_pH_6.5 and F/M_1/1_pH_6.5. This may be ascribed to the higher production of VFAs in the experiments. For BHP2, the average specific dosage of alkaline solution used for pH control was higher when the pH set point was set to 6.5, and also at the higher F/M ratio: 38.8 mL $L_r^{-1}$ (F/M_1/3_pH_5.5), 53.4 mL $L_r^{-1}$ (F/M_1/1_pH_5.5), 99.0 mL $L_r^{-1}$ (F/M_1/3_pH_6.5) and 168.0 mL $L_r^{-1}$ (F/M_1/1_pH_6.5). At pH 5.5, the experiments resulted in similar hydrogen yields suggesting the occurrence of similar metabolic pathways in the two systems (Table 23). Conversely, at pH 6.5 the hydrogen yields obtained in BHP1 tests were not achieved in BHP2. It is tempting to hypothesize that in these cases, the excessive load of sodium resulted in a toxic effect of the hydrogenase activity. Indeed, despite sodium being a micronutrient necessary for bacterial growth (Hao et al., 2006), it is demonstrated that high loads result in a suppression of hydrogen production due to a larger production of acidic metabolites deriving from non-hydrogenogenic pathways (Zumar Bundhoo and Mohee, 2016; Kim et al., 2009a, Kyazze et al., 2007). For instance, Cooney et al. (2007) observed a decrease in hydrogen production from glucose when the daily consumption of NaOH solution was increased. This phenomenon was observed together with an increase of lactate production, which is associated to a hydrogen-neutral pathway (Reaction 6, Section 3.1.1). Similarly, Kim et al. (2009a) observed a monotonic decrease of specific hydrogen production from sucrose with increasing sodium concentration.

Soluble microbial product analysis revealed that a sudden increase of the exterior sodium concentration changed the metabolic pathway such that it became favourable to lactate production while depressing butyrate production.

Concerning the process kinetics, Table 23 presents the parameters calculated using Eq. (9). The Gompertz model adopted always displayed a good degree of fitting of the experimental data ($R^2(G) > 0.99$). Similar to Batch_exp_1, it was noted that a change in the pH control method affected the process kinetics, with particular regard to $t_{95}$. Indeed, by comparing the data in Table 23, it is evident that the automatic pH control significantly enhanced the degradation rate reducing the total process duration. In particular, the $t_{95}$ values for the BHP1 runs were found to be 27-43% higher than the corresponding values for the other two sets of experiments at pH 5.5, and as much as 64-77% higher at pH 6.5. This suggests the pivotal role of accurate pH control in promoting the microbial activity of the hydrogenogenic biomass. Concerning the comparison of BHP1 and BHP2 results, a good degree of correlation was obtained for ending data such as $H_{max}$ and $t_{95}$, while, similar to Batch_exp_1, no linear correlation was found for r and λ.

**Table 23: Kinetic parameters obtained from the application of the Gompertz model: total amount of hydrogen produced ($H_{max}$ in $NLH_2$ $kgTVS^{-1}$), maximum hydrogen production rate (r in $NLH_2$ $kgTVS^{-1}$ $h^{-1}$), length of the lag phase (λ in h). The time needed to attain 95% of the maximum hydrogen yield ($t_{95}$ in h) and the linear correlation with the experimental data ($R^2(G)$) are also presented. Results of the simple regression analysis between BHP1 and BHP2 parameters are expressed by R and p-value.**

| | | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 | BHP1 | BHP2 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| pH | F/M | $H_{max}$ | $H_{max}$ | r | r | λ | λ | $t_{95}$ | $t_{95}$ | $R^2(G)$ | $R^2(G)$ |
| 5.5 | 1/3 | 81.9 | 92.0 | 5.9 | 4.6 | 3.0 | 2.1 | 23.2 | 33.1 | 0.99 | 0.99 |
| 5.5 | 1/1 | 82.0 | 93.7 | 5.3 | 3.9 | 5.0 | 0.7 | 27.4 | 34.9 | 0.99 | 0.99 |
| 6.5 | 1/3 | 44.3 | 63.8 | 6.5 | 4.2 | 4.4 | 1.6 | 14.4 | 23.6 | 0.99 | 0.99 |
| 6.5 | 1/1 | 65.5 | 84.7 | 6.6 | 3.9 | 4.3 | 1.5 | 18.9 | 33.4 | 0.99 | 0.99 |
| | R | 0.99 | | 0.01 | | - 0.92 | | 0.85 | | | |
| | *p-value* | 0.02 | | > 0.05 | | > 0.05 | | 0.05 | | | |

In conclusion, from the second set of batch experiments, it was observed that acid conditions (pH 5.5) and high organic loads (F/M 1/1 w/w) maximised the hydrogenase activity. In addition, similar to Batch_exp_1, the pH control strategy did not deeply influence the final production of hydrogen, while it played an important role in the kinetic evolution over time reducing the overall process duration. Nevertheless, when performing tests at pH 6.5, an excessive consumption of NaOH solution can lead to the inhibition of the process.

### 3.3.4 Semi-continuous trials

Semi-continuous trials were performed taking stock of batch experiments findings. Therefore, activated sludge was used as inoculum to start-up the fermentative stage, pH was set to 5.5 and the OLR was set to guarantee a high daily organic load.

Results are firstly presented by analysing process stability through pH, alkalinity and VFAs. Subsequently, single-stage and two-stage processes are compared in their anaerobic performances through biogas production, biogas quality and volatile solids removal efficiency. Finally, the energy profit is provided.

#### 3.3.4.1 Process stability

The average results of pH, IA $PA^{-1}$ ratio, TA and total VFAs obtained from the two experimental set-ups are reported in Table 24. Figure 31, Figure 32, Figure 33 and Figure 34 depict their trends over time. Figures represent the three main released organic acids: acetate, propionate and butyrate. Concerning the methanogenic reactor, the IA $PA^{-1}$ ratio is also represented and used as indicator of process stability. The trends of pH, TA, PA, IA and the daily consumption of alkaline solution in the fermentative reactor are attached as additional data in the Appendix.

**Table 24: Process stability indicators. Results are expressed in terms of averages and standard deviations.**

| Digestion (FW2) | | | | | |
|---|---|---|---|---|---|
| Scenarios | S1 | S2 | | - | |
| Reactors | R2-$CH_4$ | R1-$H_2$ | R2-$CH_4$ | - | - |
| pH | 7.33 ± 0.02 | 5.52 ± 0.02 | 7.43 ± 0.02 | - | - |
| TA ($mgCaCO_3\ L^{-1}$) | 10,557 ±424 | 6,459 ± 627 | 12,995 ± 298 | - | - |
| IA $PA^{-1}$ | 1,976 ± 307 | - | 1,840 ± 303 | - | - |
| Total VFAs ($mg\ L^{-1}$) | 1,022 ± 273 | 8,172 ± 651 | 1,033 ± 340 | - | - |
| Co-digestion (FW2+AS) | | | | | |
| Scenarios | S1 | S2 | | S3 | |
| Reactors | R2-$CH_4$ | R1-$H_2$ | R2-$CH_4$ | R1-$H_2$ | R2-$CH_4$ |
| pH | 7.02 ± 0.03 | 5.54 ± 0.02 | 7.35 ± 0.03 | 5.53 ± 0.03 | 7.44 ± 0.03 |
| TA ($mgCaCO_3\ L^{-1}$) | 8,186 ± 488 | 8,785 ±1,235 | 14,691 ± 679 | 11,696±1,073 | 17,366 ± 366 |
| IA $PA^{-1}$ | 0.16 ± 0.04 | - | 0.16 ± 0.06 | - | 0.07 ± 0.02 |
| Total VFAs ($mg\ L^{-1}$) | 267 ± 21 | 8,448 ± 884 | 364 ± 124 | 10,331 ± 518 | 269 ± 68 |

In the fermentative stage, pH was constantly kept around 5.5 all through both experimentations thanks to the addition of NaOH solution. The external control of pH was necessary to avoid the drop to values below 4, which could significantly suppress the hydrogenase activity (Zhu et al., 2011). Concerning the methanogenic stage, pH highlighted more neutral values (7.0-7.6), typical of a proper AD process (APAT, 2005).

As for the digestion of FW, IA $PA^{-1}$ ratio below 0.3 (threshold level to guarantee process stability) was reached after 28 days. This is attributable to a larger release of VFAs in the first phase of the digestion experiment with a maximum concentration that reached 3,689 mg $L^{-1}$ on day 14 (Figure 32) During this phase, propionic acid was the main product. According to Wang et al. (2009), the conversion rates of VFAs to methane vary in the order of acetic acid > butyric acid > propionic acid and an accumulation of the latter can result in a failure of methanogenesis. According to Martín-González et al. (2013), a total VFA concentration above 3,500 mg $L^{-1}$ is considered the threshold limit for process imbalance. After day 18, propionate production dropped, and stable state conditions were definitively achieved after day 28 (Figure 32).

Conversely, in the co-digestion trial, IA $PA^{-1}$ ratio was always found to be lower than 0.3 with a total concentration of VFAs in the range of 200-800 mg $L^{-1}$ (Figure 34).

As expected, fermentative reactors highlighted a significant production of VFAs. The average concentrations of the two experimentations during S2 showed comparable results of approximately 8,000 mg $L^{-1}$ while during S3 of the co-digestion trial the total concentration overtook 10.000 mg $L^{-1}$. Similar to previous studies (Cavinato et al., 2012; Lee et al., 2010; Luo et al., 2011), the prevalent acid released was butyrate, followed by acetate. This result is an indication of a proper hydrogenase activity since acetate and butyrate pathways are recognized to maximise hydrogen production yields (Section 3.1.1).

As for both the two-stage trials, the methanogenic digesters observed a progressive pH and TA increase (Table 24) together with a decrease of the IA $PA^{-1}$ ratio. These results are attributable to both the stabilisation of VFA production and to a continuous increase of alkalinity caused by an accumulation of NaOH in the reactor. As abovementioned, during the fermentative stage a 2M NaOH solution was used to avoid pH drop to values inhibiting the hydrogenase activity. Once the reactors were connected in series, the saline solution was also conveyed to the second reactor, thus increasing pH and TA. With regards to these semi-continuous experimentations, the extent of the sodium amount inside the reactors was helpful to provide stable conditions and guarantee valuable anaerobic performance. Under these conditions, sodium did not lead to any toxic effect on biogas production (Figure 37 and Figure 38). During the second scenarios, the

daily consumption of NaOH was observed being 13.4 ± 6.7 mL $L_r^{-1}$ $d^{-1}$ for the digestion of FW2 and 10.5 ± 5.6 mL $L_r^{-1}$ $d^{-1}$ for the co-digestion of FW2 and AS. By comparing these scenarios, which are characterized by the same HRT and OLR (3 d and approximately 14 kgTVS $m_r^{-3}$ $d^{-1}$), it can be highlighted that the co-digestion experiment employed on average less alkaline solution to control pH than the digestion trial. This fact is attributable to the higher intrinsic alkalinity and buffer capacity of AS (7,750 ± 55 mg$CaCO_3$ $L^{-1}$) compared to FW2 (1,300 ± 45 mg$CaCO_3$ $L^{-1}$). As stated by several authors (Jung et al., 2011; Polizzi et al., 2018), the fermentation of this protein-rich substrate (Table 19) is characterized by the release of a large amount of hydroxide ions together with ammonia ions helping to mitigate pH drop and thus consuming less external saline solution. The fermentative co-digestion of FW and AS is therefore a possibility to avoid the use of excessive amounts of sodium solution, which is an expensive solution and can result in a toxic effect (Section 3.3.3). Kyazze et al. (2007) experimented the use of a potassium solution (2M KOH), which however resulted more toxic than a sodium one. Indeed, when potassium was found above 1,420 mg $L^{-1}$ the process was totally inhibited while, at the same concentration, a sodium solution did not show any inhibitory effect. Despite the use of external alkaline solutions, the less expensive option to balance pH consists in the recirculation of the methanogenic digestate to the fermentative tank aiming at exploiting its buffer capacity (Chinellato et al., 2013; Micolucci et al., 2014). Nevertheless, this option can lead to an accumulation of ammonia, which is strongly inhibitive above 1,500 mg $L^{-1}$ (APAT, 2005). Moreover, it is a complex management solution due to the continuous changes in the recirculation ratio, which has to be continuously optimized to guarantee stable conditions inside the digester (Micolucci et al., 2014). As for the third scenario of the co-digestion trial, the higher consumption of NaOH solution (34.0 ± 9.4 mL $L_r^{-1}$ $d_{-1}$) was essentially due to the higher OLR (approximately 28 kgTVS $m_r^{-3}$ $d^{-1}$), which led to a higher release of total VFA (Table 24) that consequently promoted pH drops. A higher amount of external solution was therefore needed to maintain the pH at the desired value. Nevertheless, such sodium loads were still tolerated by anaerobic bacteria since the process did not show any inhibition and biogas production and its quality highlighted optimal results (Table 25).

In conclusion, after an initial unstable phase, both trials were characterized by process stability. The indicators (pH, IA $PA^{-1}$ ratio, VFAs) were consistent with other works showing stable performances and absence of inhibitory phenomena. Process stability was therefore also guaranteed during the periods considered as steady state, thus confirming the proper use of their data for the comparison of the scenarios.

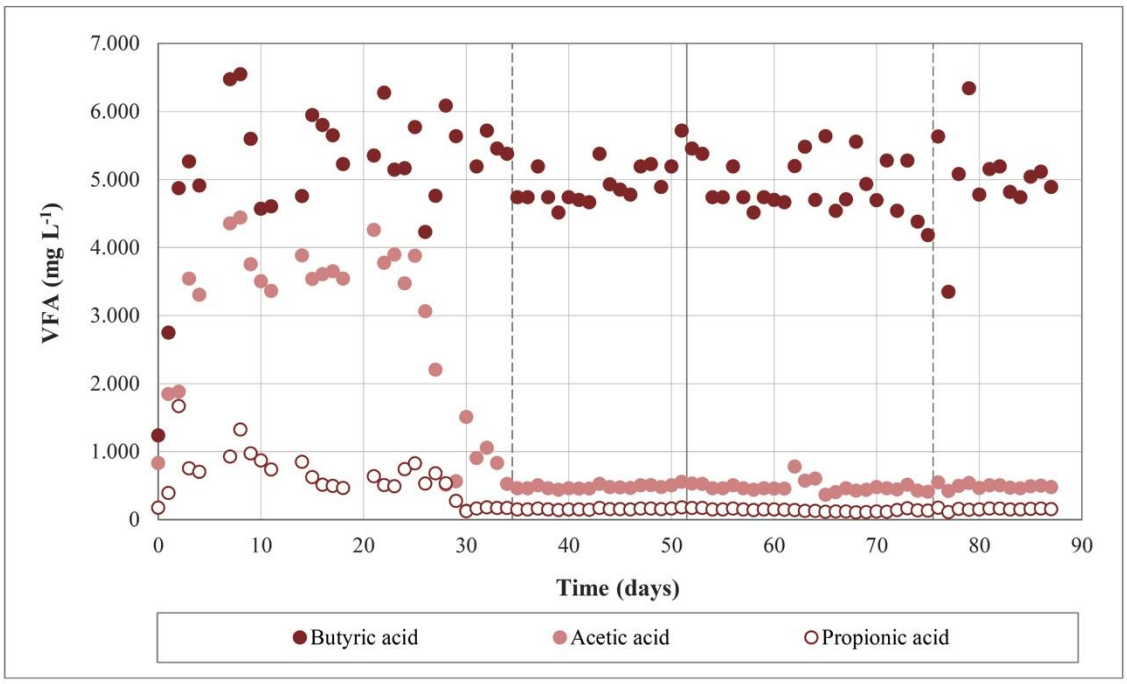


**Figure 31: Study of the two-stage technology - VFA content and AI $AP^{-1}$ ratio in R1-$H_2$ reactor during the digestion of FW2. Solid lines indicate the start of a new scenario.**

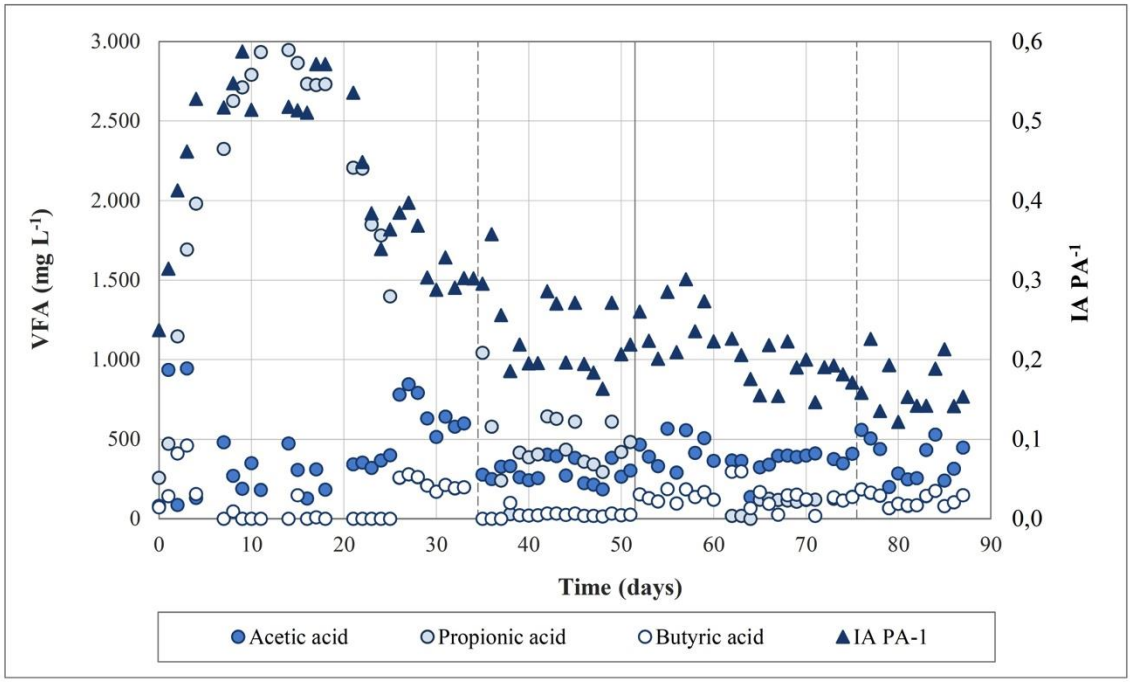


**Figure 32: Study of the two-stage technology - VFA content and AI $AP^{-1}$ ratio in R2-$CH_4$ reactor during the digestion of FW2. Dashed lines indicate the start of steady state conditions. Solid lines indicate the start of a new scenario.**

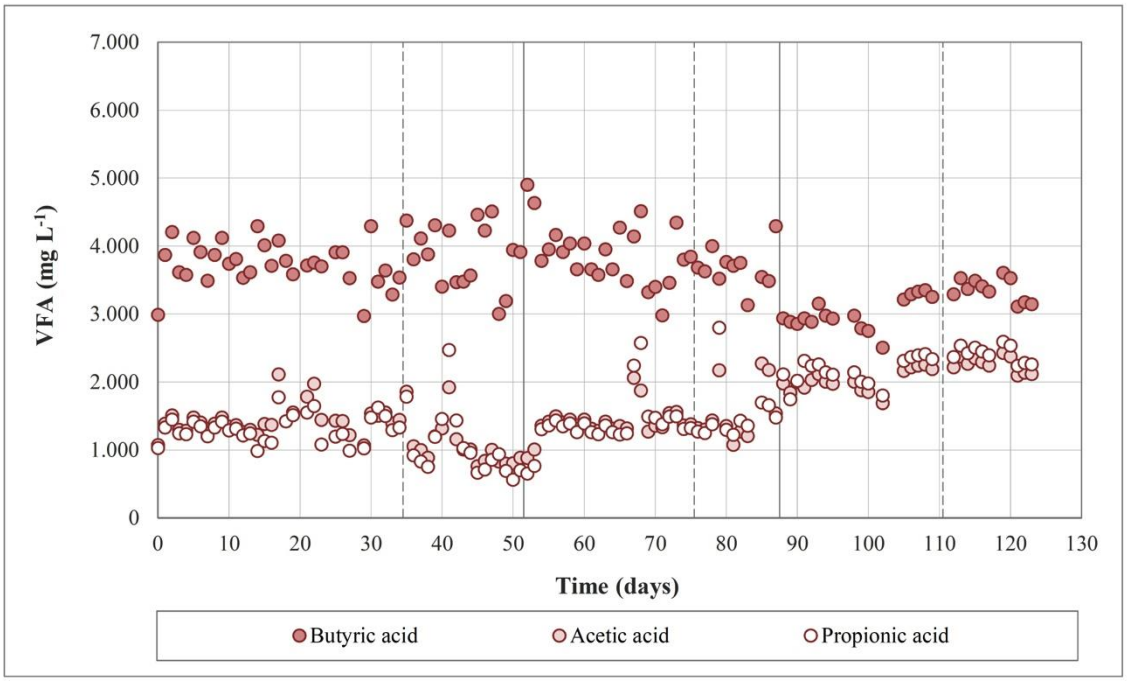


**Figure 33: Study of the two-stage technology - VFA content and AI $AP^{-1}$ ratio in R1-$H_2$ reactor during the co-digestion of FW2 and AS. Solid lines indicate the start of a new scenario.**

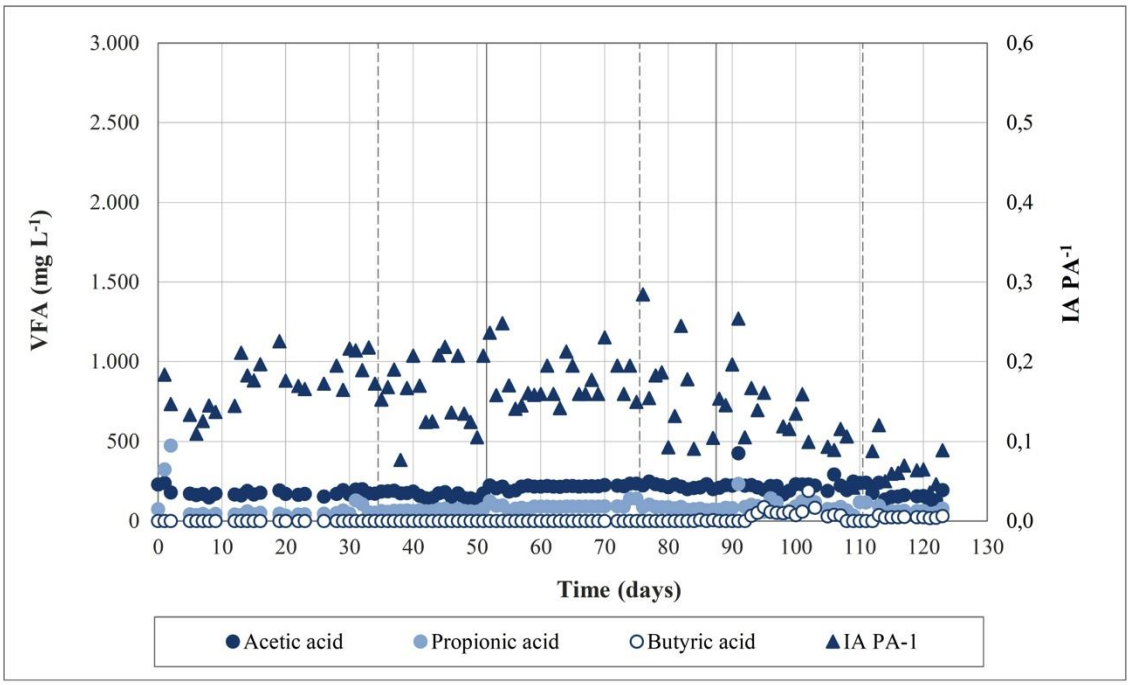


**Figure 34: Study of the two-stage technology - VFA content and AI $AP^{-1}$ ratio in R2-$CH_4$ reactor during the co-digestion of FW2 and AS. Dashed lines indicate the start of steady state conditions. Solid lines indicate the start of a new scenario.**

### 3.3.4.2 Anaerobic performances

The average results of SGP, SHP, SMP, hydrogen and methane content, and $\eta_{TVS}$ obtained from the two experimental set-ups are reported in Table 25.

**Table 25: Yields of the process. Results are expressed in terms of averages and standard deviations.**

| Digestion (FW2) | | | | | |
|---|---|---|---|---|---|
| Scenarios | S1 | S2 | | | |
| Reactors | R2-$CH_4$ | R1-$H_2$ | R2-$CH_4$ | - | - |
| SGP (NL $kgTVS^{-1}d^{-1}$) | 694.4 ± 24.6 | 43.1 ± 12.8 | 704.6 ± 28.5 | - | - |
| $H_2$ (%) | - | 22.9 ± 5.5 | - | - | - |
| $CH_4$ (%) | 65.2 ± 1.9 | - | 68.4 ± 1.1 | - | - |
| SHP ($NLH_2\,kgTVS^{-1}d^{-1}$) | - | 12.6 ± 5.0 | - | - | - |
| SMP ($NLCH_4\,kgTVS^{-1}d^{-1}$) | 453.1 ± 28.2 | - | 482.1 ± 24.0 | - | - |
| $\eta_{TVS}$ (%) | 67.0 ± 2.0 | 23.5 ± 4.0 | 62.5 ± 2.7 | - | - |
| Co-digestion (FW2+AS) | | | | | |
| Scenarios | S1 | S2 | | S3 | |
| Reactors | R2-$CH_4$ | R1-$H_2$ | R2-$CH_4$ | R1-$H_2$ | R2-$CH_4$ |
| SGP (NL $kgTVS^{-1}d^{-1}$) | 485.9 ± 25.8 | 44.8 ± 12.6 | 611.0 ± 45.4 | 61.5 ± 17.2 | 668.4 ± 50.3 |
| $H_2$ (%) | - | 18.4 ± 6.3 | - | 20.2 ± 4.5 | - |
| $CH_4$ (%) | 61.2 ± 2.2 | - | 70.1 ± 1.6 | - | 73.1 ± 1.5 |
| SHP ($NLH_2\,kgTVS^{-1}d^{-1}$) | - | 8.6 ± 4.8 | - | 12.0 ± 5.5 | - |
| SMP ($NLCH_4\,kgTVS^{-1}d^{-1}$) | 298.0 ± 24.5 | - | 428.3 ± 30.9 | - | 487.2 ± 29.5 |
| $\eta_{TVS}$ (%) | 61.0 ± 2.2 | 32.3 ± 4.4 | 54.5 ± 4.1 | 32.4 ± 4.8 | 59.8 ± 4.9 |

Figure 35 and Figure 36 show the composition of biogas in terms of methane and hydrogen contents over time.

As previously highlighted in Figure 32 and Figure 34, in the two-stage process, methanogenesis almost completely degraded the organic acids produced in the fermentative stage. The utilization ratios of acetate and butyrate were beyond 52.5% and 97.0% in the digestion trials, and beyond 84.5% and 99.0% in the co-digestion trials, respectively. These significant degradations were consistent with previous works (Lee et al., 2010; Luo et al., 2011; De Gioannis et al., 2017) and were closely linked to an increase in biogas production and in methane content that was generated following the acetoclastic pathway. During S2 and S3, methane content gradually increased with time with peaks of 70.7% for the digestion trial and 76.3% for the co-digestion experiment. The two-stage process enabled an average enrichment of

methane by respectively 3.2% (S2, digestion trial), 8.9% (S2, co-digestion trial) and 11.9% (S3, co-digestion trial) when compared to the traditional one-stage system. This is consistent with Voelklein et al. (2016) and De Gioannis et al. (2017), who stated that an acidogenic digester might serve as a carbon dioxide stripping step, thus reducing the potential costs for upgrading the biogas to biomethane. This higher methane production is essentially due to the improved hydrolysis of substrates in the first stage, with the production of relevant amounts of VFAs, which were readily available to methanogens in the second stage (De Gioannis et al., 2017).

As for the fermentative reactor, methane was never detected. The initial thermal treatment of inoculum and process conditions, such as acid pH and low HRT, were therefore efficient in inhibition and wash out of hydrogenotrophic methanogens. The average hydrogen content in biogas was 22.9% (S2, digestion trial), 18.4% (S2, co-digestion trial) and 20.2% (S3, co-digestion trial) with peaks of 42.1% and 37.0% for the digestion and the co-digestion trials, respectively. Such concentrations are comparable to previous studies. Cavinato et al. (2012) highlighted hydrogen concentrations in the range of 19-37% while Micolucci et al. (2014) reported an average content of 25 ± 9% using FW as substrate.

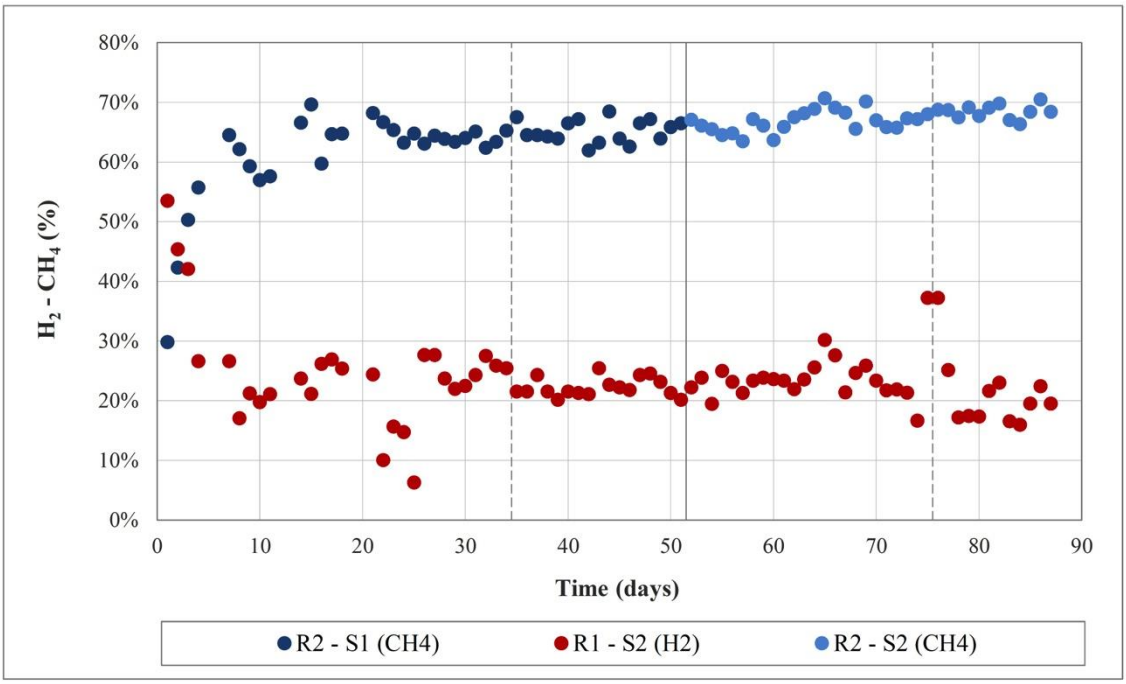


**Figure 35: Study of the two-stage technology – Hydrogen and methane content in biogas, respectively in R1-$H_2$ and R2-$CH_4$ reactors during the digestion of FW2. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

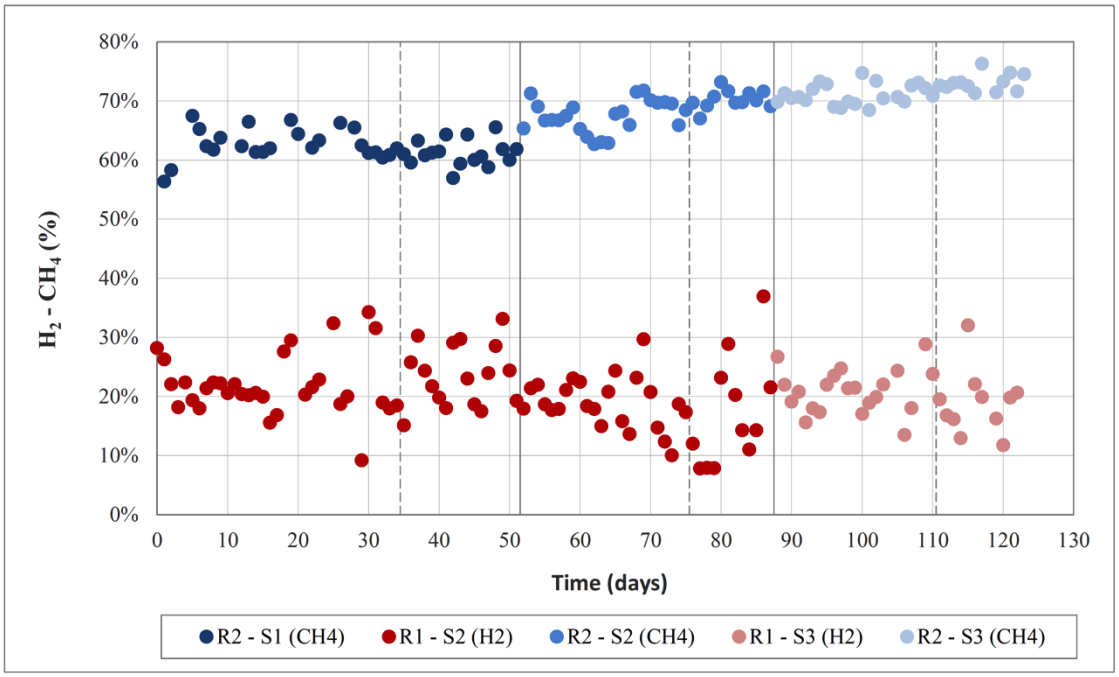


**Figure 36: Study of the two-stage technology – Hydrogen and methane content in biogas, respectively in R1-$H_2$ and R2-$CH_4$ reactors during the co-digestion of FW2 and AS. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

Figure 37 and Figure 38 illustrate the time course of biogas production in the two configurations of digestion and co-digestion. After a first unstable phase, biogas was continuously generated in both reactors without inhibition problems. This result was achieved thanks to an overall process stability previously evaluated in terms of VFAs, alkalinity and pH.

Comparing the two scenarios, the two-stage improvement in methane content was accompanied by an increase in biogas generation. The methanogenic reactor highlighted a slight improvement for the digestion study (+1.4%), while in the co-digestion experiment the average increase was around 38% in the third scenario. Considering the whole two-stage system, i.e. the sum of the biogas productions of the first and the second digester, these percentages increased up to 7.7% and 50.2%. As for the digestion of FW2, SGP and SMP results were in the range of results of previous works adopting the two-stage technology. Chinellato et al. (2013) observed a SGP of 728 NL $kgTVS^{-1}$ $d^{-1}$ and a SMP of 484 $NLCH_4$ $kgTVS^{-1}$ $d^{-1}$ using HRTs of 3 d and 12 d and OLRs of 15 kgTVS $m^{-3}d^{-1}$ and 3 kgTVS $m^{-3}$ $d^{-1}$ for the fermentative and the methanogenic reactor, respectively. Similarly, Cavinato et al. (2012) obtained a SGP of 640 NL $kgTVS^{-1}$ $d^{-1}$ with an average methane content of 65%. In this case, the two-stage technology was performed using HRTs of 3.3 d and 12.6 d and OLRs of 16 kgTVS $m^{-3}$ $d^{-1}$ and 4 kgTVS $m^{-3}$ $d^{-1}$ for the fermentative and the methanogenic reactor, respectively. Regarding the single-stage co-digestion of FW and AS, the review study of Iacovidou et al. (2012) highlighted SMP in the range of 186-

346 $NLCH_4$ $kgTVS^{-1}$ $d^{-1}$, concluding that methane production was directly related to the amount of FW in the mixture.

As for the fermentative tank, the SGP was found to be significantly lower than the methanogenic reactor, with the two experiments showing comparable results of about 45 NL $kgTVS^{-1}$ $d^{-1}$. In the matter of hydrogen generation, the co-digestion tests showed lower productions than the digestion trial. This may be attributable to the lower content of carbohydrates in the mixture FW+AS than in the FW mash. Indeed, as highlighted from Table 19 and previous studies, FW is a carbohydrate-rich substrate (Iacovidou et al., 2012; Pecorini et al., 2018), while AS is mainly composed of proteins (Pecorini et al., 2018; Wilson and Novak, 2009). The correlation between hydrogen production and the carbohydrates content of the substrate was studied by Alibardi and Cossu (2016), who found a linear relation between the two variables. Conversely, the same study highlighted that proteins and lipids did not produce significant contributions to hydrogen generation. The final SHP values were in the same order of magnitude of hydrogen yields of other studies using similar reactor conditions. As such, SHP values of 1, 51.2 and 66.7 $NLH_2$ $kgTVS^{-1}$ $d^{-1}$ were obtained by Chinellato et al. (2013), Cavinato et al. (2012), and Cavinato et al. (2011), respectively. Conversely, Chu et al. (2008), using an HRT of 1.3 d, obtained a SHP of 205 $NLH_2$ $kgTVS^{-1}$ $d^{-1}$, thus suggesting that the use of low HRT can optimize hydrogen production.

Concerning $\eta_{TVS}$, Table 24, Figure 39 and Figure 40 show an overall reduction of degradation of the organic matter in the methanogenic reactor. More specifically, the average value decreased from 67.0% to 62.5% and from 61.0% to 54.5% (S2) for the digestion and the co-digestion study, respectively. This was due to the volatile solids content of the incoming substrate of the methanogenic reactor. Indeed, while during S1 the reactor was fed with the pure substrates (FW2 and FW2+AS mashes), during S2 it was fed with the outgoing digestate of the fermentative tank that was already partially degraded. Indeed, while FW mash and the mixture FW2+AS had a TVS content of approximately 4% w/w, the outgoing digestate of the fermentative tank presented an average TVS content of around 3% w/w. Taking into account the whole two-stage process, i.e. considering $TVS_{IN}$ as the volatile content of the incoming substrate of the first reactor and $TVS_{OUT}$ as the volatile substance of the outgoing digestate of the second tank, the two final $\eta_{TVS}$ values of S2 were calculated to be 69.4% and 71.5%, 6.8% and 8.4% more than S1 while the final $\eta_{TVS}$ value of S3 of the co-digestion trial observed a reduction of the volatile matter of approximately 73.5%, with an increase of 13.9% compared to S1.

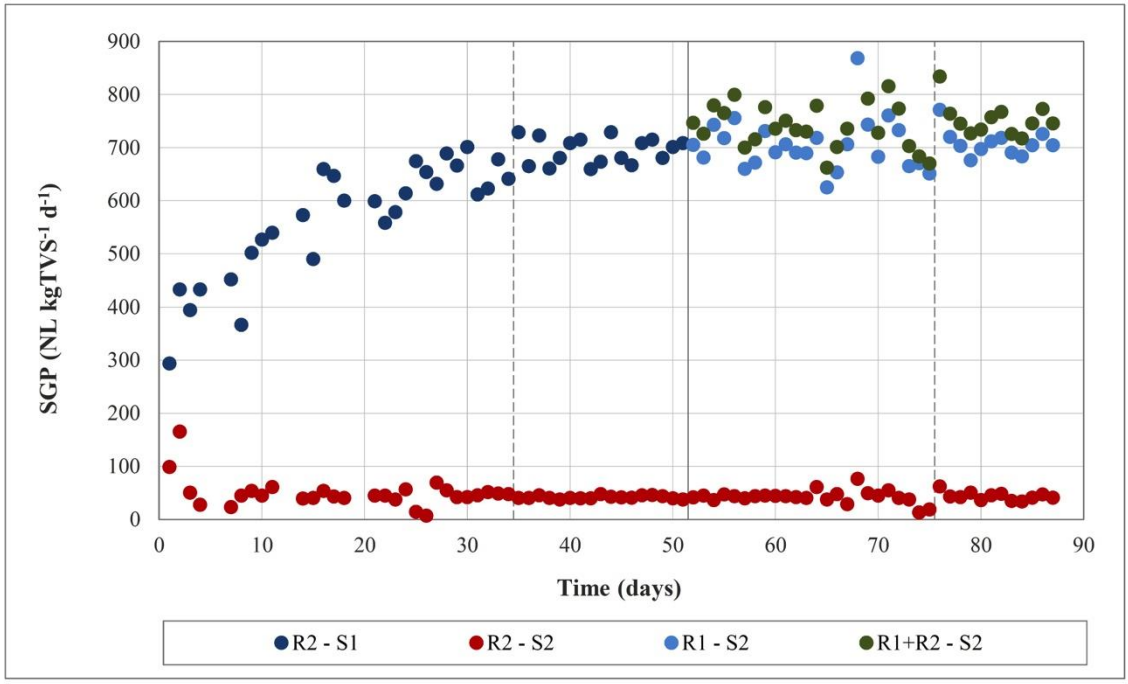


**Figure 37: Study of the two-stage technology – SGP of R1-$H_2$ and R2-$CH_4$ reactors during the digestion of FW2. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

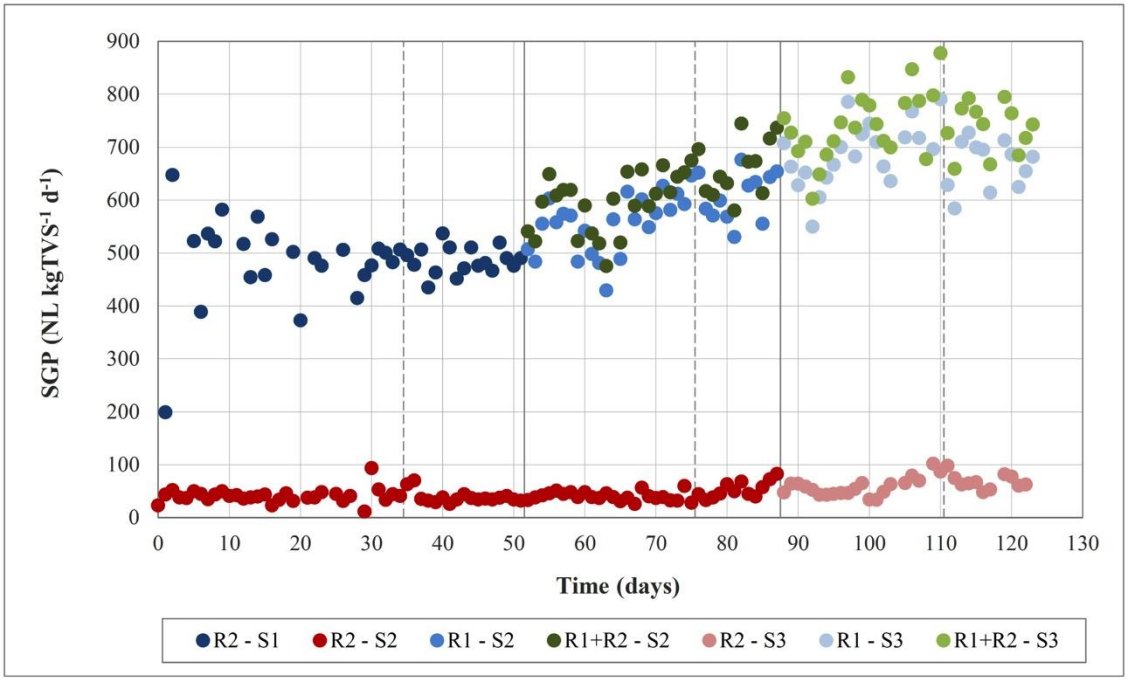


**Figure 38: Study of the two-stage technology – SGP of R1-$H_2$ and R2-$CH_4$ reactors during the co-digestion of FW2 and AS. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

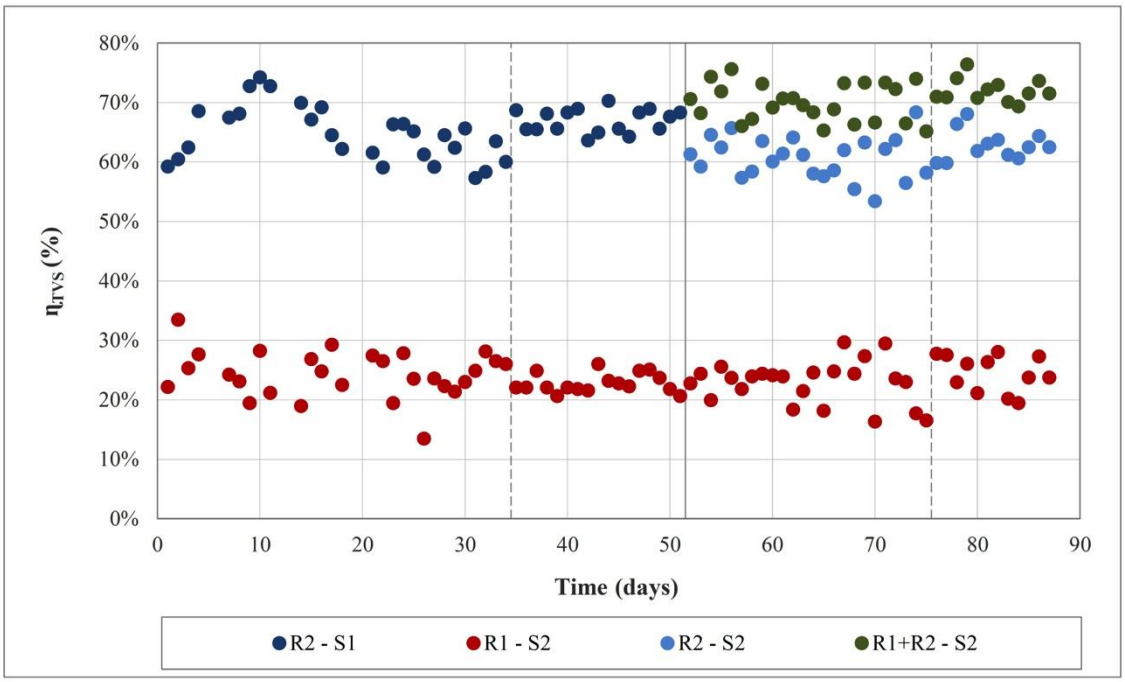


**Figure 39: Study of the two-stage technology – Volatile solids removal efficiencies during the digestion of FW2. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

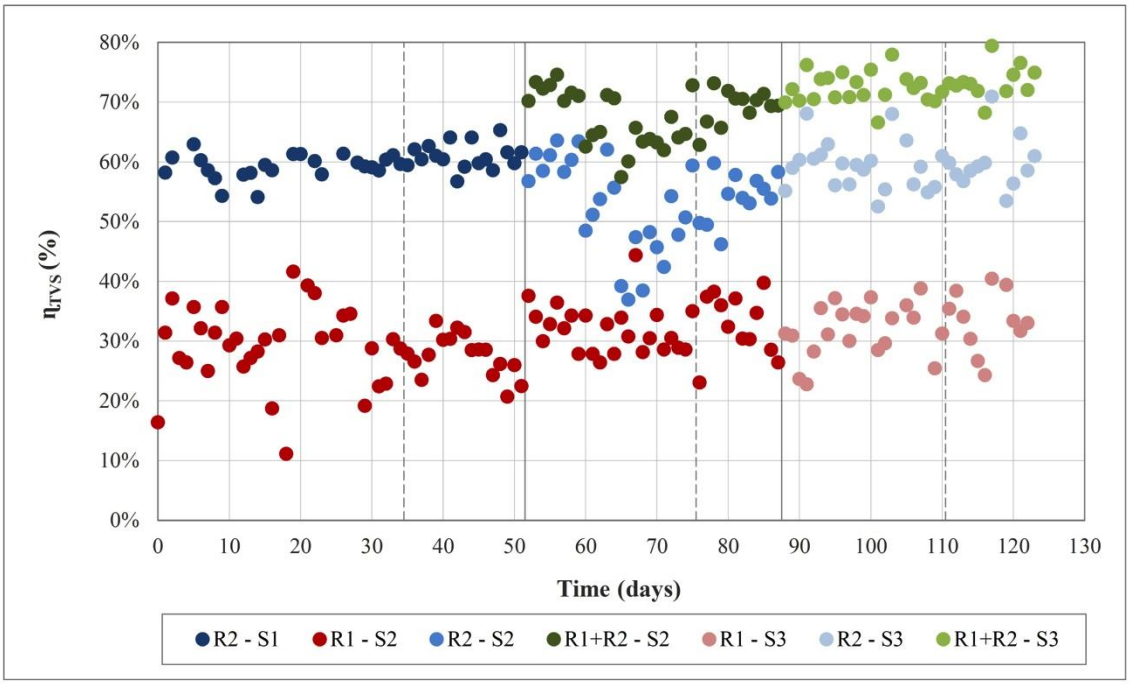


**Figure 40: Study of the two-stage technology – Volatile solids removal efficiencies during the co-digestion of FW2 and AS. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

### 3.3.4.3 Energy profit

Table 26 presents the energy profit of the two-stage technology compared to the traditional one-stage system. Despite the increases in biogas production and the significant methane and hydrogen contents in biogas, the energy balance resulted negative.

This finding was due to the high-energy demand ($E_D$) requested by the functioning of a new fermentative digester. Among the energy consumption items, mixing accounted for the 80.6% ± 7.2% of the total energy demand, followed by heating (15.4% ± 5.1%) and the energy required by the peristaltic pump (4.0% ± 2.1%). The final negative result was probably altered by the adoption of laboratory scale conditions, which can play a determinant role in energy consumptions and energy productions. Nevertheless, life cycle assessment studies (Francini et al., 2018; Albini et al., 2018) performed on the co-digestion of OFMSW and sewage sludge confirmed the energetic unsuitableness of the two-stage technology compared to the traditional process. According to Francini et al. (2018), the one-stage scenario had a better environmental performance in comparison to the case of co-fermentation, mainly due to the higher energy recovery and the higher compost production.

Considering the energy produced in the form of biogas ($E_B$), the primary contribution was provided by the increase in methane production (SMP) rather than by the production of hydrogen (SHP) in the fermentative stage. Therefore, the energy produced from the increase in methane production accounted for the 94.9% ± 5.6% of the total $E_B$ owing to the higher LHV of methane with respect to hydrogen. This statement confirms that the benefits of the two-stage technology are mainly attributable to the improvement of the second methanogenic stage, which biogas is characterized by a significantly higher content of methane compared to the traditional one-stage process. The reduction of the HRT to 1.5 d and the related increase in the OLR resulted in an additional increase of the anaerobic performances and the related energy balance, suggesting that a further reduction of the HRT can obtain even better results. For instance, Koutrouli et al. (2009) obtained the best results with a HRT of 0.6 d by performing the two-stage technology using olive pulp as substrate.

**Table 26: Energy profit of the two-stage technology.**

| Trial | Scenario | $E_B$ (kJ kgTVS$^{-1}$) | $E_Q$ (kJ kgTVS$^{-1}$) | $E_D$ (kJ kgTVS$^{-1}$) | $E_T$ (kJ kgTVS$^{-1}$) |
|---|---|---|---|---|---|
| Digestion (FW2) | S2 | 1,057 | 1,322 | - 14,082 | - 11,704 |
| Co-digestion (FW2+AS) | S2 | 4,282 | 1,360 | - 14,438 | - 8,796 |
| | S3 | 6,213 | 1,360 | - 8,484 | - 911 |

## 3.4 Conclusions

In the second line of research, the digestion of FW and the co-digestion of FW and AS were evaluated in a two-stage anaerobic system and compared to the traditional single-stage process. Batch tests were carried out as preliminary tools to obtain the best treatment conditions to be successively applied in semi-continuous mode. Therefore, hydrogen production from the fermentative stage was studied by varying pH, F/M ratio, type of inoculum and pH control strategy. Finally, the feasibility of the two-stage process was assessed through biogas production, biogas quality (methane and hydrogen content), volatile matter degradation and energy profit (Table 27).

The first set of batch experiments (Batch_exp_1) highlighted that the hydrogenic process was highly dependent on the type of inoculum. In particular, assays performed with an aerobic inoculum such as activated sludge showed higher specific hydrogen productions and volatile solids removal efficiencies compared to anaerobic inocula. The presence of high concentration of Clostridia in the aerobic media played a key role in the development of an optimal fermentative process.

The second set of batch experiments (Batch_exp_2) observed that acid conditions (pH 5.5) and high organic loads (F/M 1/1 w/w) maximised the hydrogenase activity. Moreover, similar to Batch_exp_1, the pH control strategy did not deeply influence the final production of hydrogen, while it played an important role in the kinetic evolution over time reducing the overall process duration. Nevertheless, an excessive addition of sodium solution, observed for tests performed at pH 6.5, can lead to process inhibition.

Two-stage semi-continuous trials obtained two important results in terms of anaerobic performances: in analogy with literature studies the confirmation of the improvement of the anaerobic digestion of FW and the suitability of the application of this technology to the co-digestion of FW and AS. As for the co-digestion trial, the enhancement of biogas production and biogas quality were even higher than the two-stage digestion of the sole FW. As for the specific biogas production, results observed an overall increase of 50%. Concerning gas quality, the two-stage system observed a hydrogen rich biogas in the first fermentative reactor (on average 20.2%) and an improvement of methane content in the second methanogenic digester, which shifted from 61.2% obtained for the one-stage experiment to 73.5%. Such increases were due to the improvement of substrate hydrolysis. Indeed, besides the production of hydrogen, the first fermentative stage acted as a pretreatment degrading the complex organic matter and releasing

significant amounts of VFAs being readily available in the second stage. Furthermore, the co-digestion configuration showed improved process stability compared to FW digestion, observing a lower consumption of alkaline solution for pH control due to the intrinsic buffer capacity of AS. Other additional advantages of the two-stage process are associated to the overall reduction of the HRT (shifting from 17 d of the one-stage process to 1.5 d + 12 d of the two-stage system) and the higher removal of volatile solids (+ 14%). As such, the reduction of the HRT implies a reduction of digester volume and investment costs while the increase in volatile solids removal is associated to a higher degree of digestate stabilisation, which is a relevant issue when considering its final disposal.

**Table 27: Summary of the best results obtained employing the two-stage technology.**

| Parameter | Two-stage technology |
|---|---|
| Inoculum type | Aerobic activated sludge |
| pH | 5.5 |
| pH control strategy | Automatic addition of NaOH solution |
| F/M (w/w) (for batch tests) | 1/1 |
| OLR (kgTVS $m_r^{-3}$ $d^{-1}$) | 27.6 |
| HRT (d) | 1.5 |
| Specific hydrogen production ($NL H_2$ $kgTVS^{-1}$ $d^{-1}$) | 20.2 |
| Biogas production increase – SGP (%) | + 50.2% |
| Methane production increase - SMP (%) | + 63.5% |
| Volatile matter degradation increase - $\eta_{TVS}$ (%) | + 13.9% |
| Energy balance (kJ $kgTVS^{-1}$) | - 911 |

Nevertheless, such process benefits were not able to counteract the energy expended for the operation of a new fermentative digester. The energy balance was found negative and no energy profit was obtained from any of the tested conditions. Focusing on the energy items of the balance, mixing was the prevalent energy demand item while, considering the energy produced in the form of biogas, the primary contribution was provided by the increase in methane production rather than by the production of hydrogen from the fermentative stage. This statement confirmed that the benefits of the two-stage technology were mainly attributable to the improvement of the second methanogenic stage, which biogas was characterized by a significantly higher content of methane compared to the traditional one-stage process. From this perspective, the operation of a first fermentative reactor has to be considered as a pretreatment of AD rather than a technology to produce hydrogen. Aiming at obtaining higher biogas

productions, which can balance the energy expenses, previous studies obtained significant results further reducing the fermentative HRT. Therefore, future experiments should consider performing the fermentative process with HRT in the range of hours.

# CHAPTER 4 - DISCUSSION AND CONCLUSIONS

## 4.1 Overall discussion of the results

This section aims at comparing the results of the two lines of research. Table 28 summarizes the outcomes in terms of anaerobic performances and final energy profits.

What is relevant from Table 28 is the increase in the anaerobic performances of each of the tested technologies compared to the reference scenario of the traditional one-stage digestion. Nevertheless, the results of the present research highlighted that the improvements of the anaerobic performances were not sufficient to counteract the energy demand.

The energy expenses for the thermal treatments and the mixing of an additional digester were relevant items in the energy balance of the three technologies. Concerning the produced energy, the recovery of heat after thermal treatments was the main item for autoclaving and microwaving. This result was due to the high temperature reached by the substrate after the treatment (> 95 °C) and to the limited increase of methane production. On the other hand, as for the two-stage process, the primary contribution was provided by the increase of methane production rather than by the production of hydrogen from the fermentative stage. This statement confirmed that the benefits of the two-stage technology were mainly attributable to the improvement of the second methanogenic stage, which biogas was characterized by a significantly higher content of methane compared to the traditional one-stage process. From this perspective, the operation of a first fermentative reactor has to be considered as a pretreatment of AD rather than a technology to produce hydrogen.

Proteins, polysaccharides and lipids are converted in VFAs by the fermentative microflora. In this way, the complex and often rate-limiting step of hydrolysis is performed separated from the methanogenic reactor, which therefore increases methane production thanks to the digestion of an easily biodegradable organic compounds compared to the digestion of the raw substrate.

Nevertheless, at industrial scale, the use of a CHP unit for biogas valorisation can produce enough heat to warm up the primary digester and contribute to the energy demand of a new fermentative digester ($E_h$) or a thermal pretreatment section ($E_D$). Based on this hypothesis, the energy balance can undergo positive changes and the application of the studied technologies would increase in value.

**Table 28: Comparison of the results of the two lines of research.**

| Parameter | MW | A | DF | DF |
|---|---|---|---|---|
| Substrate | OFMSW 11% fibres | OFMSW 25% fibres | OFMSW 100% FW | Co-digestion FW+AS |
| Biogas production increase – SGP (%) | + 19.0% | + 8.9% | + 7.7% | + 50.2% |
| Methane production increase - SMP (%) | + 18.1% | + 11.5% | + 6.4% | + 63.5% |
| Volatile matter removal increase - $\eta_{TVS}$ (%) | + 10.5% | + 11.1% | + 6.8% | + 13.9% |
| Energy balance (kJ $kgTVS^{-1}$) | - 338 | - 2,291 | - 11,704 | - 911 |

In order to define the best solution to improve the anaerobic performances of the digestion of the OFMSW, a first rapid answer could be given taking into account the energy outcomes provided in Table 28. From this table it is evident that, despite the negative results, MW highlighted the best energetic response. Nevertheless, the pretreatment choice would be short-sighted if it was based only on the energy assessment. As such, the composition of the OFMSW plays a fundamental role in the selection of the proper technology. MW and DF would not be equally effective on a highly lignocellulosic substrate and, on the contrary, autoclaving would not be equally effective on a more biodegradable substrate such as FW or the mixture of FW and AS. In fact, from the present research it was noted that autoclaving is preferable when OFMSW is highly lignocellulosic (commonly when it is originated from garden waste), microwaving when the concentration of lignocellulosic fraction is limited and dark fermentation when OFMSW is mainly composed of FW, which is a carbohydrate-rich substrate, suitable for fermentative bacteria. Concerning the DF, the research highlighted that it is a suitable option to improve also the co-digestion of FW and AS and it is characterized by a higher versatility compared to the other two thermal treatments. Under this perspective, the relevant production of organic acids during the fermentative stage can be also used for other purposes of the anaerobic biorefinery rather than to produce methane. One of these is the conversion of VFAs into bio-polyesters, as for instance the polyhydroxyalkanoates.

## 4.2 Conclusions

The aim of the present research was to evaluate the improvement of the AD of the OFMSW adopting microwave and autoclave pretreatments and the two-stage technology. Such overall objective was evaluated through semi-continuous trials, which were performed according to the achievement of specific objectives, i. e. the determination of the optimal process conditions. As such, preliminary batch tests were performed to investigate treatment duration, amount of substrate to be processed and adequacy of the treatment to substrate composition for the study of pretreatments and, inoculum type, food-to-microorganism ratio, pH and pH control mode for the study of biohydrogen production.

As for the specific objectives:

1. Pretreatments: batch experiments observed that, even if the most severe treatment obtained the highest methane production, the best energetic response was found for the lighter treatment due to its lower energy demand. As for microwaving, 4 minutes on a substrate sample of 0.5 kg. Concerning the adequacy of the treatment to substrate composition, results in terms of methane production, macromolecules solubilisation and energy balance highlighted a better response of microwaving on a substrate with low lignocellulosic content (M1, 11% w/w fibres) and autoclaving on a substrate rich in fibres (M2, 25% w/w fibres). Furthermore, the increase in methane production was found directly related to the release of soluble COD after the treatments.
2. Two-stage technology: batch experiments highlighted that the hydrogenic process was highly dependent on the type of inoculum, pH control strategy, pH and F/M conditions. In particular, assays performed with an aerobic inoculum such as activated sludge showed higher specific hydrogen productions and volatile solids removal efficiencies compared to anaerobic inocula. The presence of high concentration of Clostridia in the aerobic media played a key role in the development of an optimal fermentative process. Furthermore, acid conditions (pH 5.5) and high organic loads (F/M 1/1 w/w) maximised the hydrogenase activity. As for the pH control strategy, the automatic addition of a 2M sodium hydroxide solution, did not deeply influence the final production of hydrogen, while it played an important role in the kinetic evolution over time reducing the overall process duration.

As for the overall improvement of the anaerobic performances:

1. Pretreatments: Semi-continuous trials confirmed the adequacy of the application of microwaving on a substrate with low lignocellulosic content (M1) and autoclaving on a substrate rich in fibres (M2). The adoption of semi-continuous trials amplified the methane and biogas production increases found in batch tests. Therefore, SMP observed an increase of 18.1% for microwaving on M1, and 11.5% for autoclaving on M2. This could be attributable to the optimal pH and alkalinity conditions observed inside the digester. The buffer capacity developed into the semi-continuous reactor was able to minimize acidification problems and total ammonia nitrogen loading. Concerning the volatile solids removal efficiency, results reflected the outcomes obtained for the SMP. Microwaving highlighted an increase of the degradation of the volatile matter of approximately 10.5% for M1. Similarly, an increase of about 11.1% was obtained for autoclaving on M2. As for the energy balance, thanks to the higher increase of methane production, the energy profit observed a relevant improvement for semi-continuous trials compared to batch tests. Nevertheless, the balance was found negative. The improvement of the anaerobic performances did not counteract the energy expended for the treatments. Among the forms of produced energy, $E_Q$ was the prevalent. As for MW, $E_Q$ accounted for approximately the 65% of the total energy produced while for A, this percentage increased up to 83-87%. This result was mainly due to the high temperature of the media after the treatment and to the limited increase in methane generation. As such, the heat of the organic mass after the treatment is a relevant item to take advantage. By comparing the two treatments, it can be highlighted that MW resulted in a better energetic response than A mainly due to the lower energy demand, which was approximately 30% less than A.
2. Two-stage technology: semi-continuous trials obtained two important results in terms of anaerobic performances: in analogy with literature studies the confirmation of the improvement of the anaerobic digestion of FW and the suitability of the application of this technology to the co-digestion of FW and AS. As for the co-digestion trial, the enhancement of biogas production and biogas quality were even higher than the two-stage digestion of the sole FW. As for the specific biogas production, results observed an overall increase of 50%. Concerning gas quality, the two-stage system observed a hydrogen rich biogas in the first fermentative reactor (on average 20.2%) and an improvement of methane content in the second methanogenic digester, which shifted from 61.2% obtained for the one-stage experiment to 73.5%. Such increases were due to the improvement of substrate hydrolysis. Indeed, besides the production of hydrogen, the first fermentative stage acted

as a pretreatment degrading the complex organic matter and releasing significant amounts of VFAs being readily available in the second stage. Furthermore, the co-digestion configuration showed improved process stability compared to FW digestion, observing a lower consumption of alkaline solution for pH control due to the intrinsic buffer capacity of AS. Other additional advantages of the two-stage process are associated to the overall reduction of the HRT (shifting from 17 d of the one-stage process to 1.5 d + 12 d of the two-stage system) and the higher removal of volatile solids (+ 14%). As such, the reduction of the HRT implies a reduction of digester volume and investment costs while the increase in volatile solids removal is associated to a higher degree of digestate stabilisation, which is a relevant issue when considering its final disposal. Nevertheless, such process benefits were not able to counteract the energy expended for the operation of a new fermentative digester. The energy balance was found negative and no energy profit was obtained from any of the tested conditions. Focusing on the energy items of the balance, mixing was the prevalent energy demand item while, considering the energy produced in the form of biogas, the primary contribution was provided by the increase in methane production rather than by the production of hydrogen from the fermentative stage. This statement confirmed that the benefits of the two-stage technology were mainly attributable to the improvement of the second methanogenic stage, which biogas was characterized by a significantly higher content of methane compared to the traditional one-stage process. From this perspective, the operation of a first fermentative reactor has to be considered as a pretreatment of AD rather than a technology to produce hydrogen.

In conclusion, microwaving, autoclaving and dark fermentation are suitable pretreatments that improve the anaerobic digestion of the OFMSW. The adoption of such technologies is useful the anaerobic process making the most complex organic substance easier to bacteria attack. In this way, the anaerobic microflora is able to degrade a greater quantity of organic matter, showing, as a result, an increase of methane production and volatile solids removal. Their application is closely related to the characteristics of the incoming substrate. As such, autoclaving is preferable when OFMSW is highly lignocellulosic (commonly when it is originated from garden waste), microwaving when the concentration of lignocellulosic fraction is limited and dark fermentation when OFMSW is mainly composed by FW, which is a carbohydrate-rich substrate, suitable for fermentative bacteria. Concerning the dark fermentation, it is a suitable solution to improve also the co-digestion of FW and AS and is characterized by a higher versatility compared to the other two thermal treatments. Under this perspective, the relevant production of organic acids during the fermentative stage can be also used for other purposes of the anaerobic biorefinery rather than the

production of methane. One of these is the conversion of VFAs into bio-polyesters as for instance the polyhydroxyalkanoates.

Nevertheless, the results of the present research highlighted that the improvement of the anaerobic performances was not sufficient to counteract the energy expended for the operation of the three tested technologies. In order to close the energy balance positively, the future studies have to be carried out aiming at evaluating new process conditions to further increase biogas production and to minimise energy expenses. Concerning the application on industrial scale, the adoption of a CHP unit can be a key tool for valorising the studied technologies. As such, the production of heat from the CHP unit can be used to warm up the primary digester and contribute to heat a new fermentative digester or a thermal pretreatment section. Based on this hypothesis, the energy balance can undergo positive changes and the application of the studied technologies would increase in value.

## 4.3 Major constraints of the research

The major constraint of the present work is the difficult comparison between the two lines of research with the intent of defining the best solution for the improvement of the AD. Such constraint is due to the use of different substrates and different experimental set-ups in the two experimentations. As for the first issue, the two lines of research address to OFMSW with distinct characteristics. While pretreatments address to lignocellulosic OFMSW with a significant percentage of garden waste, the two-stage technology address to carbohydrate-rich OFMSW with a relevant content of FW. Furthermore, the adoption of different substrates depends on the long duration of the research and the biodegradability of the materials, which therefore needed to be periodically renewed. The use of different experimental set-ups is due to the different funds that supported the researches. Indeed, the study of the two-stage technology was supported by the Bio2Energy project, which was funded by MIUR-Regione Toscana DGRT 1208/2012 and MIUR-MISE-Regione Toscana DGRT 758/2013 PAR FAS 2007-2013 in sub-programme FAR-FAS 2014 (Linea d'Azione 1.1). Such funds provided new equipment, among with the gas-chromatograph for the VFAs measurement and new reactors which mixing was continuously guaranteed by electric gear motors while mixing inside the reactors used for the pretreatments study was manually provided a few times a day.

## 4.4 Future developments

Future developments of the two lines of research are needed to overtake the energetic issue. Concerning the pretreatment study, new experiments should be carried out with lower treatment intensities aiming at reducing the energy demand. As for the two-stage technology, new tests should be performed reducing fermentative HRT aiming at obtaining higher biogas productions and higher energy recovery. With the aim of further maximizing the increase in methane production in the second reactor, recent studies focused on the flushing of the hydrogen-rich biogas of the first stage through the methanogenic digester (Bassani et al., 2015; Yan et al., 2016). In this way, methane is not only produced following the acetoclastic pathway through the degradation of VFAs, but is also significantly generated by hydrogenotrophic bacteria that anaerobically oxidize hydrogen an carbon dioxide (Reaction 8, Section 0). Adopting this configuration, the methane content in biogas can overtake the 89% v/v thus notably increasing its energy yield (Bassani et al., 2015).

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# APPENDIX

## Additional data - Chapter 2

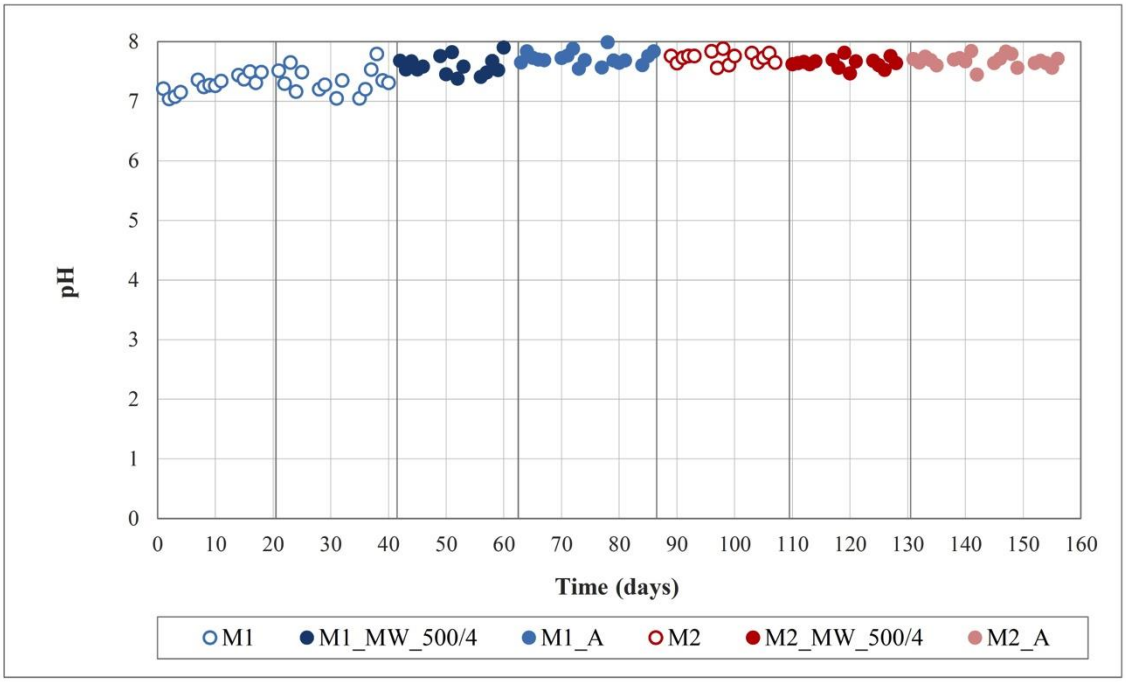


**Figure 41: Semi-continuous trials on substrate pretreatments - Process stability indicators over time: pH.**

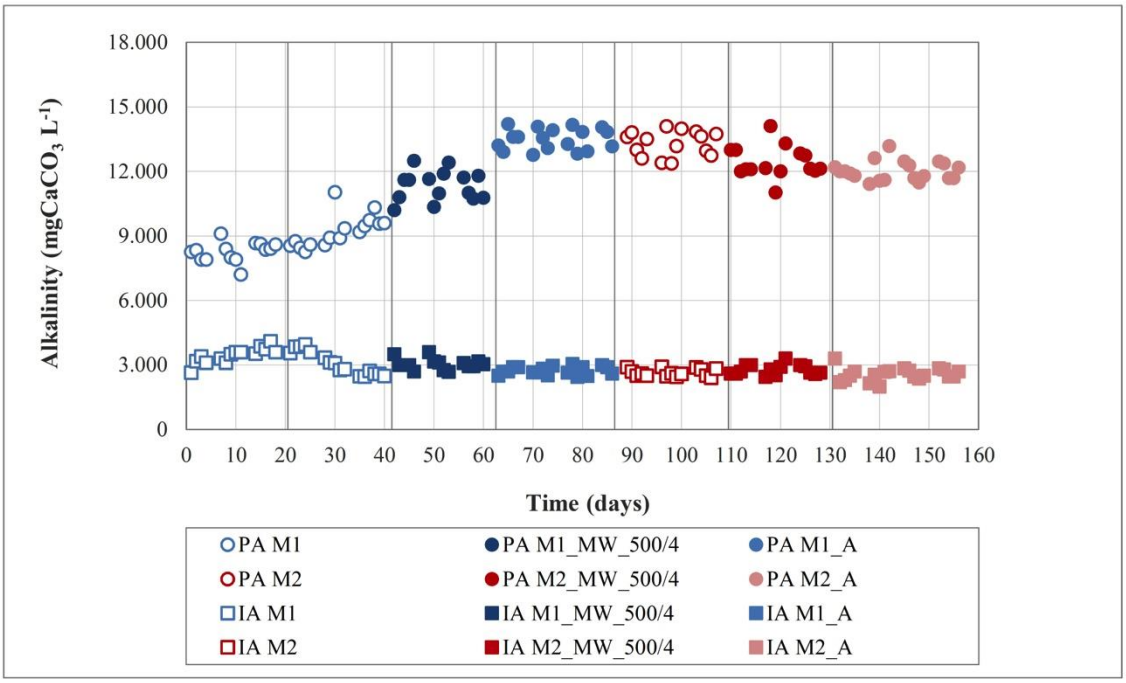


**Figure 42: Semi-continuous trials on substrate pretreatments - Process stability indicators over time: intermediate and partial alkalinity.**

# Additional data - Chapter 3

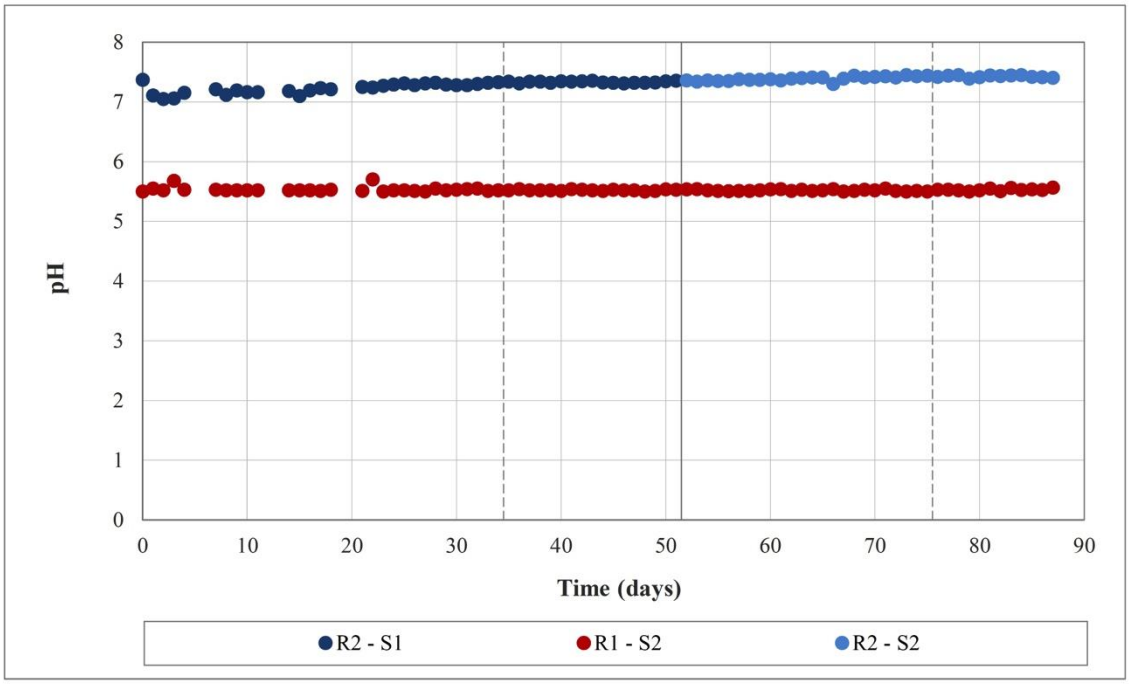


**Figure 43: Study of the two-stage technology – pH during the digestion of FW2. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

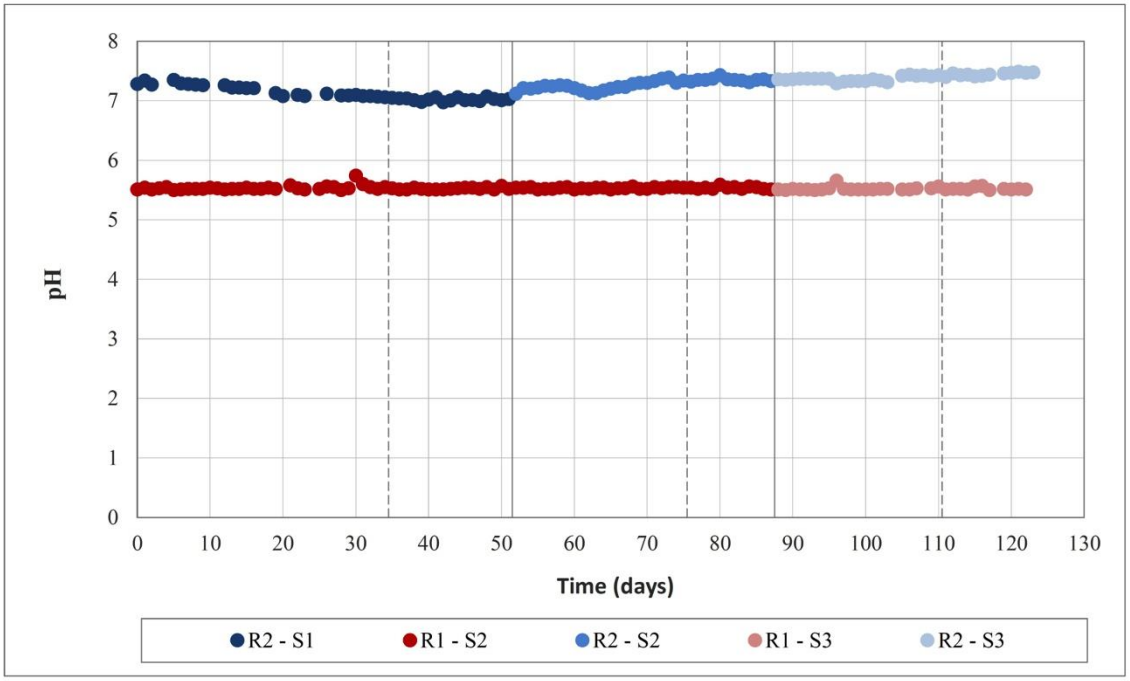


**Figure 44: Study of the two-stage technology – pH during the co-digestion of FW2 and AS. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

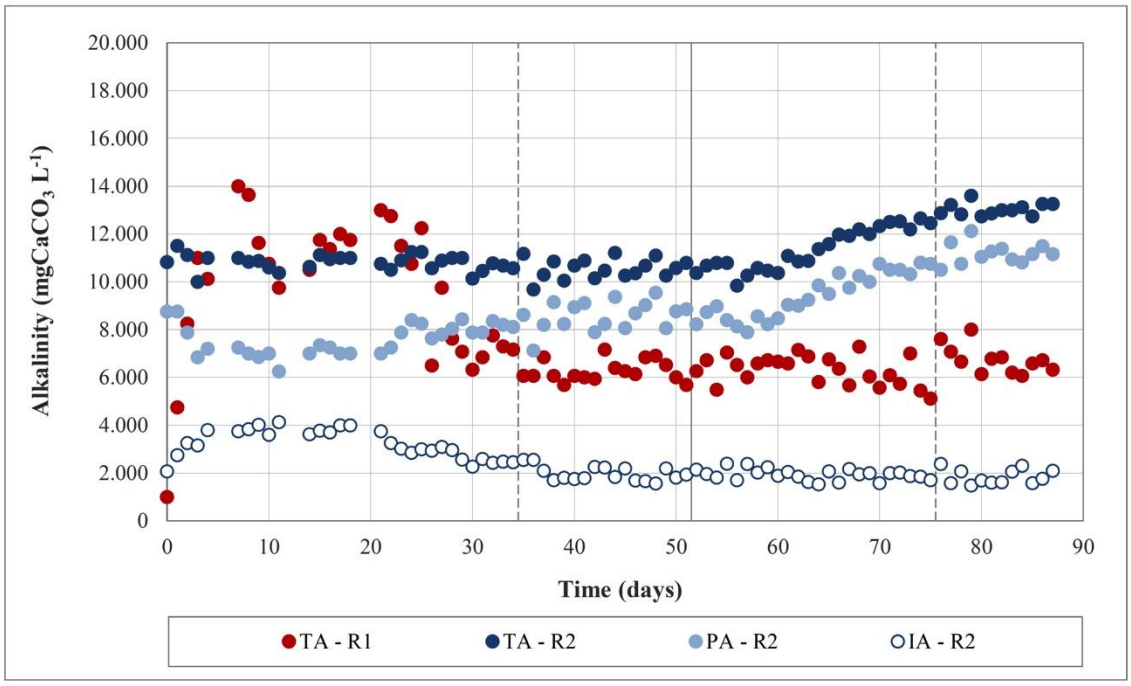


**Figure 45: Study of the two-stage technology – alkalinity during the digestion of FW2. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

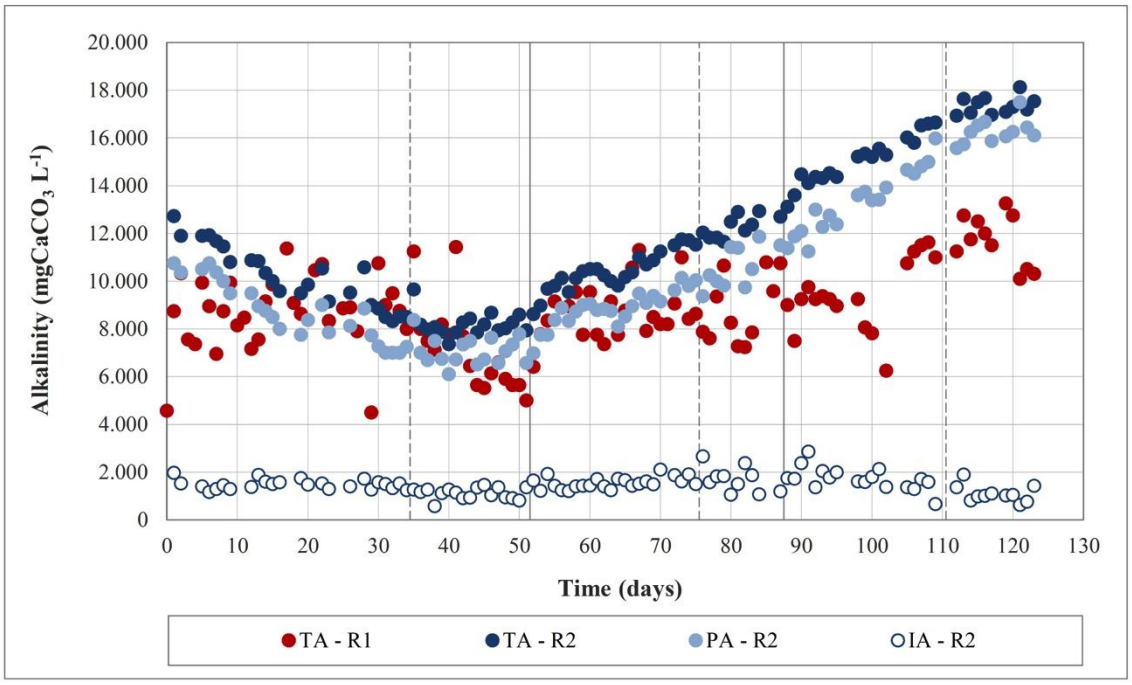


**Figure 46: Study of the two-stage technology – alkalinity during the co-digestion of FW2 and AS. Dashed lines indicate the start of steady state conditions for R2-$CH_4$ reactor. Solid lines indicate the start of a new scenario.**

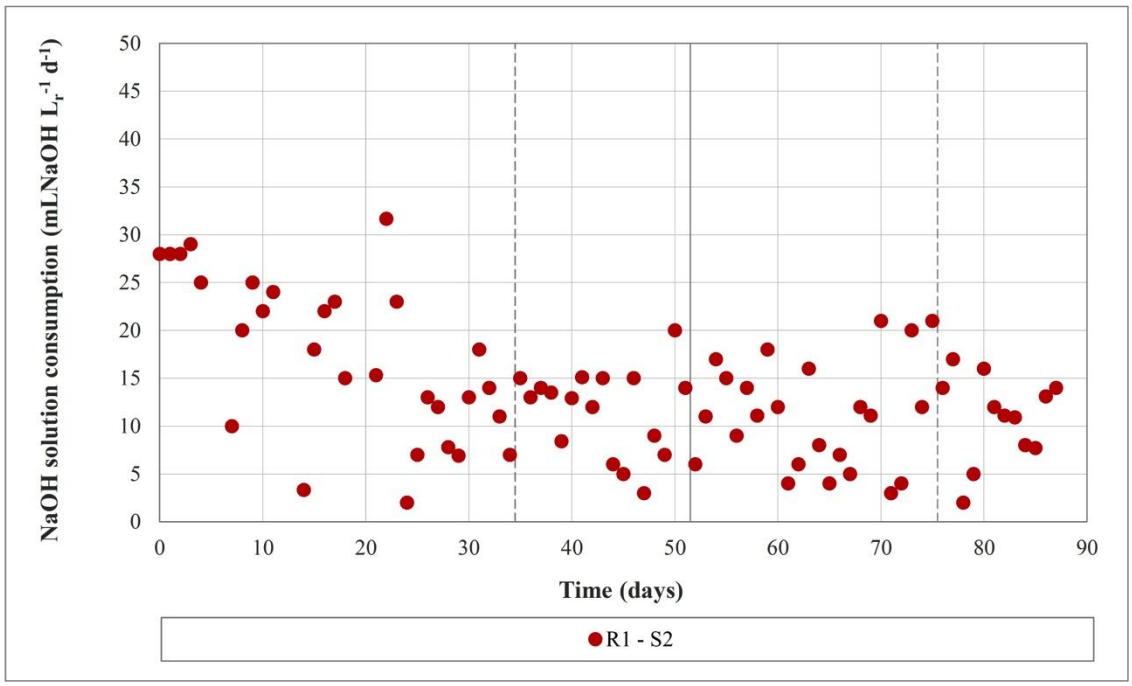


**Figure 47: Study of the two-stage technology – Daily consumption of NaOH solution in R1-$H_2$ reactor during the digestion of FW2. Solid lines indicate the start of a new scenario.**

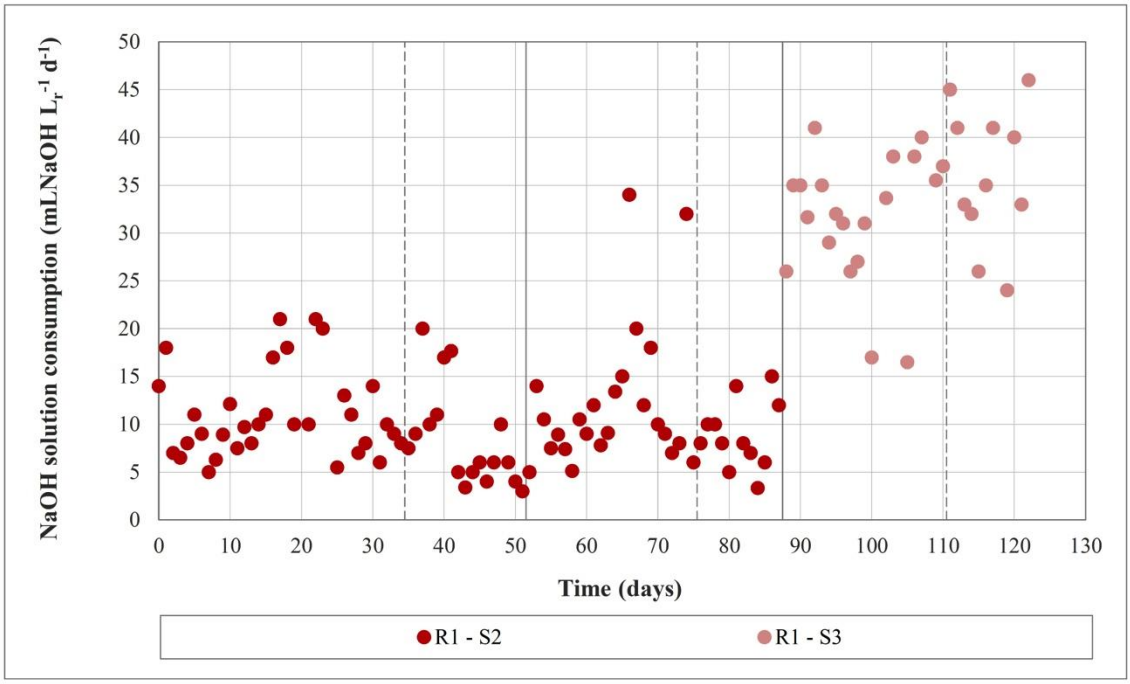


**Figure 48: Study of the two-stage technology – Daily consumption of NaOH solution in R1-$H_2$ reactor during the co-digestion of FW2 and AS. Solid lines indicate the start of a new scenario.**

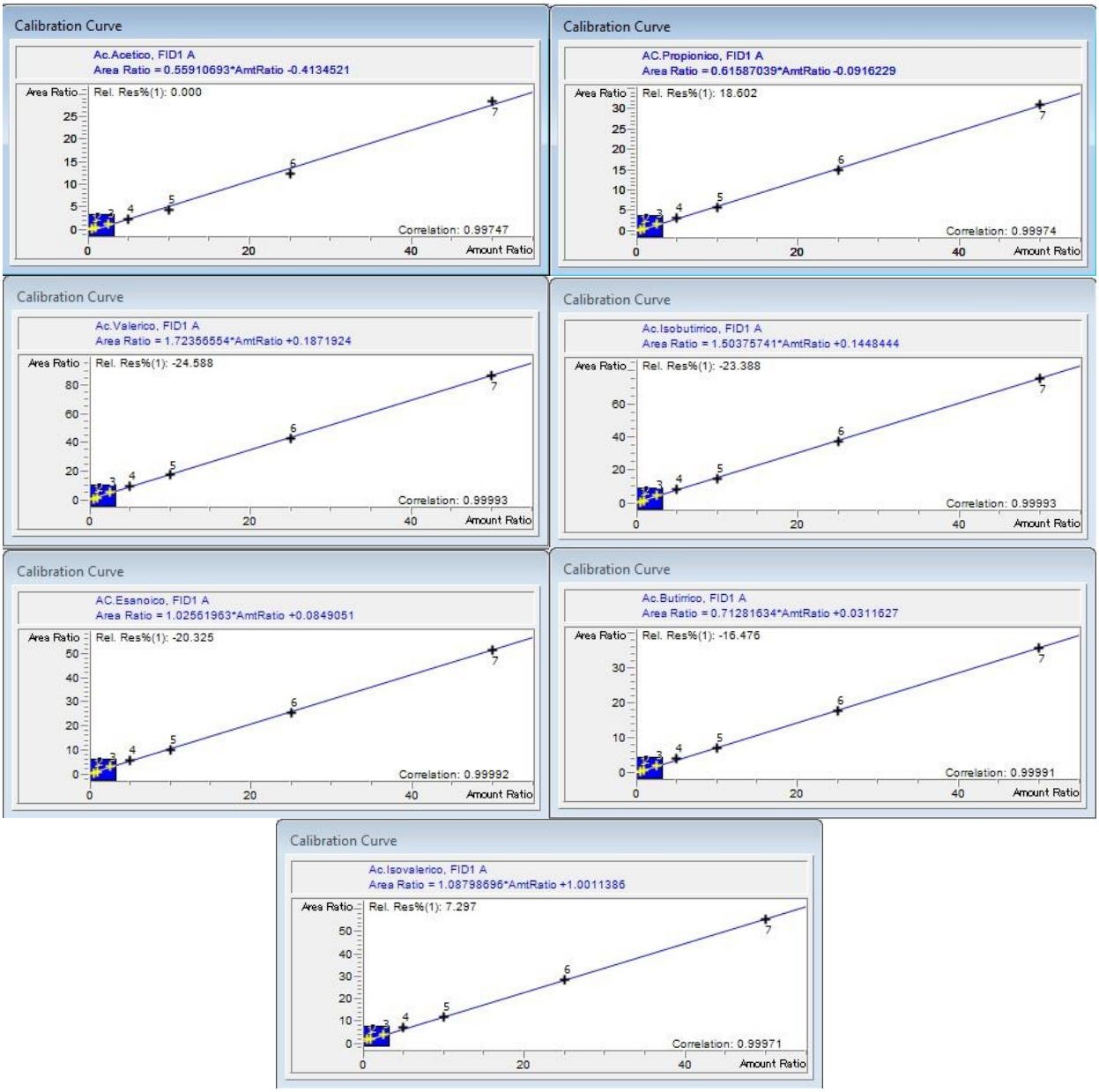


**Figure 49: Study of the two-stage technology – VFA measurement: calibration curves.**