UNIVERSITÀ DEGLI STUDI DI PADOVA
DIPARTIMENTO DI INGEGNERIA CIVILE, EDILE E AMBIENTALE
Department Of Civil, Environmental and Architectural Engineering

Corso di Laurea Magistrale in Environmental Engineering

MASTER THESIS

Enhancement of the anaerobic digestion of nitrogen substrate by implementing the approach of a microbial electrolysis cell to force a microbial change

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ACADEMIC YEAR 2022-2023

ACKNOWLEDGEMENT

First, I would like to thank the supervisor in Padova PhD. Maria Cristina Lavagnolo, my supervisor at host university Prof. Dr.– Ing. habil. Christina Dornack, and the PhD students Pascal Otto and Philipp Witkabel, who assisted me with my thesis work abroad through the Erasmus+ project in Germany. I would like to thank them for their support and for patiently guiding me through the master's thesis. I am beyond grateful to have had such helpful and understanding professors that were considerate of all my surrounding conditions.

I would also like to thank the Institute of Waste Management and Circular Economy, Secretary Diana Hempel, and the staffs Cornelia Heinz and Jacqueline Rohde for providing the chemical analysis results. My gratitude also extends to the Erasmus department at the University of Padova, and TU Dresden and all professors that helped me throughout the mobility, with their help the exchange process was much more manageable.

My gratitude goes to Prof. Maria Cristina Lavagnolo and Prof. Roberto Raga for the incredible classes during the Pandemic times and many other professors. I appreciate all the fellow students and friends I have made at UNIPD, mainly Lana Sobral, Daniel Bonello, Winny Puteri, Aidil Zuhri and Andri Lutchenko, who were a crucial part of my studying groups and friendships developed. My study life was much more pleasant with you.

Finally, my gratitude goes to my parent's s and sister, the most important people in my life. You have always been by my side, cheering for my success and never stopped believing in me and my dreams. Thank you for supporting me on this journey of studying abroad, over 9.182 km away and always surrounding me with love and giving.

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List of Abbreviations

AD	Anaerobic digestion
CH_4	Methane
CHP	Combined Heat and Power Basis
CO_2	Carbon dioxide
COD	Chemical Oxygen Demand
DIET	Direct interspecies electron transfer
EU	The European Union
FAN	Free Ammonia
FOS	Volatile organic acids
GAC	Granular activated carbon
GHG	Greenhouse gas
HIET	Interspecies Electron Transfer
HRT	Hydraulic retention time
IEA	International Energy Agency
LCFA	Long- Chain fatty acids
MEC	Microbial Electrolysis Cell
MIET	Mediated Interspecies Electron Transfer
NH_4-N	Ammonium nitrogen
OLR	Organic loading rate
SEM	Scanning Electron Micrograph
SRT	Solid Retention Time
TAC	Total inorganic carbon
TAN	Total Ammonia Nitrogen
TEM	Transmission Electron Micrograph
TKN	Total Kjeldahl nitrogen
TS	Total solids
VFAs	Volatile Fatty Acids

ABSTRACT

Anaerobic digestion is a natural biochemical process that can convert organic materials into combustible biogas. However, high ammonia levels can inhibit the process of methanogenesis and cause the failure of biogas production. For that reason, this study aimed to improve the stability and efficiency of anaerobic digestion in the presence of high ammonia concentrations and the production of biogas, through the implementation of microbial electrolysis cells (MEC) and direct interspecies electron transfer (DIET). The application of MEC to anaerobic digestion can accelerate the degradation of a substrate by enriching exoelectrogens and methanogens thus increasing biogas production. DIET has been recognised as faster and more stable means to transport reducing equivalents between bacteria and archaea, demonstrating the potential to enhance the rate-limiting steps during anaerobic digestion. The study was conducted with two independent experiments, one lasting 92 days and the other 34 days following the VDI 4630 and DIN 38414-8. Four reactors in each experiment were supplied with electrical energy, while two served as controls with no energy supply. The substrate used in the first experiment was aged, while the substrate in the second experiment had higher ammonia concentrations. The results suggest that the implementation of microbial electrolysis cells had a slightly positive effect on both experiments. However, these technologies did not significantly increase methane production compared to the control reactors.

Keywords: anaerobic digestion, biogas production, nitrogen inhibition fermentation, direct interspecies electron transfer (DIET), and microbial electrolysis cells (MEC).

1. Introduction

The energy crisis and global warming have become the highest issues worldwide (Singh, 2021). Meanwhile, the world population are continually growing, as reported by world meter (World Population Clock, n.d.), and the primary energy resource that supplies the world's needs comes from non-renewable fossil fuel. However, non-renewable fossil fuels cause environmental issues linked to global warming, where CO_2 is a primary greenhouse gas (GHG). Thus, aiming to reduce the effects of climate change and the energy crisis, the European Union (EU) decided in 2008 to reduce GHG emissions by producing energy from renewable sources (United Nations, n.d.).

The highest amount of GHG released into the atmosphere comes from the increase in energy consumption by the rise in the energy use from the manufacturing industry, which was equal to 740 million tons of CO_2-eq, 21% of total greenhouse gases emitted. Then, it is followed by the supply of electricity, gas, steam and air conditioning and the total activities by households divided between transportation, heating and other purposes (EU Economy Greenhouse Gas Emissions, n.d.). Among that, fossil fuels such as coal, oil and gas are the most significant contributor to global climate change, accounting for over 75% of global greenhouse gas emissions and almost 90% of all carbon dioxide emissions (United Nations, n.d.).

For that, the EU has developed targets to achieve net zero emissions, which means declining the use of coal, oil and gas through legislation or policy documents by 2050 (EU Economy Greenhouse Gas Emissions, n.d.)., By contrast, one of the EU options was to invest in sustainable bioenergy, which could reduce emissions in a wide range of areas, such as fuels for transportation, and mainly replace natural gas with biomethane to provide heating and electricity (International Energy Agency, n.d.). It is expected that energy technologies will reduce emissions in the electricity sector, which is the largest source of CO_2 emissions. For that reason, with the net zero emissions plan, the EU union aim to obtain 90% of global electricity generation in 2050 from renewable sources (International Energy Agency, n.d.).

The implementation of net zero by 2050 targets will need further development of available technologies and new technologies that are still being studied to speed up the process. The international energy agency -(IEA) mentions that even with the technologies available that are helping reduce CO_2 emissions through 2030, in 2050, half of the CO_2 reduction will come from technologies that are still being studied or prototype phase (International Energy Agency, n.d.). Therefore, the European Commission has created the REPowerEU Plan, which has the aim to develop a new geopolitical and energy market to accelerate the clean energy transition by supporting higher investment in renewable energy to Europe’s independence from uncertain supplies and volatile fossil fuels such as gas, oil, and coal before 2030 (European Commission, n.d.).

One of the plans is to propose develop a biogas and biomethane industrial partnership to promote the sustainable production and use of biomethane (European Commission, n.d.). The proposed action aims to support the production of a sustainable potential volume of biogas to further upgrade it to biomethane and direct biomethane production from waste and residues, thus avoiding the use of food and feed feedstocks leading to land use change issues (European Commission, n.d.).

Therefore, considerable has been conducted in the energy sector to find a carbon-neutral and renewable energy resource to replace fossil fuels. Hence, to create energy from renewable energy, the current study was conducted with the implementation of microbial electrolysis cells

(MEC) and direct interspecies electron transfer (DIET) to improve the anaerobic digestion system under a substrate with higher ammonia values. High ammonia is reported as the primary cause of digester failure in anaerobic digestion because it direct inhibits microbial activities (Rajagopal et al., 2013). For that reason, an optimal ammonia concentration ensures sufficient buffer capacity of the methanogenic medium in AD, which helps increase the stability of the digestion process (Rajagopal et al., 2013).

From that, it could be further used to produce biogas with a higher share of CH_4 that could be used for electricity and heat production. Furthermore, the greenhouse gas CH_4 produced is captured and not released into the environment. Meanwhile, the CO_2 produced during anaerobic digestion and combustion of methane is released into the atmosphere. This can be considered climate-neutral in this context, as it is part of the natural carbon cycle.

2. Problem and objective

AD leads to the formation of biogas, and it is composed of a four-stage biochemical process that occurs under anaerobic conditions. The product of each process is used in the next stage, so all of them must occur efficiently. Otherwise, biogas production can decrease if one of the stages is inhibited, and that can occur due to the substrate composition, inoculum and the operating conditions of the digester (Yellezuome et al., 2022). Some substrates such as animal manure, the food industry, organic fractions of municipal solid waste and agriculture have been studied to be utilised in AD. However, some of those substrates contain high amounts of organic nitrogen, which during their degradation, raises high concentrations of ammonia, causing inhibition of the AD digester (Yellezuome et al., 2022).

Consequently, leading with a substrate with a higher protein content can occur the inhibition of ammonia that is released during the digestion of proteinaceous material, imposing higher ammonium concentration (Jiang et al., 2019). Ammonium ion (NH_4+) and free ammonia nitrogen (NH_3) are the two primary forms of inorganic ammonia nitrogen in an aqueous anaerobic solution (Rajagopal et al., 2013). The inhibition of ammonia might inhibit the methanogenesis phase in AD in two ways: ammonium ion might directly inhibit the methane production enzymes or hydrophobic ammonia molecules that could diffuse into bacterial cells, which could cause proton imbalance (Rajagopal et al., 2013). In addition, the NH_3 in the cell could cause an imbalance in pH due to the conversion to ammonium NH_4 while absorbing protons in the process. The inhibition effects have mainly been observed by the accumulation of volatile fatty acids and a pH drop in AD digestion.

For this reason, fermentation experiments according to VDI 4630 will be carried out in this master's thesis. The experiments focus on investigating DIET and MEC in connection with the degradation of difficult substrates. The implementation of DIET could facilitate the interactions between the bacteria and the degradation of organic because they don't need hydrogen to be produced for use as an electron carrier. They can realize between different species through biological electrical connections using c-type cytochrome OmcS electrically conductive pili and conductive material (Baek et al., 2018). MEC has been studied for the capability of improve decomposition of organic matters by consuming VFAs produced during AD and converting H_2 though an electrochemical reaction (Wang et al., 2018).

Thus, the objective is to observe if both systems with an electrical voltage and electrode to enhance the AD process with a substrate with higher nitrogen. It also investigates, in a second phase, the performance of an enriched sludge adapted to high ammonia values to observe the adaptability of MEC reactors compared to the control.

3. Theoretical Background

The second chapter aims to overview the essential terms and topics to elaborate on the project work.

3.1. Terms

The analysis made in this work requires a broad range of theories, background and application models. In order to help readers to understand the study aim, it is necessary to present some fundamental and theoretical background. Thus, this chapter will introduce some concepts of anaerobic digestion, direct interspecies electron transfer (DIET), microbial electrolysis cell (MEC), and fermentation process.

3.1.1. Anaerobic Digestion

Anaerobic digestion is a biological process in which microorganisms break down organic materials. AD occurs in closed spaces where there is no oxygen, and it is composed of four steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, as shown in Figure 1(Mostafa et al., 2020). The process can treat a wide range of organic wastes, including wastewater, energy crops, agriculture residues, municipal solid waste, industrial, and complex solid wastes, where organic matter is converted into biogas (Robles et al., 2018). Biogas is typically rich in 50-75% CH_4 and 25-50% CO_2, with a small amount of water vapour and other gases (Mostafa et al., 2020).

Each step of the AD process can be different depending on the type of treated feedstock because the microorganism has distinct characteristics in physiology, nutrient metabolism, and environmental sensitivity, which occur through complex interactions between them. In general, the process is composed of breaking down the biomass into a gaseous mixture of CH_4, CO_2, H_2S, NH_3, and the product of one step is used as an input for the following stage (Uddin and Wright, 2022). Therefore, in the hydrolysis process, it occurs the breakdown of complex biopolymers into basic monomers or oligomers. In acidogenesis, the hydrolysis products are further broken down by acidogenic bacteria and mainly converted into short-chain volatile fatty acids, alcohol, and ketones. In acetogenesis, the bacteria transform the products of the acidogenesis stage in some long-chain fatty acids into acetate, H_2 and CO_2.

Then, it comes to the methanogenesis phase, where the intermediate products such as H_2, CO_2 and acetate of the previous stage generate CH_4 and CO_2 (Uddin and Wright, 2022). This last stage is the most sensitive stage of AD because it occurs in the slowest biochemical reactions and methanogenic bacteria cannot survive in the presence of oxygen. Moreover, the methanogenesis stage has a stronger influence from the operations conditions such as the composition of the raw material, hydraulic retention time (HRT), temperature, agitation and pH (Trisakti et al., 2017). Therefore, the efficiency of syntrophic methanogens and bacteria has an essential role in the digestion process. Otherwise, the methanogenesis stage can affect the anaerobic digestion process (Chen et al., 2022).

Along that, the disadvantages of the AD process are related to the slow growth of anaerobic microorganisms in the methanogenic process and the lower efficiency of fermentation degradation of critical intermediates such as propionate and butyrate for the AD efficiency (Yiwei Liu et al., 2021). Anaerobic degradation bacteria are sensible for different conditions such as temperature, pH, Carbon/Nitrogen (C/N) ratio, organic loading rate (OLR), and HRT (Uddin

and Wright, 2022). Therefore, the sludge properties and surrounding environment affect AD digestion and optimising these parameters, improves the efficiency and speed the digestion.

Nevertheless, the improvement of AD through the implementation of some technologies such as DIET and MEC, can increase their performance and efficiency of degrading materials. Such technologies will be further discussed in other chapters (M. Liu et al., 2021). Furthermore, AD is considered one of the most economical methods to produce energy recovery technology from the waste (Moreno, 2016). That's because biogas produced can be used directly or converted into energy by a combination of heat and power (CHP) units, and it also generates by-products that can be used as organic amendment rich in nutrient sludge that can be used as a fertiliser (González et al., 2018).

Thus, biogas production could be an option for sustainable development goals of climate change and clean energy production by reducing the dependence on fossil energy, which is critical nowadays. The avoidance of fossil fuels is because they are non-renewable and produce harmful gases such as sulphur dioxide, nitrogen oxides and carbon dioxide, which can generate impacts on human health and environmental pollution (M. Liu et al., 2021). The depletion of fuel reserves can generate high energy prices and increase environmental pollution. Therefore, the displacement of fossil fuel for biogas helps reduction of emissions, sometimes resulting in carbon-negative systems (Tanigawa and Stolark, n.d.). Besides the numerous potential benefits of organic waste utilization, including environmental protection, and investment.

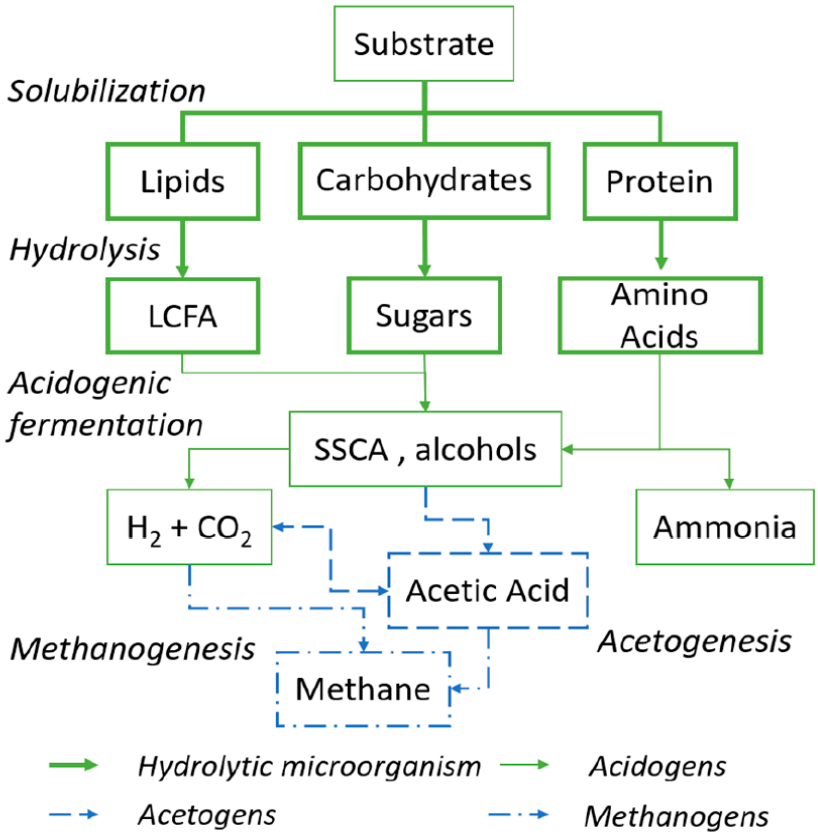

Figure 1 Metabolic pathways of anaerobic digestion and involved microorganism. LCFA-long-chain fatty acids, SCCA-short chain carboxylic acid (Menzel et al., 2020).

3.1.2. Hydrolysis

The hydrolysis stage is an important step in the anaerobic digestion process because polymers are not directly used by fermentative microorganisms (Abdelgadir et al., 2014). Therefore, in this stage bacteria break down lipids, complex polymerics such as carbohydrates, proteins, and particulate organic matter into soluble components such as fatty acids, peptides, amino acids, sugar, glycerol and oligosaccharides by extracellular enzymes (Menzel et al., 2020). Then, they are secreted by the microbial community and made them available for other bacteria.

Hydrolysis can occur faster if the substrates are easily degradable and enough physical contact between the enzymes and the substrate is provided. However, in the case of a substrate with a more recalcitrant structure, it requires a long time for degradation and, most of the time it is not complete (Abdelgadir et al., 2014). Thus, substrates with a complex structure, such as lignocelluloses, are not totally accessible to the enzymes, so hydrolysis is often considered the rate-limited step. Nevertheless, the tiny particles produced in the further conversion to short-chain VFAs, alcohols, carbonic dioxide and hydrogen are converted in the acidogenesis stage (Abdelgadir et al., 2014).

In addition, the hydrolysis stage as the further stages, can be affected by substrate, inoculum, and environmental conditions. If they do not have optimal conditions, volatile fatty acids and alcohols are formed at a high partial pressure of hydrogen, which could accumulate into the system and reduce AD effectiveness. Those products are more reduced than the products generated under optimal conditions. Thus, they need to be modified before being converted to biogas.

Among them, the intermediates by-products are ammonia, long-chain fatty acids (LCFA), humic acid and hydrogen partial pressure in higher concentrations cause inhibition during the hydrolysis process. Therefore, higher concentrations cause inhibition effects which can generate inhibition effects on AD and need more attention to keep digestion efficient. shows an example of a hydrolysis reaction where a polysaccharide is broken down into a glucose (Abdelgadir et al., 2014).

Hydrolysis reactions are followed by:

$$\text{Lipids} \rightarrow \text{Fatty Acids}$$

$$\text{Polysaccharides} \rightarrow \text{Monosaccharides}$$

$$\text{Protein} \rightarrow \text{Amino Acids}$$

Equation 1

$$C_{24}H_{40}O_{20} : H_2O + 3H_2 \rightarrow 4C_6H_{12}O_6$$

3.1.3. Acidogenesis

In this stage, the hydrolysis products, such as organic monomers of sugar, amino acids, and acetate, are degraded by acidogenic bacteria. As a result, VFAs, H_2, CO_2, sulfide and ammonia are produced (Kamran, 2021). In the acidogenesis stage, the concentration of the products generated during the degradation should be balanced to maintain a stable and efficient process. Otherwise, hydrogen concentration as an intermediate product during acidogenesis could impact the type of the final product produced (Córdova and Chamy, 2020).

The main acidogenesis products are acetic acid (CH_3COOH), propionic acid (CH_3CH_2COOH), butyric acid ($CH_3CH_2CH_2COOH$); and ethanol (C_2H_5OH) (Abdelgadir et al., 2014). When there is an equilibrium system, most organic matter is converted into an available substrate for methanogenic microbes such as acetate, hydrogen, and carbon dioxide. Still, a significant amount is transformed into alcohols and short-chain fatty acids. Therefore, those products, such as hydrogen, carbon dioxide, and acetic acid, would skip the acetogenesis stage and be used directly in methanogenic bacteria at the last stage (Abdelgadir et al., 2014).

By contrast, the partial pressure of the hydrogen regulates the products in the hydrolysis step. The most accessible primary fermentative bacteria are acetate production via pyruvate with hydrogen production. The increase of hydrogen partial pressure led to an increase in VFA production and a decrease in pH below 6, causing inhibition of the biomass hydrolysis (Preethi et al., 2022).

Moreover, the acidogenic stage includes many different fermentative genera and species, such as *Lactobacillus, Escherichia, Staphylococcus, pseudomonas, Desulfovibrio, Selenomonas, Sarcina, Streptococcus, Desulfobacter* and *Desulforomonas* (Mara & Horan, 2003). They convert amino acids to fatty acids, acetate and NH_3 (Kamusoko et al. 2016). In contrast, *Eubacterium limosum, Clostridium*, and *Streptococcus* transform sugar into intermediary fermentation products (Kamusoko et al. 2016). Equations 3 and 4 represent three typical acidogenesis reactions where glucose is converted to ethanol, propionate and acetic acid, respectively (Abdelgadir et al., 2014).

Equation 2 $$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$$

Equation 3 $$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O$$

Equation 4 $$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

3.1.4. Acetogenesis

In that stage, the anaerobic digestion of the products of acidogenesis, such as propionate, butyrate, iso-butyrate, valerate, iso-valerate, and ethanol, is converted to acetic acid, H_2 and CO_2. Then, the products of acetogenesis can further be used to form methane by the methanogens process (P. Wang et al., 2017).

At the acetogenesis phase occurs the anaerobic oxidation reactions with methane-forming microorganisms. In this reaction, protons are used as the final electron acceptors, leading to hydrogen production. However, oxidation reactions can occur with a lower hydrogen partial pressure, and that's why the interactions in the reactions are essential otherwise, the hydrogen is consumed (Córdova and Chamy, 2020).

The hydrogen produced in this stage plays a role for intermediates products such as VFAs and alcohol since the reactions need a lower hydrogen partial pressure to convert all acids thermodynamically. The decrease of partial pressure can occur due to the hydrogen-depleting bacteria (Córdova and Chamy, 2020).

By contrast, the acetogenesis stage plays an essential role in the rate-limiting step to maintain a rapid, stable AD system because the VFAs, mainly propionate, inhibit methanogenesis at higher concentrations even when there is a neutral pH (P. Wang et al., 2017). Equation 5 represents the conversion of propionate to acetate, achievable at lower hydrogen pressure. Equation 6 is glucose converted to acetate during the anaerobic digestion in the acetogenesis stage (Abdelgadir et al., 2014).

Equation 5

$$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + H^+ + HCO^-_3 + 3H_2$$

Equation 6

$$CH_6CH_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

3.1.5. Methanogenesis

In the last stage of anaerobic digestion, methane is produced by methanogenic archaea, where they decompose hydrogen, carbon dioxide, and acetic acid into methane and carbon dioxide (Kamran, 2021). In General, carbon metabolism is distinguished by the type of methane synthesis depending on the substrate, which can occur from CO_2, methanol, or acetate. Additionally, this stage is a critical process in anaerobic digestion because microorganisms are sensitive to environmental factors, and their growth is slow compared with the acetogenic bacteria (Córdova and Chamy, 2020).

The anaerobic digestion in methanogenesis can be divided into acetate and H_2/CO_2 consumer, and some of them are both. Therefore, methane is generated in two ways: by acetoclastic methanogens, a group of archaea which uses acetic acid molecules to generate carbon dioxide and methane, as shown in (Córdova and Chamy, 2020). The second way is from hydrogenophilic methanogens, which occur by reducing carbon dioxide with hydrogen to produce methane, as shown in equations 7 and 8 (Abdelgadir et al., 2014). The archaea community responsible for the methane conversion are Methanobacterium, Methanosarcina, Methanomicrobium and Methanospirillium (Córdova & Chamy, 2020). Although methane production is higher from reducing carbon dioxide, the limited hydrogen concentration in digesters results in acetate reaction being the primary producer of methane (Abdelgadir et al., 2014).

Equation 7 $CH_3COOH \rightarrow CH_4 + CO_2$

Equation 8 $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$

Acetoclastic methanogenic archaea are responsible for 70% of the methane production in the digester. In contrast, the remaining 30% of methane production comes from the conversion of hydrogen to methane by hydrogenotrophic methanogenic archaea (Córdova and Chamy, 2020). Thus, acetoclastic and hydrogenophilic are the most critical microorganism in the deg-

radation of anaerobic digestion. They are important for the efficient functioning of the intermediate trophic groups and syntrophic bacteria responsible for transforming the intermediate products of direct methane precursors such as organic acids and alcohols.

Furthermore, Kamran, 2021 mentions that the primary constituents of biogas are CH_4 and CO_2, with a minimum amount of nitrogen and hydrogen. Normally, the composition of biogas is methane with: CH_4 around 50% - 75%, CO_2 is 25% - 50%, and 1% - 10% of water content and traces of ammonia, oxygen, hydrogen sulfide and nitrogen. However, the percentage of each constituent of biogas varies depending on the feedstock composition used in the digestive process. Among that, the amount of methane depends on the energy content of the biogas. Therefore, the higher the concentration values of biogas, the higher the energy content.

Additionally, to maintain the activities of the acidifying and methane-producing bacteria, the methanogenesis stage should have a pH around 6.8 to 7.2 (Ibro et al. 2022). The microorganisms in this stage are relatively sensitive to environmental elements such as temperature and feedstock composition. For that reason, it is important to control and monitor the operation parameters.

3.2. Factors influencing the anaerobic degradation process

3.2.1. Temperature

It can be considered one of the most important environmental factors influencing the growth of microbes in the anaerobic degradation process because, depending on the type of microorganism species, it needs a specific temperature range to grow. Based on the temperature, the AD microorganism can be divided into psychrophiles (<25°C), mesophilic (25-45°C), and thermophilic (45 -75 °C) (Trisakti et al., 2017).

Temperature is an important factor in the AD process as it affects the microorganism activities in the digester. Hydrolytic and acidogenic bacteria are not totally sensible to temperature changes because of the higher diversity of bacterial communities. However, the acetogenic and methanogenic processes are significantly influenced by temperature. The optimal growth temperature for most methanogenic archaea is 30-40 °C, with a few genera growing and best conditions between 50-60°C. Therefore, the variation in operation temperature also changes the dominant species present in the process.

The appropriate operating conditions vary from 35-37 °C because the mesophilic occur with a wide variety of microbial capable of dealing with environmental change (Robles et al., 2018). Hence, if happen any change in temperature from mesophilic to thermophilic, it will slow down the biogas production rate. However, biogas production will increase again with the shift in the microbial population (Robles et al., 2018). In contrast, mesophilic digesters are also easy to operate and maintain, resulting in a lower investment cost, but the retention time can be higher in lower biogas production (Mir, et al. 2016).

On the other hand, thermophilic conditions have a rapid conversion of organic acids due to higher temperatures. That also means short retention times and smaller reaction volumes which can result in a pathogen-free of higher quality due to the higher degradations (Robles et al., 2018). However, higher temperatures can affect the biogas yield due to the increased concentration of free ammonia. When ammonia dissolves in the liquid, it inhibits AD which may inhibit the gas production (Mir, et al. 2016).

By contrast, CO_2 can dissolve at lower temperatures and produce carbonic acid by reacting with water, which increases acidity. Thus, resulting in slower microbial growth and substrate

degradation, decreasing biogas production. In conclusion, for the growth of the microorganism and biogas production, it is essential to control the temperature from the AD process.

3.2.2. pH value

The pH values vary along the different stages of anaerobic digestion caused by VFAs, bicarbonates, alkalinity, and CO_2. The variation in pH could cause the change of dominant microbial populations affecting the main organic acids products. Nevertheless, maintaining the values stable is important for AD digestion, which can be made by buffers such as calcium carbonate, lime and chemicals such as NaOH and $NaHCO_3$ (Mir, et al. 2016).

Producing methane requires a neutral to slightly alkaline environment pH of 6.8 to 7.5. In this range, microorganisms are active and consequently, digestion is efficient. For methanogenesis, an optimal pH value is between 6.8 to 7.2, while hydrolysis and acetogenesis need a pH of 5.5 to 6.5 (Mir, et al. 2016). Therefore, the buffer capacity or alkalinity is used to maintain the equilibrium and reduce variation in pH. The buffer capacity is the equilibrium between carbon dioxide and bicarbonate, with ammonia as the important cation which can cause resistance to pH changes. (Robles et al., 2018). The higher the bicarbonate concentration in the digester, the greater the alkalinity and the resistance to change pH.

Nevertheless, maintaining a stable system can be challenging due to many factors, such as depletion in the buffering capacity and higher H_2 partial pressure, which may increase pH and VFA accumulation. VFA is produced from acidogenesis in the AD process, and maintaining the conditions for acidogenesis and methanogenesis is difficult because of the different metabolic characteristics of the microorganisms. Among that, an accumulation of VFAs can cause a drop in pH and cause inhibition of the methanogenesis (Baek et al. 2019). The dominates and this form is more inhibitory than the ammonium ion (NH_4). The pH influences the chemical equilibria of NH_3, H_2S and VFA's, which could inhibit the activity of the microorganisms (Baek et al. 2019).

3.2.3. Inhibitory Substances

For a stable and well-balanced microbial community to be operational and efficient in biogas production, the chemical and characteristics of the substrate are essential to meet the nutrition requirements involved in the degradation process, such as protein, lipids and carbohydrates (Robles et al., 2018). Therefore, macro- and micronutrients should be provided for the microbial community in the digester to avoid inhibit effects (Robles et al., 2018).

Carbohydrates are a common component in substrates, mainly from plant-derived substrates that are also enriched with sugar decomposed through acidogenesis to form VFA (Siddique & Wahid, 2018). Among that, substrates rich in protein can also produce good values of methane in biogas. In contrast, the substrate containing a large amount of lipids can develop problems such as blockages, adsorption into biomass, and microbial inhibition (Siddique and Wahid, 2018).

The microelements such as sodium, potassium, cobalt, iron, and calcium are required in small amounts than macroelements by the microorganism in their nutrition. In some cases, the microelements' function is to support the growth of microorganisms. For example, sodium is used to help transport sugar into the cell of the colon bacterium, while iron is an energy source, component to cytochromes, and electron transport compound (Mara and Horan, 2003).

The fundamental macronutrients to microbial growth are carbon (C), nitrogen (N), phosphorus (P) and sulfur (S) because they are the primary source of energy and the basic building block of the cell material. For example, nitrogen and phosphorus are used in the protein and nucleic

acid synthesis (Siddique and Wahid, 2018). For optimal gas production, the C/N ratio is suggested between 15:1 and 25:1, where the amount of phosphorus should be 6-7 times less than for the nitrogen (Siddique and Wahid, 2018). While sulfur requires numerous enzymes, potassium is essential in cellular transport and cation balancing. Furthermore, iron, nickel, cobalt, molybdenum, and tungsten are necessary because they are co-factors for the unique enzyme systems of anaerobic microorganisms. For that reason, the presence of metals is essential for methane production.

3.2.4. Space Loading and Retention time

The retention time represents the time the feedstock stays in the reactor to degrade completely (Robles et al. 2018). Furthermore, retention time is divided into two types: SRT, meaning the average time bacteria spend digesting the biomass, which can variate, conforming to the type of substrate, organic loading rate and temperature (Mao et al., 2015). Meanwhile, HRT depends on ORL and substrate composition.

Furthermore, the organic loading rate (OLR) defines the amount of raw material fed into the digester over time, and it is an important parameter affecting the biogas production rate. It regulates the number of volatile solids needed to input the anaerobic digestion and can affect the methanogenic activity and kinetics (Gautam et al., 2022). OLR is normally maintained in a digester by manipulating HRT, but the optimum value depends on the substrate characteristics such as biodegradability, C/N ratio and other factor (Barua and Dhar, 2017).

The rate of feedstock added into an AD reactor has to be adapted for the growth rate of methanogens archaea. The increase in biogas is a result of the improvement in methanogens forming. If the OLR in AD is overloaded, methanogenesis pathways become inhibited, which could result in the accumulation of VFA in the reactors, generating a drop in the biogas production (Orhorhoro and Sadjere, 2018). Moreover, it cause an increase of acidogenesis, a decrease in pH, and mass death of methanogenic archaea (Mir et al. 2016). Therefore, the process will become acidic if more feedstock material is added than the microorganisms can degrade.

Given this context, a decrease in HRT could cause an accumulation of VFA, leading to an insufficient chemical oxygen demand (COD) removal, making the process inefficient. By contrast, a longer retention time results in insufficient utilisation of digester components. In addition, the variation of retention time causes a destabilisation and degrades the performance of the anaerobic system (Mao et al., 2015). Therefore, a lower OLR and a long optimal HRT would be an optimal strategy to achieve higher methane yields. Jain et al. 2015 mention that retention time should be at least 2-4 days, or the bacteria may come out with the slurry, affecting the biogas production. For that reason, continuous feeding is important to achieve maximum gas production. Mir, et al. 2016 mention that the optimal retention time for biological conversation is 12-24 days for thermophilic and 15-30 days for the mesophilic digester. Still, the substrate type, organic load, and temperature must be considered.

3.3. Nitrogen Inhibition in Biogas Production

Nitrogen is an essential macronutrient for microbial growth because new cells mass needs nitrogen to be produced. Nitrogen also serves as a buffering agent neutralisation for acidogenesis effects in the production of VFA, raising process stability. It is found as organic nitrogen in the form of protein, urea, amino, and uric acids (Yellezuome et al., 2022). They are present in the substrates and disintegrate into ammonia nitrogen, reacting as inhibition when an overloading

concentration occurs, which causes the reduction of the microbial growth (Yellezuome et al., 2022).

This happens because higher ammonia concentrations increase volatile fatty acids, facilitating foaming issues due to the imbalanced microbiological activities (Resch et al., 2011). In addition, AD with higher ammonia concentrations can lead to an accumulation of VFA, resulting in a decline of pH, mainly when the buffer capacity of the fermenter sludge is insufficient. The critical ammoniacal nitrogen concentration variates from 1,0 to 14 g/L, but it depends on the substrate, inoculum, process conditions, acclimatisation period, and mainly pH and temperature (Yellezuome et al., 2022).

Microorganisms need nitrogen for the absorption of nitrogen taking place in the form of ammonium. Ammonium is an essential parameter for the buffer capacity in an anaerobic reactor. It is released during the anaerobic hydrolysis of organic nitrogen compounds, causing an increase in pH value. Nevertheless, ammonification reduces the pH value resulting from the acidification step of anaerobic digestion (Fricke et al. 2007).

Nevertheless, the optimal concentration of ammonia provides an efficient methanogens activity and increases the stability of AD. However, a lower concentration of synthesising amino acids and nucleic acids is essential for microbial growth (Harirchi et al., 2022). Among that, ammonia reacts as the base to neutralise the organic acids provided by the fermentative bacteria, thus helping with buffering capacity and maintaining pH in neutral conditions around 6.5 to 7.5, which is necessary for cell growth and obtaining maximum methane yield (Harirchi et al., 2022).

The ammoniacal nitrogen (NH_3-N) is a measure of the amount of ammonia. It is all the ammonia forms such as ammonia (NH_3), ammonium (NH_4+), nitrogen gas (N_2), nitrates (NO_3), nitrites (NO_2–), and organic nitrogen such as proteins (Madhu, 2018). Therefore, it measures the amount of ammonia at its toxic levels. In an aqueous solution, ammoniacal is present in two forms: ammonium ions NH4+-N and free ammonia (FAN, NH_3-N), causing direct and indirect inhibition in the AD process (Yellezuome et al., 2022). Nevertheless, FAN has been suggested to be the main ammonia inhibition because FAN is freely permeable to microbial cell membranes, where they pass through the cell membranes of methanogens and cause a proton imbalance. Therefore, a higher concentration of ammonia in AD reactors leads to the inhibition of methanogens. The inhibition could cause instability in the reactors due to the accumulation of VFA decreasing pH, which could result in lower biogas production or failure of the system in the worst case (Morozova et al., 2020). By contrast, environmental effects such as substrate concentration, pH, inoculum, process conditions and temperature might inhibit the methanogens and synergise the inhibition effect of ammonia. Yellezuome et al. 2022, mention that pH and temperature are the main factors influencing ammonia inhibition rather than TAN concentrations. The pH induces the microorganism growth and compound of total ammonia nitrogen (Harirchi et al. 2022).

In contrast, the total ammoniacal nitrogen is mostly converted to free ammonia at higher pH, and the form is toxic in the system (Harirchi et al. 2022). The temperature could also influence microbial growth and free ammonia concentration because the high free ammonia concentration represses methanogens more efficiently in thermophilic temperatures than in mesophilic (Harirchi et al. 2022). The chemical balance between NH_3 (free ammonia) and NH_4^+ (ammonium) is shown in Equation 9.

Equation 9 $$NH_4^+ + OH^- \leftrightarrow NH_3 + H_2O$$

The inhibition effect occurs under the undissociated form of intermediate catabolic product on microorganisms which depends on pH and temperature in the reactor. Even small changes in pH values and temperature on the dissociation balance of ammonia and ammonium could cause an inhibition, as shown in Figure 2 (Fricke et al., 2007). When pH increases, there is a possibility of inhibition by hydrosulphide, volatile fatty acids decline, and the inhibition from ammonium nitrogen increases. While temperature could impact microbial growth and free ammonia concentration by increasing process temperature affecting the metabolic rate of microorganisms positively, but also growing ammonia levels (Morozova et al., 2020). Therefore, the free ammonia concentrations correlate with temperature and pH.

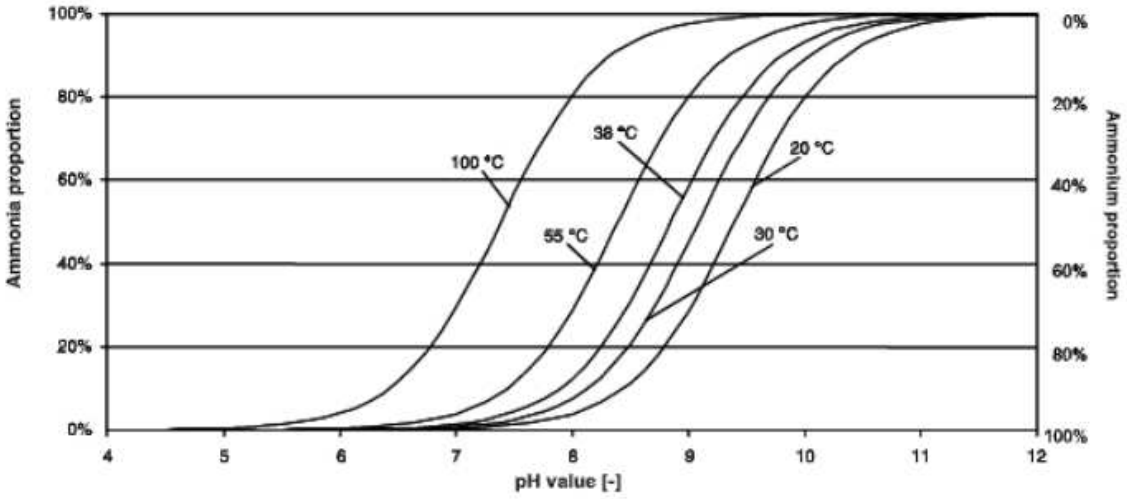

Figure 2 Dissociation balance between ammonia/ ammonium conforming pH and temperature (Fricke et al. 2007).

Depending on the substances present on the substrate, the values can variate. For example, in the presence of hydrosulphide and carbon dioxide, the dissociation balance of ammonia/ammonium would occur in the direction of ammonium, and the inhibition by ammonia is reversed (Fricke et al., 2007). Among that, bacteria in the system can adapt to various environmental conditions and higher concentrations when ammonium or ammonia values increase slowly in the reactors. However, that also makes evaluating the inhibition and toxicity difficult because of the variation. Thus, the concentrations that can occur in inhibition are given in broad ranges.

Based on Morozova et al. 2020 studies, ammonia inhibition in the AD process could also be indicated by the decrease in specific methane yields along with the increase of VFA concentrations and pH dropping due to the inhibition of bacteria growth. In addition, to obtain higher biogas production, the C/N rate is used as an effective parameter for evaluating the performance and stability of the AD because the rate is a parameter to characterise fermentation materials.

The C/N ratio reflects the nutrient level of the digestion substrate. For that reason, the digestion systems are sensitive to the C/N ratio. Among that, higher values of the C/N ratio reflect a low protein solubilisation rate and cause low TAN and FAN concentrations in the system (Mao et al., 2015). Among that, the optimal value of the C/N ratio should range between 20 to 30 to keep enough nitrogen for cell production, avoiding carbon degradation and reducing the possibility of exciting nitrogen, which could lead to a toxic ammonium concentration (Xue et al., 2020). Given this context, a higher C/N ratio provides insufficient nitrogen to keep cell biomass

and causes fast nitrogen degradation by microbials, which results in a lower biogas production (Mao et al., 2015).

A lack of nitrogen in the AD system can cause an insufficient utilisation of carbon sources that can limit AD performance in anaerobic digestion. At the same time, limited C/N ratios lead to the accumulation of ammonia in the fermentative stage, which could generate an inhibition (Resch et al., 2011). Thus, the proper balance of nutrients and ammonia is critical to reacting to the optimal biogas yield.

Furthermore, the possibilities to recover the biological process when occurring inhibition could be by stopping feeding with the substrate, adding substrate with low nitrogen content, refeeding digester material or lowering the pH value. However, those measures can only eliminate lower degrees of inhibition. When dealing with nitrogen-rich substrates, a strategy should be made to keep the C/N ratio to reduce the concentration of TAN and FAN in the digestate to maximum biogas and methane yields (Morozova et al., 2020).

3.4. Direct interspecies electron transfer (DIET)

This chapter will provide information about the different types of interspecies electron transfer. It will mainly focus on the DIET mechanism between microorganisms and how DIET enhances the rate and efficiency of the AD process and, consequently, CH_4 production.

AD process of complex organic materials highly depends on syntrophic interactions between bacteria and archaea, which are built to overcome energy barriers and break down compounds (Z. Wang et al., 2021). The syntrophy provides proximity between organisms which generates the efficiency of H_2 transfer, leading to the conversion of VFAs to methane under AD conditions. In contrast, syntrophy is based on the transfer of reducing equivalents, where hydrogen and formate are electron shuttles for interspecies electron transfer between the microbes (Z. Wang et al., 2021).

Therefore, the interspecies electron transfer occurs in the bacterial production of H_2 from organic substrates and H_2 consumption by methanogens. Additionally, hydrogen or formate need to be maintained at lower concentrations because their metabolism occurs from compounds such as short and long fatty acids, alcohols, and the metabolism of these compounds with the production of H_2 is only thermodynamically feasible when H_2-consuming methanogens maintained with the H_2 production (Zhao et al., 2020).

DIET is a syntrophic metabolism that is found naturally, where electrons are transferred directly from donors to acceptors via microbial nanowires, cytochromes, and conductive materials, as shown in Figure 3. The process occurs during acetogenesis, acetate oxidation and methanogenesis stages, where electrons flow directly from one cell to another without requiring reduced molecules such as H_2 or formate (Gahlot et al., 2020).

Implementing DIET with conductive material in the AD process could facilitate dealing with complex and concentrated wastes and other solids wastes. The conductive materials work as a conduit to the bacterial flagellum that maintains the syntrophy between fermentative bacteria and methanogens archaea. The syntrophy provides proximity between organisms which generates the efficiency of H_2 transfer, leading to the conversion of VFAs to methane under AD conditions. Since the conventional AD process has some limitations, such as the accumulation of VFAs, and the accumulation of H_2, causing an increase of partial pressure of hydrogen and generating inhibition of acetogenic bacteria as described in chapter 2.2. Implementing DIET with AD could improve the efficiency of AD digestion and the CH_4 production (Gahlot et al.,

2020). In order to give a better overview of the advantages of the AD process only with DIET and via conductive material, Table 1 was developed based on Gaholt et al. (2020).

Table 1 Comparative advantages of AD alone, with DIET and DIET via conductive material (Gaholt et al. 2020

Anaerobic Digestion only	AD with DIET	DIET via conductive materials
Environment Protection . GHG quenching . Waste reduction . Prevent soil/ air/ water pollution . Maintaining the global carbon cycle **Energy Recovery** . Biogas regeneration . Carbon neutral **Nutrient Recovery** . Nutrient-rich organic manure .Transformation into marketable productions providing employment and profits . Reduce usage of synthetic fertilises	. Increase rate and efficiency of digestion of organic waste . Requirement of hydrogen/formate shuttle system bypassed . DIET has biogeochemical significance in terrestrial wetlands . Increase efficiency of overall AD process . Make AD more economical for solid waste maanagment . Lower VFA concentrations in reactors . Prevents souring of digesters caused due to organic overloading	**Conductive materials provide:** . High surface area . Increased number of active sites . High reactivity . High specificity . Self-assembly . Mitigate ammonia inhibition in AD . All the above characteristics significantly increase the bio-methanation rate and leads to stable process functioning

The electron transfer methods present in Figure 3 in (a) represents the transmission electron micrograph (TEM) visualisation of a syntrophic acetogen surrounded by methanogenic archaea for interspecies electron transfer via hydrogen/formate (b) is the scanning electron micrograph (SEM) of the potential interspecies electron transfer via pili like structures (c) TEM shows the haem group reactivity in interspecies electron transfer microbes and (d) TEM of interspecies electron transfer microbes in the presence of magnetite. Further explanation about the mechanism will be provided in chapter 2.4.1.

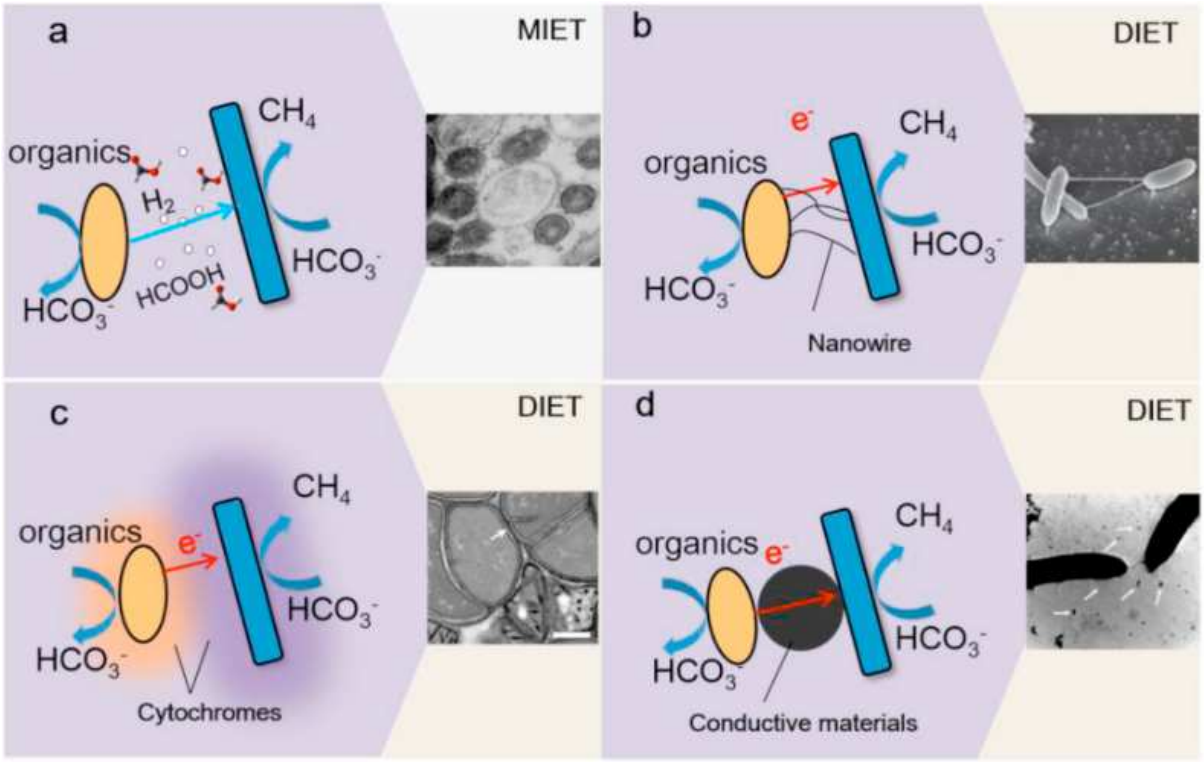

Figure 3 Possible interspecies electron transfer mechanism (Z. Wang et al., 2021).

Another mechanism for electron transfer can occur by hydrogen interspecies electron transfer (HIET), a complect microbial metabolic process that requires different microorganisms in which H_2 is an essential substrate to produce CH_4. H_2 reacts as a carrier to transfer electrons to VFAs or alcohol to CO_2. At the same time, propionate and butyrate are the two main sources of H_2, and their degradation occurs thermodynamically when H_2 partial pressure is low (Zhang and Zang, 2019). However, the HIET process is affected by hydrogen concentration, when the hydrogen partial pressure is higher, the reactions might not be sufficient to support energy conservation, cell maintenance and microorganism growth (Z. Wang et al., 2021). Besides that, hydrogen and formate suffer from inefficient diffusion and strict requirements for intercellular distance, such as higher hydrogen and formate concentrations which lead to a thermodynamical limitation for the propionate and butyrate degradation (Z. Wang et al., 2021). Although HIET is usually higher used as a transfer mechanism for syntrophic propionate oxidation via acetogenis and methanogenesis, the interspecies transfer via formate allows for an increased electron transfer rate (Z. Wang et al., 2021). Comparing both processes, DIET has faster electron transfer than HIET and has lower possibilities to inhibited by higher concentrations of substrates and intermediates. Thus, DIET could enhance AD degradation for complex substrates and reduce the start-up period.

Likewise, the most understanding of interspecies electron transfer in anaerobic digestion is based on mediated interspecies electron transfer (MIET), which occurs via hydrogen and formate. However, MIET to occur in standard conditions is viable only at lower metabolite concentrations due to the thermodynamic conditions (Lin et al., 2017). Comparing both interspecies electron transfer, DIET has been proven more efficient because it does not require various enzymatic steps to produce hydrogen as an electron carrier (Lin et al., 2017). Nevertheless, DIET

system, hydrogen and formate electron carriers are not involved in the transfer between organisms, which results in faster biological conversion. Then, the hydrogen and formate concentrations do not inhibit the metabolism process and overcome the thermodynamic limitation under higher hydrogen partial pressure as occurs with MIET and HIET in the anaerobic digestion process (Z. Wang et al., 2021).

To conclude, the advantage of DIET is still being studied, but it has demonstrated that the DIET process is less sensitive to accumulating H_2 because electron transfer occurs faster than diffusive electron exchange by soluble electron shuttles. Thus, the process is beneficial when higher rates of organic loading occur and rapid metabolism of fermentable substrates releases H_2 (Zhao et al., 2020).

3.4.1. DIET via conductive pile and cytochromes

This chapter will describe the different types of DIET mechanism, their functionality, and some microorganism present. From that, it is possible to understand how DIET performs and which mechanism can enhance AD digestion.

There are three types of DIET mechanisms, as shown in Figure 4 identified as transfer of electron connections via e-pili and OmcS (a multiheme c-type cytochrome) of electron-donating microorganisms, DIET via membrane-bound electron transport proteins and via abiotic conductive materials. However, the mechanism from e-pili helps DIET with two bacteria by the association in which electrons are released during the oxidation (J.H. Park et al. 2018).

The first mechanism is the E-pili which are protein filaments produced by microorganisms during electron transfer under specific conditions (J.-H. Park et al., 2018). Extracellular electron acceptors can obtain electrons from c-type cytochromes generated from exo-electrogenic microorganisms, such as *Geobacter* and *Shewanella.* In contrast, OmcS cytochrome in *G. sulfurreducens* might be an essential electron mediator to facilitate electron transfer from e-pili to electron acceptors (L. Li et al., 2021). The longer-range electron transfer can occur through the conductive pili independent of the indirect mechanism, solid electrodes, other microorganisms and electrically conductive biofilm (Rasapoor et al., 2020).

The species that have worked well with DIET are *Geobacter species* which are abundant and metabolically active in the methanogenic environment, as well in anaerobic digestion digesters. Thus, *Geobacter* is considered to establish DIET-based syntrophic associations with methanogens via conductive e-pile and OmcS, which accept electrons and reduce CO_2.

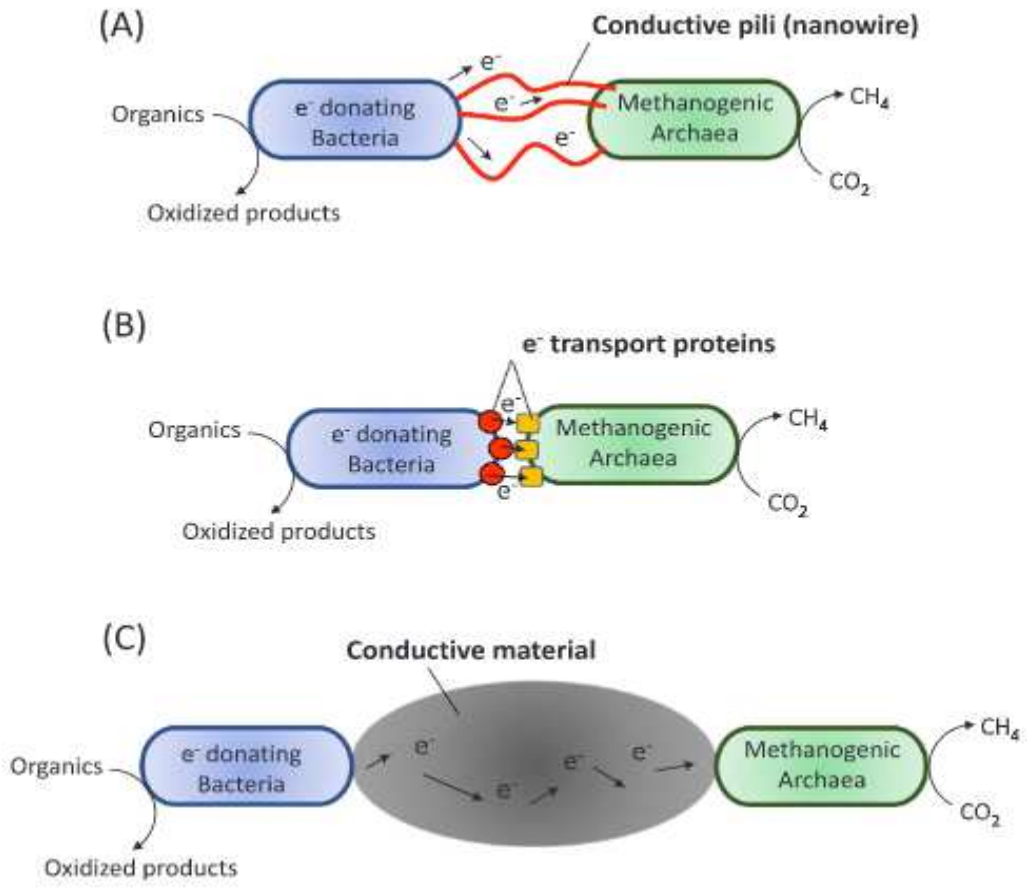

Figure 4 Mechanisms of DIET between organics-oxidising bacteria and archaea. (A) DIET via conductive pili. (B) DIET via membrane-bound electron transport proteins. (C) DIET via abiotic conductive materials (Lovely, 2017b).

The species that have worked well with DIET are *Geobacter species* which are abundant and metabolically active in the methanogenic environment, as well in anaerobic digestion digesters. Thus, *Geobacter* is considered to establish DIET-based syntrophic associations with methanogens via conductive e-pile and OmcS, which accept electrons and reduce CO_2.

The second mechanism of DIET is via membrane-bound electron transport proteins (B), where a photophic bacterium *Prosthecoholris aestaurii* could accept electrons by *G. sulfurreducen*s in a co-culture of two bacteria. The transfer of electrons occurs by the multiheme outer-surface cytochrome OmcZ, realise the transfer of electrons to electrodes (J.-H. Park et al., 2018). Zhang and Zang (2019) mention studies where DIET by membrane-bound electron transport proteins such as OmcZ might be a mechanism for methane production. However, there are few studies about the second mechanism that could suggest that DIET by membrane-bound electron transport protein is not typical for methanogens (Zhang and Zang, 2019).

In the third mechanism, DIET via abiotic conductive materials (C) establishes an electrical connection, e.g. between *Geobacter* and *Methanosaeta* species with the objective of increasing methane production. Therefore, carbon-based conductive materials such as biochar, granular activated carbon (GAC), powdered activated carbon, carbon cloth, carbon nanotubes, graphite and graphene have been widely used in the DIET stimulation (Zhang and Zang, 2019). Those

materials have high electron conductivity, large specific surface area and porosity (Zhang and Zang, 2019). It is reported that anaerobic digestion reactors with conductive materials have higher resistance by strengthening DIET to resist the effects of acid shock. Hence, it is beneficial to the growth and electron transfer of microorganisms.

Furthermore, Z. Wang et al. (2021) mention a combination of studies that focus on DIET for butyrate and propionate oxidation which could probably be established between syntrophic microbes such as *Syntrophaceae, Syntrophomonas, Anaerolineacea, Smithella* and methanogens. Also, bacteria such as Bacteroides and *Syntrophomonas* species that occur in a diverse mix of bacteria were related to increased sludge conductivity that could result from the electric conductivity of the filaments. Among them, co-cultures with *G. metallireducens* as the electron-donating partner and *Geobacter sulfurreducens* as the electron acceptors partners show that propionate and butyrate, as well as acetate, propanol and butanol, could be metabolised via DIET with fumarate as electron acceptor (Zhao et al., 2016). Moreover, they have generated electricity from butyrate oxidation with the combination of *Geobacter* and have sped up methane production with the conductive materials (Z. Wang et al. 2021). Nevertheless, there needs to be more evidence showing how microbes functionate with DIET.

Although DIET, related to bacteria and methanogens on anaerobic digestion has great potential to accelerate and stabilise biochemical reactions exchanging electrons through a biological electrical connection. There is a need to understand better the DIET process related to volatile fatty acids and their relation with microbes (Z. Wang et al., 2021).

As far as known, the organic conversation reported by Wang et al. (2021), syntrophic oxidation of variable fatty acids accelerates DIET due to the concentration of VFAs. Then, after oxidation in a DIET reactor was 63.5% of the value of the control, amounting to a maximum VFA consumption rate of 57.5% and a methane production rate increased by 27%. Peng et al. (2018) also reported that using GAC and magnetite enhanced the reduction of COD, proteins, and polysaccharides, while Lizama et al. (2019) observed an increase in protein by 23.2% and carbohydrates by 17.8%. Thus, indicating that DIET improves the acetogenesis-methanogenesis process and anaerobic digestion performance.

3.4.2. DIET via a conductive material

The objective of including conductive materials such as granular activated carbon (GAC), biochar, magnetite, and graphite are made to enhance anaerobic digestion from instabilities and increase methane production. That occurs because the conductive material serves as an electrical conduit between bacteria, helping direct electron transfer and enriching the specific microbial populations such as *Geobacter* and *Methanosaeta* species in an anaerobic degradation (J.-H. Park et al., 2018). Therefore, some organic compounds are metabolised faster with methanogenic mixed species in DIET, such as glycerol, glucose, sucrose, phenol, and hydrocarbons. Still, it requires more microbiological investigation, as Zhao et al. 2020 reported.

Conductive materials have been reported to provide similar functions as OmcS and e-pili by stimulating DIET in co-cultures of *G. metallireducens* and *G. sulfurreducens* (Li et al., 2021). They can work as a cytochrome in a co-culture which contains an OmcS-free *G. sulfurreducens* that could mutate as an electron-accepting bacterium. Therefore, *Geobacter spp*. might have the genes that code for the Pila pilin monomer of e-pili and the homologues of genes that code for the cytochrome and protein involved in extracellular electron transfer. In addition, using conductive materials could avoid the energy consumption related to the production of extracellular conductivity pili and c-type cytochromes to supply the biological electrical connections between cells (Lin et al. 2017).

Furthermore, DIET might give more energy for transferring electrons than MIET because less energy is lost related to the formation of intermediates and the reactions to oxidise them, which could explain the acceleration of methanogenesis in the presence of conductive materials (Lin et al., 2017). The simplified electron transfer mechanism for DIET and MIET with hydrogen as electron carriers is illustrated in Figure 5(a) the electron transfer in anaerobic digestion is mediated from interspecies electron transfer, and (b) is direct interspecies electron transfer via graphene, as an example.

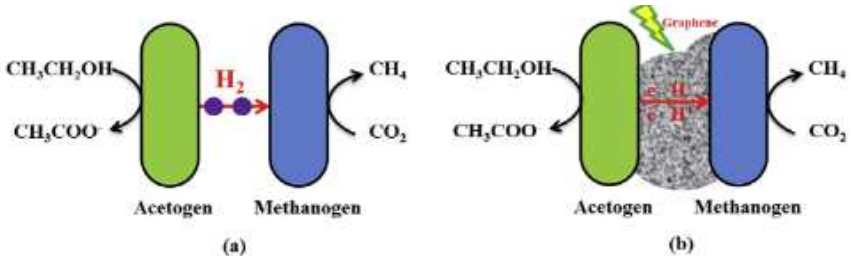

Figure 5 Mechanism for an extracellular cell-to-cell electron in anaerobic (Lin et al. 2017).

Although obtaining the efficiency of anaerobic digestion with DIET and conductive materials requires two types of microorganisms: electron-donating bacteria and methane-forming archae. Where electron-donating bacteria oxidise the organics, making them release the electrons to the conductive material. In contrast, methanogenic archaea use electrons transmitted from electron-donating bacteria through the conductive materials to reduce the carbon dioxide to methane (Gahlot et al., 2020).

Bacteria such as flagellum are needed in the anaerobic process to maintain syntrophy (fermentative bacteria) to ensure efficient hydrogen H_2 transfer. When that occurs, the syntrophy and methanogens convert VFAs to methane under anaerobic conditions (Gahlot et al., 2020). Thus, conductive materials with DIET could electrically connect *Geobacter* and *Metallireduces* species by metabolising ethanol when the co-culture is supplemented with conductive materials. Consequently, it would accelerate the substrate degradation and subsequently increase the production of methane yield and lower VFA concentrations in the anaerobic digestion (Gahlot et al., 2020). The acceleration of organic acid degradation and tolerance with higher hydrogen could also be attributed to the nature of conductive materials. Hydrogen was adsorbed by the added material and used as aggregated hydrogenotrophic methanogens (Z. Wang et al., 2021).

Nevertheless, bacteria need time to adapt to the new environment, which is known as lag time. Consequently, some fast-grown bacteria display a short lag phase compared to the slow-growing bacteria (Gahlot et al., 2020). Park et al., 2018 mention a decrease in the lag phase by 10%-75% when the reactor has conductive materials. In contrast, they also have shown an improvement in methane production rate and methane yield between 79%-300% and 100%-178% because they have guided in faster electron conduction by diffusible carriers. Additionally, short-term and long-term operations have provided different results despite having the exact dosage of materials. Tian et al. (2017) mention that long-time exposure to a higher concentration of nano-graphene has suppressed yield, while short-term exposure increased output.

Among that, the use of conductive materials is made more efficient during methanogenesis because of the higher conductivity of the material used, large surface, and long electron transfer distance of the conductive material (Z. Wang et al., 2021). Therefore, carbon-based materials such as GAC, biochar, and graphene produced 13% to 140% more methane (Z. Wang et al.,

2021). However, the composition of the microbial community and reaction conditions could have an impact on enhancing methane production. Z. Wang et al. (2021) described a study where DIET was implemented with GAC addition, and the digestion system could resist OLR, COD and VFA removal and maintain efficiency in methane production. That occurs in anaerobic digestion of raw incineration leachate with inhibition due to higher OLR that could take over 100 days to start up and quickly deteriorate.

In an experiment of the short term, using graphene as conductor material on the AD system resulted in an enhancement in methane yield, a production rate of 17% with a 30 mg/L and later to 120 mg/L, which generated an increase of 51.4% higher than the control value (Tian et al., 2017). The implementation of graphene could also increase chemical oxygen demand (COD) removal efficiency and can change the syntrophic association between acidogenic microbes and methanogenic archaea and (M. Liu et al., 2021). In addition, the changes in graphene could improve electrical conductivity and increase biogas production since graphene is rich in hydroxyl, carboxyl, and epoxy groups which can be modified by esterification and amidation (M. Liu et al., 2021).

Furthermore, glucose has been shown to be fast converted into small organic compounds where VFAs were produced from glucose fermentation, such as propionate, acetate, and butyrate (Tian et al., 2017). In addition, it was demonstrated that glucose methanogenesis was improved by adding graphene in the long term in anaerobic digestion under lower temperatures between 10-20°C. Thereby, methane production and the changes in acetate and butyrate production were associated with the graphene dosage, which concluded that graphene is capable of inducing substrate utilisation, converting acetate to methane (Tian et al., 2017).

By contrast, comparing graphene and activated charcoal as conductive materials in anaerobic digestion resulted in significant growth in biomethane production from graphene than activated charcoal. It could be due to the electrical conductivity of graphene being higher than activated charcoal and the micro size of graphene being smaller, resulting in a higher specific surface area and better interactions with microbes (Lin et al., 2017). In an experiment using ethanol, it was noticed that 32% of ethanol was consumed in the absence of conductive material and adding 1.0 g/L graphene and 20.0 g/L activated charcoal obtained 50.3% and 43.5% of ethanol consumed. Hence, the results may prove that graphene has a significant role in the rapid degradation of ethanol, which can be related to the higher electrical conductivity and specific surface area (Lin et al., 2017). These results provide evidence that conductive materials can promote syntrophic reactions between acetogenic bacteria and methanogenic archaea, facilitating substrate degradation and utilisation for supply methanogenesis.

Lin et al. (2017) mention in the study the microorganisms in the presence of graphene in AD digestion of ethanol with DIET. The microbial dominant was the *Geobacter* population of 9.9%, and an abundance of *Pseudomona*s increased by 6.9%, while only 1.9% was without graphene. Moreover, *Pseudomonas* species are electrogenic bacteria responsible for converting VFAs to electric current in microbial fuel cells. However, they cannot transfer electrons derived from central metabolism to the outside of the cell, which is a weak conductive pili (Lin et al., 2017). However, adding graphene in AD might react as conductive pili and electron transfer from *Pseudomonas* to the methanogenic during AD, contributing to their increase. As a result, *Pseudomonas* and *Geobacter* could be considered responsible for the process on a DIET in AD of ethanol due to their significant enrichment with the addition of graphene.

3.4.3. Microbial electrolysis cells (MEC)

This chapter and chapter 2.4.4 will further explain the MEC functionality with the implementation of a supply voltage. The aim is to facilitate the bioelectrochemical reactions to enhance the degradation of AD reactors and methane production.

MEC is a microbial electrochemical technology which can use supply voltage to improve bioelectrochemical reactions. Such reactions result in efficiency in biogas production in AD reactors by faster degradation of highly concentrated wastes, VFAs, toxic materials and non-degradable organic matters (J. Park et al., 2018). Therefore, a MEC cell has the potential to stimulate the growth of hydrogenotrophic, which leads to methane production by hydrogen consumption or electron supply at the cathode. The H_2 electron form cathode has been proven to increase methane production and enhance stability in anaerobic digestion, which is related to the hydrogenotrophic methanogenesis (Cai et al., 2016).

The advantage of using MEC to produce methane is that the process can occur at ambient temperature, avoiding the heating cost (Y. Zhang and Angelidaki, 2014). In contrast, MEC can work with a large variety of substrates, from pure compounds to complex substrates of organic matter in the wastewater (Kadier et al., 2016). Some of them are glucose, glycerol, cellulose, acetic acids, livestock, food processing convert by-products of fermentation, such as acetate and butyrate and many others (Kadier et al., 2016). Those compounds can be synthesised at the cathode by combining the electrochemical production of hydrogen as an electron donor. Hydrogen is often used as the primary electron donor. However, the microbial electrosynthesis pathway is also used to involve direct electron transfer from the cathode to the microorganism. For example, hydrogenotrophic methanogenic *Archaea* can locate in the cathode, where they will use the hydrogen produced to reduce CO_2 to CH_4, as shown in reaction 10 (Rousseau et al., 2020).

Equation 9 $$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$$

Among that, the methane production from the MECs system is composed of an anode, cathode, and electricity source, as shown in Figure 6. Furthermore, the energy supply is connected to the anode and cathode under anaerobic conditions. At the anode occurs, oxidation reactions, which are essential to provide electrons for reducing CO2 in the cathode region (Amrut Pawar et al., 2020). On the other hand, in the cathode area, a mix of microorganisms uses the electrons supplied from the oxidation reaction from the anode to produce methane (Kadier et al., 2016). Hence, microorganisms on the anode can convert chemical energy in organic substrates into electrical energy, which is then used to generate products such as H_2 and CH_4 at the cathode.

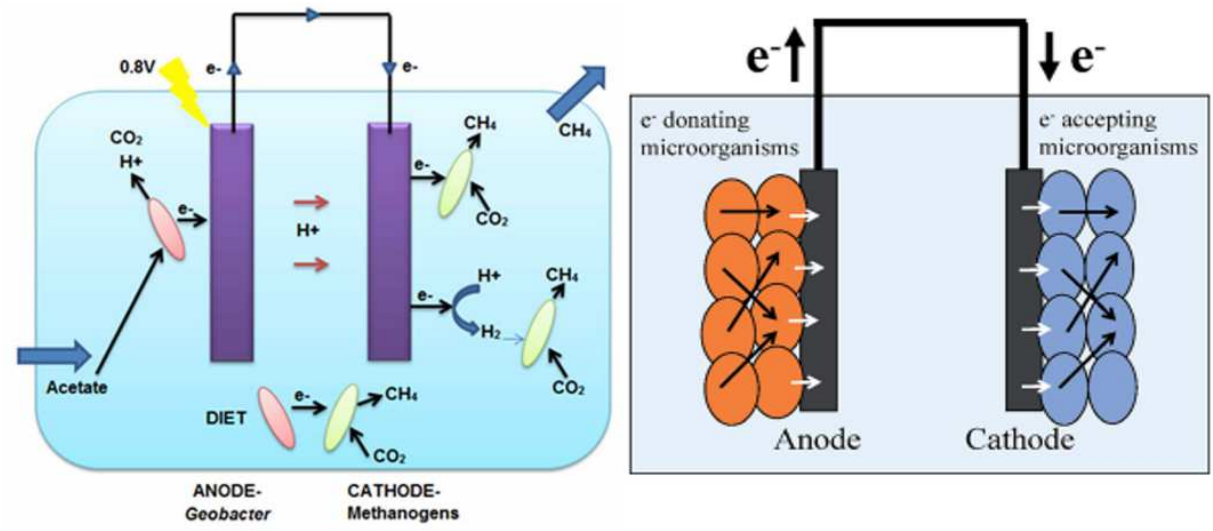

Figure 6 Typical MEC cell with the various pathways of bacterial methane production presented (Vipond and Rahman, 2018). In the image from the right side shows the potential electron transfer rountes through biofilm via DIET with black arrows. Extracellular electron transfer to/from the anode/cathode shown with while arrows (Cheng and Call, 2016).

In the MEC system, the electrochemical activated microorganisms spontaneously form the electroactive biofilm on the electrode surface. The main populations at the anode convert organic compounds to protons, CO_2 and electrons. Those electrons generated are transferred to the anode and then to the cathode through an electrical circuit where the hydrogen is generated (Rousseau et al., 2020). By contrast, there is an energy barrier or resistance in the electrochemical process when producing hydrogen from water, called the systems over potential. Due to thermodynamic limitations, organic compounds such as volatile acids, butyrate, acetate and propionate and solvents such as ethanol and butanol cannot be used in the fermentative H_2 production (Dange et al., 2021). Therefore, to achieve higher energy efficiency for the water-slitting electrochemical reaction use of specific catalytic is essential because they help to reduce the overpotential of the hydrogen production system and contribute to the charge transfer reaction at the surface of the cathode electrode (Amrut Pawar et al., 2020).

The electrogenic microorganism aggregated on the anode surface and decomposed the organic matter into CO_2, electrons (e-), and protons (H+) as part of its metabolism. The microorganism transfers the electrons to the anode, and the protons are liberated into the MEC solution. The equations related to the electron moving from the cathode to the anode representing sodium acetate (Kadier et al., 2016):

Equation 10

$$\text{Anode:} \qquad CH_3COO^- + 4H_2O \rightarrow 2HCO^{3^-} + 9H^+ + 8e^-$$

Equation 11

$$\text{Cathode:} \qquad 8H^+ + 8e^- \rightarrow 4H_2$$

Nevertheless, the reactions do not occur spontaneously. MEC requires an external supplied voltage under a biological condition to occur spontaneously and produce hydrogen at the cathode from a combination of protons and electrons. Hence, for any reaction to happen spontaneously, the Gibbs free energy of the reaction (ΔGr) should be negative, but the conversion of most of the organic compounds to hydrogen yields a positive ΔGr. An example, under standard biological conditions (T= 25°C, PH2 = 1 atm, pH= 7), the Gibbs free energy of reaction (ΔGr°′) for acetate oxidation to hydrogen is positive, as shown in the reaction (Logan et al., 2008):

Equation 12 $$CH_3COO^- + 4H_2O \rightarrow 2HCO^{3-} + H^+ + 4H_2$$

($\Delta Gr^{\circ\prime}$ = + 104.6 Kj/mol)

In a reaction with a positive $\Delta Gr^{\circ\prime}$, the acetate cannot be fermented to hydrogen. For that to occur, extra energy has to be added to the system to overcome the thermodynamic limit for hydrogen evolution. In MEC, the added voltage supply can be done by the power supply (Logan et al., 2008). Therefore, the microbial electrolysis process requires an applied voltage higher than $\Delta Gr^{\circ\prime}/nF$, where *n* corresponds to the amount of electrons involved in the reaction. This value represents the equilibrium voltage (Eeq), which for acetate under standard biological conditions is represented in Equation 14 (Kadier et al., 2016). Among that, to obtain the equilibrium voltage, it is necessary to determine the individual half-life potential using the Nernst equation (Dange et al., 2021).

Equation 13

$$E_{cat} = E^{\circ}_{cat} - \frac{RT}{2F} 1n \frac{P_{H_2}}{[H^+]^8} \qquad = 0 - \frac{8.314 \times 298.15}{2 \times 96485} 1n \frac{1}{[10^{-7}]^8} = -0.414\ V$$

E_{cat}= is the standard electrode potential for hydrogen (0 V);

R= 8.314 J/K*mol is the universal gas constant.

T = 298,15 (K) is the absolute temperature.

F= 96485 C/mol e^- is the Faraday's constant.

In addition, to the anode reaction, the theoretical reduction potential is represented according Equation 14 with the reaction of equation 13:

Equation 14

$$E_{an} = E^{\circ}_{an} - \frac{RT}{8F} 1n \frac{[CH_3COO^-]}{[HCO^{3-}]^2 [H^+]^9}$$

$$= 0.187 - \frac{8.314 \times 298.15}{8 \times 96485} 1n \frac{0.0169}{[0.005]^2 [10^{-7}]^9} = -0.3000\ V$$

F= 96485 C/mol e^- is the Faraday's constant

As described before, the negative result indicates that the reaction is not spontaneously and a voltage has to be applied in order to overcome the limitation and produce hydrogen (Logan et al., 2008). The power source needs to have a higher applied potential than the equilibrium

potential (E_{eq}) which can be calculated by the theorical anode (Ean) and cathode potential (Ecat) as given (Logan et al., 2008):

Equation 15 $$Eeq = Ecat - Ean$$

E_{eq} represents the equilibrium potential of the EC.

E_{cat} is the cathodic half-life potential.

E_{an} is the anodic half-life potential.

$E°_{an}$ = 0.187 V is the standard electrode potential for acetate oxidation for a solution of HCO^{3-} = 0.005M, CH_3COO^- = 0.0169M, pH = 7. Thereby, the equilibrium for a MEC to produce H_2 at cathode under those conditions is:

$$E_{eq} = E_{cat} - E_{an} \Rightarrow (-0.414V) - (-0.300V) = -0.114V$$

The equation 14 shows that the cathode and Eeq depends on the PH_2, thus lowering PH_2 it is possible to decrease the need of potential at the cathode. Hence, when extracting biogas that contains higher H_2 content it will also support the oxidation of VFA like acetic acid. Logan et al., (2008) mention that each 10-fold increase of the hydrogen partial pressure increases Eeq by 0.03V. It is also mentioned that producing hydrogen at a partial pressure of 10 or 100 bar, instead of 1 bar, theoretically requires only an additional voltage of 0.03 or 0.06V, which is equal to an additional energy requirement of 0.06 and 0.13 kWh/m3 H_2. Therefore, this leads to electrochemical pressurization which might made possible to produce hydrogen at pressures much higher than atmospheric pressures, which could also work the reverse and thus, could reduce the use of voltage for lower hydrogen partial pressures (Logan et al., 2008). In the case of using supply voltage to produce H_2, the voltage should be higher than 0.114 V (Dange et al., 2021). Generally, the applied voltage is considered higher than the theoretical due to the mass transport loss in the MEC system, ohmic loss, and active loss.

3.4.4. MEC with energy supply

The implementation of MEC with an energy supply is used to provide extra energy during the AD process. The acceleration of electron transfer might improve the methane production rate and yield (Guo et al., 2022). Researchers have observed that the standard voltage values might vary between 0.2 – 0.8 V in the AD reactors for bioelectrochemical reactions where exoelectrogenic bacteria degrade organic matter and release electrons to the anode (J. Park et al., 2018).

The results showed that the bioelectrochemical responses might increase methane production by improving microbial activities and removing organic matter and VFAs (J. Park et al., 2018). Another experiment by Cerrillo, Viñas, and Bonmatí (2016) used an AD reactor in MEC with organic and nitrogen overload from pig slurry. The results indicate that AD with MEC stabilises organic and nitrogen overloads. Therefore, the studies suggested that MEC could generate better results when implemented in the AD process with a supply voltage.

Furthermore, Choi et al. (2017) mention an increase in methane production in a glucose substrate when the external voltage increases from 0.5 V to 1 V, as shown in Table 2 at an operation 1.0 V with 408.3 mL CH_4/g COD glucose, which has 30.3% higher than the control reactor without a supply voltage. They have observed that the bioelectrochemical methane fermentation of CO_2 to CH_4 was enhanced due to the external energy supply. However, when rising

to 1.5 V, it was noticed a decrease in methane production, which could be a suggestion that at higher voltage, microorganisms were being destroyed (Choi et al., 2017). In comparison, Linji et al. (2013) have found that the optimal external voltage for energy recovery from waste-activated sludge is 0.8 V.

By contrast, Choi and Lee (2019) observed methane yield and production rates by 1.2 and 1.3 times higher in an AD reactor coupled with MEC for AD in food waste substrate under an electric field of 1.2 V. In comparison, Linji et al. (2013) have found that the optimal external voltage for energy recovery from waste-activated sludge might be 0.8 V.

Table 2 Maximum current generation in MECs reactors with variation in applied voltages (Choi et al. 2017).

Parameters	**0.5 V**	**0.7 V**	**1.0 V**	**1.5 V**
Current density (A/m^3)	5.84 ± 0.16	12.28 ± 0.94	19.04 ± 0.29	2.36 ± 0.07
Total coulomb (C)	377.8 ± 12.6	616.8 ± 77.1	1,354 ± 145.7	159.1 ± 38.2
Cathode potential (V)	-1.08 ± 0.02	-1.13 ± 0.01	-1.14 ± 0.02	-1.03 ± 0.02

Table 2 results could suggest that different voltage values affect electron transport between electrodes, resulting in different oxidation and reduction reaction rates on the electrodes. By contrast, J. Park et al. (2018) experimented on the effect of MEC on methane production rate and the stabilisation time of highly concentrated food waste. The methane production daily increased to 12.1 ± 2.2 L/d, and in the final stage, generating values of 17.0 ± 1.6 L/d, as represented in Figure 7.

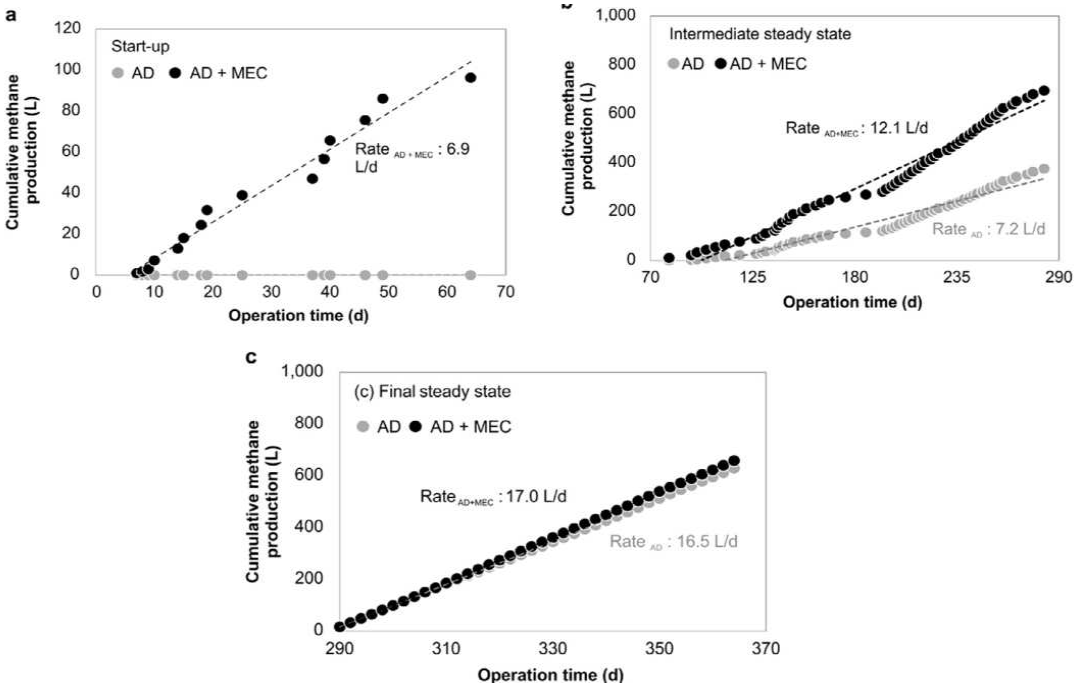

Figure 7 Methane production rates of AD + MEC reactors (J.Park et al. 2018).

The experiment was divided into three phases (a) start-up, (b) intermediate steady state and (c) final steady state. Thereby, the results reported that COD removal and methane production efficiency in the final steady state was almost the same as AD. Still, it has sped up methane production rate without a drop in pH or accumulation of VFAs by MEC. That has occurred because the bioelectrochemical reactions prevented the factors that cause inhibition during start-up.

4. Materials and Methods

This chapter will detail the experiment design conducted in this study. The study was a long-term fermentation test following the German standards DIN 38414-8 and VDI 4630. The objective of the test is the quantitative and qualitative influence of a gradually increased nitrogen concentration on the anaerobic microbial degradability of the fermentation substrate under a defined electrical voltage.

The DIN method was initially applied in the wastewater sector, describing the use of eudiometer tubes for gas collection. VDI 4630 method is aggregate to DIN 38414-8, and it stands for "Fermentation of organic substances", which addresses the use of small-scale digesters and provides specific guidelines for the duration of the digestion. Therefore, the laboratory-scale digesters used in this study were operated in a eudiometer batch system composed of six identical batch fermenters. Two were referential batches without electrical voltage and two with electrical voltage. The experiments were performed at room temperature under psychrophilic conditions.

In this study, a laboratory-scale semi-continuous experiment was composed of two independent experiments. One for 92 days and the second for 34 days, with a feeding interval of 1-2 days (three times a week), and urea was feed around 25-50 ml of digested sludge. Moreover, the same volume added to the system was taken for analytical determination, such as microbiology analyses, pH, temperature, COD, nitrogen, acetic acids, and others. In addition, the biogas produced during the period was recorded and taken for analysis of the composition using the mobile multi-gas measuring device *Dräger x-am 8000.*

4.1.1. Experiment design

This chapter will describe the components used to set up the lab-scale batch experiment during anaerobic digestion in the eudiometer. The experiment was set up on the frame of the manufacturer *MiniTec,* composed of single aluminium profiles that could be screwed together and supported levels of solid wood where it was a support area for the reactors. The middle level of the aluminium frame was used as a storage level for the laboratory power supply units, and the top above was occupied by vessels with the sealing liquid for the eudiometer Figure 8 shows a schematic diagram of the individual components in the overall setup of the experiment.

For the execution of the experiment shown in Figure 8 the schematic diagram of the components is composed of (A) the level vessel with barrier liquid, (B) the current transmitter, which is the laboratory power supply, and (C) the glass reactors with graphite electrodes. Each part of the components will be described in more detail below.

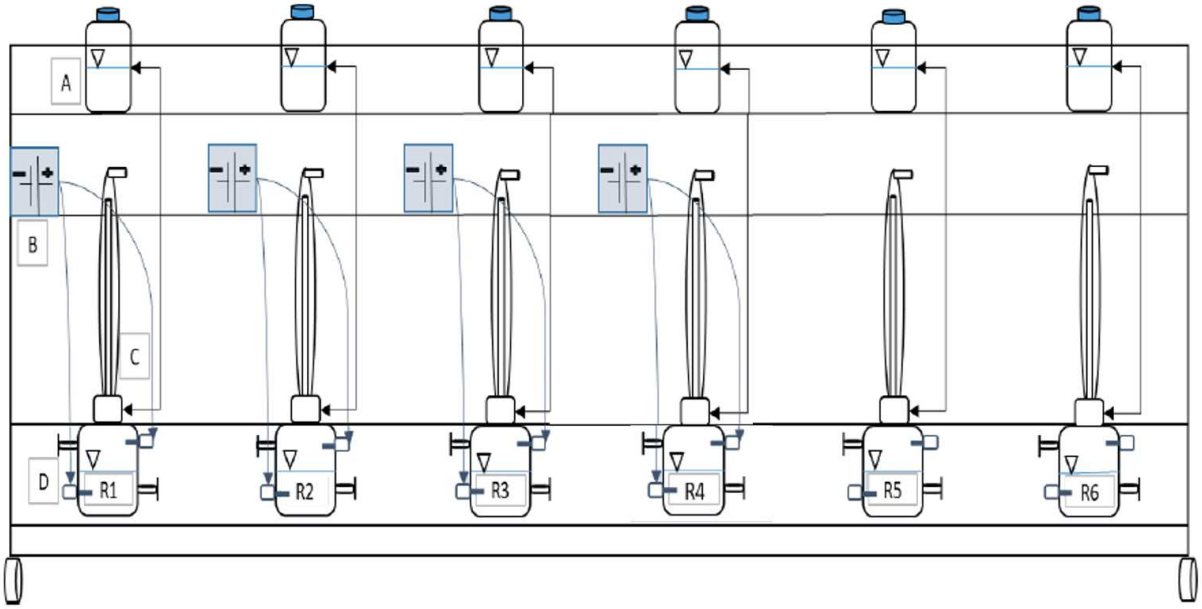

Figure 8 Modified Schematic diagram of the components in the overall setup of the lab-scale experiment (Witkabel, P. 2022).

Therefore, (D) represents the glass reactors with a volume of 1.0 L developed by the manufacturer *TOPAS*. Those glass vessels were specially manufactured for the research project conducted by the Institute of Waste Management and Circular Economy at TU Dresden, Germany. They contain a central opening used for feeding and sampling, located at the top through which the vessels are connected to the eudiometer (C), as shown in Figure 9.

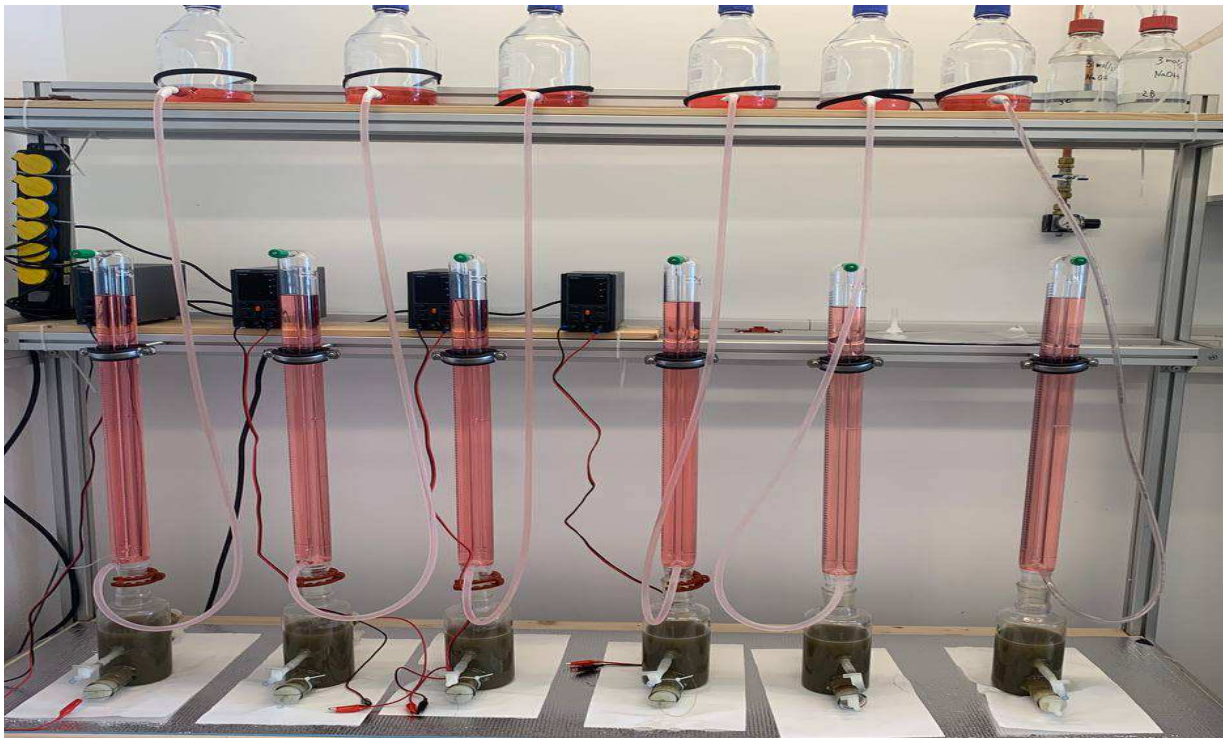

Figure 9 Experiment Setup of the long-term test, own representation.

4.1.2. Reactor design

The six batch reactors of AD were conducted in glass fermenters with a capacity of 1.0 L, as shown in Figure 10. They are composed of three opening areas, one on the top was used to connect with the eudiometer, and the other two were aggregated with the graphite electrode and further sealed. Subsequently, there are two 12 mm glass nozzles which could be used for continuous operation. However, only the nozzle close to the electron on the top has been used for feeding and sampling. All the openings and nozzles were properly tight or sealed to avoid leaking.

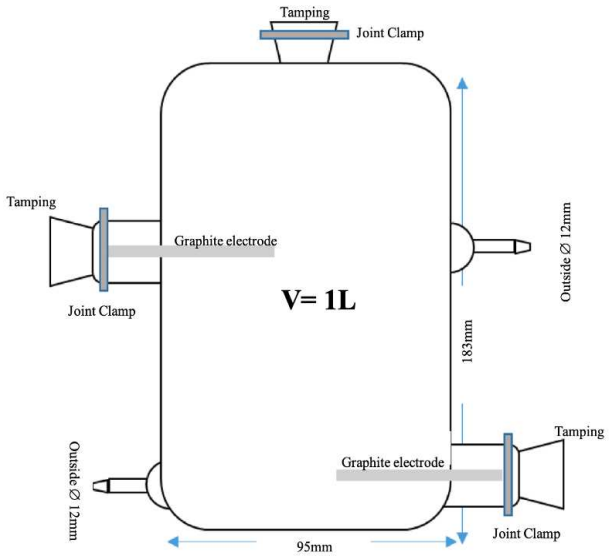

Figure 10 Modified batch reactor from the lab-scale experiments (Witkabel, P. 2022).

Moreover, the graphite electrodes were included in each reactor in a gas and liquid-tight manner using two plugs. Additionally, the titanium wires were wrapped with heat shrink tubing and insulated, then attached to the electrodes and allowed through an opening in the plugs to the power supplies (B).

The eudiometer is a glass tube used to qualify digester gas volumes over a liquid in a reactor (Brody, et al., 2010). Among that, it is composed of ground glass and has a capacity of 1,000 ml volume on each reactor, graduated from the top down from 0-1,000 ml in 5 ml intervals steps which allows reading the barrier liquid levels in the eudiometer. They are placed into an upright bottle with a ground joint fermenter of 1.000 ml. In contrast, there is a connecting tube inside the eudiometer that permits the digester gas to enter without any barrier liquid entering the reactor. It is located on the upright bottle into the measuring tube and runs through the eudiometer bottom as shown in Figure 11. The reactors are connected from the top to the eudiometer and levelling bulb in which the production of digester gas displaces. The sealing liquid from the eudiometer enters the level vessel via a hose line, making it possible to read the quantity of the biogas produced. In addition, the manufacturer Neubert- Glas GbR produced the eudiometer used in this study.

Therefore, the change in the sealing liquid provides information about gas production. By contrast, the connecting surface between the eudiometer and the ferments was designed to remain fixed because the glass was designed to rub with the laboratory grease to guarantee proper

gas tightness. Nevertheless, the ground joint clamp attached from the outside was provided to reinforce the tightness and stability of the tamping.

Figure 11 Eudiometer for determination of gas from substrate, own representation.

Among that, on the top level of the eudiometer have the level vessels (A), which were filled with sealing liquid. Based on the DIN 38414-8, the sealing liquid consisted of 30 ml hydrochloric acid (H_2SO_4) in 1L distilled water, 200 mg sodium sulphate decahydrate ($H_{20}Na_2O_{14}S$) and a few drops of methyl orange.

4.1.3. Graphite as electrode

For the production of methane in a MEC system, the electrode should have high electrical conductivity, chemical stability, anti-corrosiveness, good biocompatibility, slower density, large surface area, founding resistance, easy to construct and lower cost for implementation (Amrut Pawar et al., 2020). Therefore, graphite has been used in many literatures as an electrode in MEC experiments due to the higher surface area in a small volume, which could lead to a high volumetric current density since more surface is available for biofilm attachment (Sleutels, 2010).

Furthermore, the indirect interspecies transfer of electrons via electrons which conduct hydrogen or formate in conventional AD is not as efficient as DIET with conductive materials (Gahlot et al., 2020). Nevertheless, the efficiency of DIET with conductive materials requires electron-donating through conductive material and methane-forming archaea where the electron-donating bacteria oxidise the organics that release the electrons to the conductive materials.

Meanwhile, the methanogenic archaea use those electrons from conductive materials and reduce the carbon dioxide with hydrogen to methane (Gahlot et al., 2020). Moreover, Amrut Pawar et al. (2020) mention that using graphite as a cathode could produce more methane than a precious metal-based cathode.

Among that, a study conducted by M. Zhang et al. (2019) shows that the use of graphite as an electrode in AD digester has increased CH_4 production, enhancing the degradation of propionate and butyrate and may have accelerated the electron transfer. They also suggested that graphite could reduce the lag time of the start-up phase and increase the degradation of ethanol. That could a result from a higher electrical conductivity and graphite surface area, which facilitate the growth metabolism of the AD process.

In general, carbon-based conductive materials can promote the syntrophic metabolism of alcohols and VFAs in DIET, which results in a decrease in the lag phase and an increase in methane yield. Hence, graphite has been reported to stimulate the syntrophic conversions of alcohols and VFAs to methane through DIET in defined co-cultures of *Methanosacina barkeri* and *G.metallireducens* with populations of *Methanosaeta* and *Methanosarcina sp.* in which their get attached on the conductive material (Gahlot et al., 2020). The presence of those bacteria on the MEC reflects that they may donate electrons to the conductive materials.

4.1.4. Urea as an input substrate

The fermentation experiments conducted in this study investigated the influence of an electricity feed on the degradation of the nitrogen-rich substrate in biogas production by the implementation of the approach of MEC to force microbial change. Therefore, urea (CH_4N_2O) reacted as the nitrogen supplier in the study, and it was chosen because it is an end product of the metabolic breakdown of proteins in all mammals and some fishes (Britannica, n.d.). Thus, it is essential nitrogen in animal excretions resulting from agriculture and is widely used as a fertiliser, feed supplement, and starting material for manufacturing plastics and drugs.

Urea was added as a nitrogen source into the digester, which would be decomposed during fermentation and generated ammonia (NH_3) and ammonium (NH_4^+) (Lin et al., 2017). Then, the bacteria involved in biogas production can use ammonia and ammonium as nitrogen sources. However, as already described, large amounts of them can cause inhibition or toxicity for bacterial growth. In addition, when raw materials are used in the urea manufacturing process, there is ammonia and carbon dioxide which generate urea in the ammonia plant because it yields ammonia as a product and carbon dioxide as a by-product that can be used directly for manufacturing urea process (‘Urea Manufacturing Process’, 2020). The chemical reactions are primarily involved in the process:

The reaction of ammonia and carbon dioxide reacts and form ammonium carbamate:

Equation 16 $$2NH_3 + CO_2 \rightarrow NH_2COONH_4$$

Decomposition of ammonium Carbonate to form Urea and Water:

Equation 17 $$NH_2COONH_4 \rightarrow H_2O + NH_2 + NH_2CONH_{2\,(urea)}.$$

4.2. Experiment procedure

In this chapter, the methodology and the development of the long-term tests will be explained in detail. Thereby, the lab scale experiment started with the reactor build described in the previous chapter and was conducted by two individual experiments, which will be explained separately.

4.2.1. Start-up experiment I

The experiment lasted 92 days, as long as a small quantity of gas was still produced. The main amount of biogas was developed in the first week on experiment day 10 and increased after turning on the supply power energy on experiment day 17. At each reading of gas volume in the eudiometer tube, the temperature and air pressure were estimated to calculate the volume of gas in the normalised state.

Nevertheless, in the building phase, a beaker of 1000 ml was used to add the digested sludge to each of the reactors named R1-R6 with a permanent pen, the level of 1000 ml was marked, and each reactor was categorised with their proper name, such as R1-R6. Subsequently, the glass sections of the upper opening were rubbed with laboratory grease, and the eudiometer was connected to the reactor, as shown in Figure 12.

After setting up the reactors, a purge with nitrogen was used in each reactor three times to remove the oxygen in the ambient air from the system and create an anaerobic condition. Among that, the level of the barrier liquid in the eudiometer was reported at the beginning because the volume of the biogas formed could be determined between the difference graded in levels at the next reading, which occurred 1-2 days (three times a week). Finally, a zero sample of the input digested sludge was taken from the sludge from the tank as a reference for the analysis carried out at the laboratory at the department.

By the end of the start-up phase, the new levels graded on the eudiometers were reported on the lab book and the volume of biogas formed, and their composition was taken for analysis. The biogas of a stable biogas process mainly comprises the product components CH_4 and CO_2. These components could be assumed as 100 % of the gas formed, while the gas component, N_2, does not occur in elemental form during anaerobic degradation because of the scavenging process. Meanwhile, O_2 could also be detected in a lower concentration representing 0.8 to 1.6% of the gas composition, resulting from a presumable not completed gas tightness of the system. Hence, all the reactors were operated under the same conditions, and the biogas composition was transferred to a gas bag containing CH_4, CO_2, H_2 and H_2S. Then the gas composition was analysed with the multi-gas measuring device Dräger x-am 8000.

Figure 12 Set up of the reactors, own representation.

By the gas composition measurement, it is possible to determine and calculate the quantity of biogas produced from the composed substrate in accordance with standard DIN 38414. However, the composition of a gas from a digester depends on the substrate, the organic matter load, and the feeding rate of the digester. Andriani et al. (2014) mentions that the levels of CH_4 from the decomposition of different substrates ranging might be from 50-70 %, and the level of CO_2, is around 30-50 %. However, the large amount of CO_2 lowers the heating value of biogas, reducing the combustion efficiency, effectiveness and economic value of biogas equipment generated.

The first week of the feeding process in experiment I occurred with 0.4 g of urea and 0.8 g of sugar added to the system. Nevertheless, the amount of sugar and urea added into the reactors conform to the system's need, as represented in Table 3. Those amounts of sugar and urea were prepared by weighing with a precision balance, mixed with the substrate, stirred, and then introduced into the reactors on the valve area between the anode and cathode electrode with a lab needle of 60 ml volume.

During the feeding, 40 ml of the total substrate was added to each reactor (R1-R6). Nevertheless, the same amount was taken for analysis before feeding. Then, measured pH and temperature. The sample was saved in a plastic bottle, labelled with the name of each reactor (R1-R6), and stored at 4°C in the laboratory fridge to avoid degradation or loss of the integrity of the biological sample over time when stored at room temperature. Therefore, limiting the effect of interfering with microbial community composition relative to the variation observed between different samples. Subsequently, after collecting four samples that generated an amount of 150 ml from each reactor, they were used for chemical parameters.

At the beginning of the experiment test, the reactors were running for a week without an energy supply to give time to the microorganism to adapt to the new environmental conditions. During this time, it was not observed any volumetric biogas formation in the eudiometers from

all the reactors that the measurement equipment could register. Thus, after a week of the experiment running, it was turned the energy supply from reactors R1-R4, where the voltage introduced was 1.0 V in reactors R1 and R2. In reactors R3 and R4, the voltage introduced was 1.5 V.

Table 3 Operation process from the experiment I, own representation.

Start-up of Experiment I		
Start of experiment and filling the reactors with 1,000 ml	Collecting zero sample in each reactor Temperature and pH from each sample recorded	no sampling was collected. Temperature and pH from each sample recorded
	From experiment day 8: 0.8g of sugar and 0.4 g of urea with 40 ml of an old sludge containing all needed nutrients, but with a lower COD was added in each reactor Slowing stirring the reactors by hand to try mixing the new substrate added.	Experiment till day 13: 0.8g of sugar and 0.4 g of urea with 40 ml of an old sludge containing all nutrients needed, but lower COD was added in each reactor Slowing stirring the reactors by hand to try mixing the new substrate added.
Main Phase		
40 ml of sample collection of each reactor and feeding of 40 ml of substrate diluted with urea and sugar. Temperature and pH from each sample recorded. Slowing stirring the reactors by hand to try mixing the new substrate added		
From experiment day 13: 1.0 g of sugar and 0.6 g urea with 40 ml of an old sludge containing all nutrients needed, but lower COD was added. Turn on the voltage 1.0 V in reactor R1-R2 and 1.5 V to reactor R3-R4.	From experiment day 27: 1.0 g of sugar and 0.8 g urea with 40 ml of an old sludge containing all nutrients needed, but lower COD was added of substrate in each reactor.	From experiment day 40: 2.0 g of sugar and no urea with 40 ml of an old sludge containing all nutrients needed, but lower COD was added of substrate in each reactor.
40 ml of sample collection of each reactor, feeding of 40 ml of substrate diluted with urea and sugar. Temperature and pH from each sample recorded; Slowing stirring the reactors by hand to try mixing the new substrate added.		

From experiment day 44:	From experiment day 47:	From experiment day 90:
1.0 g of sugar and no urea with 40 ml of an old sludge containing all nutrients needed, but lower COD was added of substrate in each reactor.	1.0 g of sugar and 0.4 g of urea with 40 ml of an old sludge containing all nutrients needed, but lower COD was added of substrate in each reactor.	0.6 g of sugar and no of urea with 40ml of an old sludge containing all nutrients needed, but lower COD was added of substrate in each reactor.

4.2.2. Start-up of the Experiment II

The experiment II lasted for 34 days, with an energy supply of 0.8 V to R1-R2, 1.0 V to reactor R3-R4 and reactors R5-R6 as the control. The voltage in experiment II was based on the evaluation of the previous experiment I, in which the reactors with 1.0 V had better performance over the experiment test than the ones with 1.5 V. For that reason, it was decided to use the supply voltage 0.8V to reactors R1-R2, 1.0 V to R3-R4 and reactors R5-R6 as control. Furthermore, experiment II aimed to analyse the performance of an enriched sludge adapted to higher concentrations of ammonia under the adaptability of the MEC reactors compared to the control.

As mentioned in chapter 3.4.4, Park et al. (2018) had obtained good results in the MEC experiment with a supply voltage of 0.8 V. Thus, based on a better performance on experiment I and the results obtained from other authors which worked with the voltage 0.8 V, it was decided in this experiment to work with the same voltage of 0.8 V to the reactors R1-R2. While 1.0 V was based on the Choi et al. (2017), which worked with a glucose substrate under a voltage of 1.0 V. Hence, it was implemented in the reactors R3-R4 a voltage of 1.0 V, whereas the reactors R5-R6 maintained as control.

Moreover, to obtain an enriched sludge with high ammonia concentration, two separate reactors were built with the capability of 6,0 L. From day 27 of experiment I, the separated reactors were prepared and filled with 4,5 L of sludge. Initially, the reactors were fed 1,0 g of sugar and 1,0 g of urea per reactor. On day 44, one extra litre of sludge was added to each reactor and fed with 1,0 g of sugar and 1,0 g of urea per litre. On the same day, it also included a steering machine with a velocity of 65 i, min under the two reactors for the entire preparation period of obtaining an enriched sludge with high ammonia concentration. From day 47, it was fed only 1,0 g of sugar per reactor. Among that, the pH and temperature of each respective reactor were measured, and on day 61, the first reactor had a pH value of 7,75 and a temperature of 26,2 °C, while the second reactor had a pH of 7,75 and a temperature of 25,7 °C.

Since the substrate of this experiment was prepared during experiment I, the microorganisms present on the substrate was already adapted to the environment with higher ammonia concentration. Consequently, on the first week of the experiment day 8, it was notable an amount of biogas by the measuring device *Dräger x-am 8000*. However, the biogas production has dropped, and after turning on the energy supply on experiment day 11, biogas production increased again. At each reading of gas volume in the eudiometer tube, the temperature and air pressure were estimated to calculate the volume of gas in the normalised state. Table 4 gives an overview of the experiment operation process.

Furthermore, it was collected 35 ml of the total substrate from each reactor and recorded the pH, and temperature of each sample. Then, it was stored at 4 °C in the laboratory fridge to avoid degradation or loss of the integrity of the biological sample over time when stored at room temperature. The reason for that, is to limit the effect of interaction with microbial community

composition relative to the variation observed between different samples. Subsequently, the samples were stored until obtained a volume of 150 ml from each reactor which would be further used to analyse the chemical parameters of each reactor. Afterwards, it was added in each reactor the same amount of substrate that has been taken for analysis. In addition, Table 4 illustrate the amount of substrate, quantity of urea and sugar-fed in each time which gives an overview of the operation process of experiment II.

Table 4 Operation process of experiment II, own representation.

Start-up of the Experiment II		
Building the experiment and filling the reactors with 1,000 ml of sludge	Collecting zero samples in each reactor Temperature and pH from each sample recorded.	No sampling was collected Temperature and pH from each sample recorded
	From experiment day 1: Diluted 0.6 g of sugar and 0.4 g of urea with 35 ml sludge adapted to higher concentrations of ammonia. Slowing stirring the reactors by hand to try mixing the new substrate added.	Experiment till day 8: 0.6 g of sugar and 0.4 g of urea with 35 ml of sludge adapted to higher concentrations of ammonia. Slowing stirring the reactors by hand to try mixing the new substrate added
Main Phase		
35 ml of sample collection of each reactor, and feeding of 35 ml of substrate diluted with urea and sugar. Temperature and pH from each sample recorded; Slowing stirring the reactors by hand to try mixing the new substrate added		
From experiment day 11: It was added 0.6 g of sugar and no urea with 35 ml of the sludge adapted to higher concentrations of ammonia. The urea feeding was stopped because the concentration has already reached the inhibition level; Turn on the voltage to 0.8 V in R1-R2 and 1.0 V in R3-R4 to analyse the performance of reactors under the MEC system.		

4.2.3. The main operation of the fermentation experiments

The start-up of the main operation process of both experiments was developed as described above. In experiment I, the start-up of the primary process was composed of the addition of 0.4 g of urea and 0.8 g of conventional refined sugar in each reactor. That amount was diluted in the 40 ml of digested anaerobic sludge that was regularly being fed to the open valve between the cathode and anode of the reactors three days a week. By contrast, in the main phase of the

fermentation experiments, a volume of 40 ml of anaerobic sludge was regularly collected on the feeding day from the open valve between the cathode and anode of the reactors over one week.

The samples were collected for each reactor until they reached a volume of 160 ml per week. That volume was a mixed sample from the regular collection over four days of the experiment, representing an average value over a week. The parameters taken to analysis were dry substance (DS) in [%], Chemical oxygen demand (COD) unfiltered [mg/l], total organic acids [g/l], dissolved organic carbon DOC [mg/l], ammonium nitrogen (NH_4-N), and Total Kjeldahl nitrogen (TKN) in [mg/l].

Subsequently, collecting the samples were maintained in a fridge to keep them frozen until the time of the analyses to reduce the possibility of any microbial activity outside the reactors. Nevertheless, the samples were maintained in a thermostatic cabinet collected at 4 °C, corresponding to a standard storage temperature. By contrast, after a week that the lab scale experiment was running and the microorganism of the system was adapted to the new conditions, the voltage of 1.0 V in R1–R2 and 1.5 V to reactor R3–R4 was turned on.

At the start-up of experiment II, the amount of sugar was reduced to 0.6 g and maintained 0.4 g of urea. Those amounts were diluted in 35 ml of enriched sludge that was adapted to high ammonia values and fed into each reactor three times a week. However, it was overserved that it was not producing enough gas on the eudiometer to be analysed with the measurement equipment. Thus, it was decided to main only feeding with 0.6 g of sugar and no urea to allow the microorganism to degrade the substrate. As a result, it was observed in the eudiometer that an increase in the gas formed, and it was possible to measure the composition with the equipment. It was also noticed that after tuning in the energy supply on day 11, the amount of gas produced has increased.

In the main phase of experiment II, a volume of 35 ml of anaerobic sludge was regularly collected on the feeding day from the open valve between the cathode and anode of the reactors. The samples were collected for each reactor until it reached a volume of 160 ml per week and further analyses to the parameters described above. At the end of both phases, a sample was collected from each reactor to describe and distinguish the microbiome present on the sludge in each reactor by 16S rRNA sequencing. Those samples were transferred to the centrifugate tubes, where 25 ml of the sludge was taken, and 25 ml of ethanol was added to dilute them. Then, they were frozen at -20 °C. However, the constraints cannot present the results in this study.

4.3. Measurement Methods

This chapter will describe in detail the methods, parameters and devices used to analyse the samples collected during the experiment, as shown in Table 5.

Table 5 Parameters used in the study, own representation.

Parameters	Method	Objective
COD	. COD: The test is measured from dichromate reflux.	. COD: Bacteria convert organic compounds to biogas in an environment without oxygen (*Anaerobic COD Removal - PAQUES*, n.d.); . The reduction of COD could represent the amount of degradation that occurred within anaerobic digestion, which means the consumption of organic and inorganic compounds (Meegoda et al., 2018).
Conductivity	. It can be measured the conductive of the solution studied in a beaker where the conductivity is proportional to the current that flows between the electrodes; . Use of conductivity electrode to analyse the solutions (*How to Measure Conductivity in Liquid*, n.d.).	. The ability of a solution to conduct electrical current (ions) that has been found for microbial cells (Marín-Peña et al., 2020); . Represent the characteristics related to direct interspecies electron transfer that correlate with the measured electrical conductivity in DIET (Caizán-Juanarena et al., 2020).
Gas Analysis	. Quantitative and qualitative	. Obtain the composition of the gas formed in the reactors, such as CO_2 and CH_4.
Organic acids	. Organic acids use the cuvette test LCK365 and the Nordmann titration method which show the total of the individual acids as an acetic acid equivalent.	. Organic acids are produced in the first stage of anaerobic digestion, which is important in-process fermenter monitoring in fermentation plants, values higher than 3000 mg/L could change pH values.

		. The ratio is used to monitor the stability of the substrate used, the digester; . This parameter is based on the measurements of the ratio of VFAs to alkaline buffer capacity, giving the fermentation status (Nkuna et al., 2021).
pH-value	. It consists of measuring the potential difference between two points, where one is the electrode contacting the internal solution. The second point is connected to a reference electrode, immersed in the studied solution (Vanysek, 2004); . The reference electrode is normally built in the glass electrode in a centric double barrel body of the device (Vanysek, 2004)	. Define the concentration of hydrogen in a solution. . Control the availability of nutrients, biological functions, microbial activity, and the behavior of chemicals. . It affects the activity of biogas production during the acidogenic and methanogenic microbial communities (Vongvichiankul et al., 2017).
Redox- value	. It is measured by using redox probes, and the difference between them represents a metal electrode and a reference which are both immersed in a solution (Kölling, 2000).	. It's used to describe a system's overall reducing or oxidation capacity because redox potential is sensitive to the presence of O_2 in an aqueous solution (Gummert et al., 2020). . It could be used to predict changes in pH of the digester because substrates including oxygen, nitrate and sulfate promotes oxidation which that could cause change in the redox potential and causing changes in pH (Gummert et al., 2020). . It can be used as an indicator of the process of methane fermentation, as methanogenic archaea growth requires a low redox potential. The redox potential for growth of anaerobic digestion has been reported to a range from -200 to -400 mV (Naik et al., 2014).
TKN/ Ammonia	. It is composed of three steps: digestion, distillation, and titration;	. The total Kjeldahl Nitrogen is used determinate the quantitative of organic nitrogen plus ammonia and ammonium in the chemical analysis of a compound.

	. The same is digested with concentrated sulfuric acid in the presence of a mercury catalyst to convert organic nitrogen to ammonium sulfate; . The ammonium ion is released as ammonia using a titration (Hicks et al., 2022). . Ammonia can be measured by probe which is connected to an electronic ion meter that measures and displays the voltage resulting from ammonia, that converted to concentration using the standard curve (United States, Environmental Protection Agency- EPA, 2015); . The fraction of unionized ammonia (NH_3) can be calculated using measures of total ammonia, pH, temperature, and ionic strength (measured either in terms of total dissolved solids or conductivity) (United States, Environmental Protection Agency- EPA, 2015); . Colorimeter: one or more reagents are added in intervals to the sample, until the intensity of the color produced is proportional to the ammonia that reacts with the reagent. . This color absorbance then is measured using a colorimeter or spectrophotometer. Blanks and standards are used to generate a standard curve from where the sample absorbance reading is converted to ammonia concentration.	. It gives the possibility to adjust the quality of a wastewater treatment because it helps to understand how the biological system is leading with the applied ammonia load (*Simplified TKN (Total Kjeldahl Nitrogen) s-TKN™	Hach*, n.d.); . NH_4-N gives the possibility to evaluate the share nitrogen present in the form of NH_4-N nitrogen in bound in organic matter. . The value recorded in the NH_4-N is the sum of both forms NH_3 and NH_4+ and is reported as total ammonia or simply - ammonia. The relative proportion of the two forms present in water is highly affected by pH (Madhu, 2018).

5. Results

This chapter shows the lab-scale experiment's results. Therefore, the biogas formation and the composition analysis values will be evaluated from experiments I and II, including the results obtained from each reactor are graphically represented and introduced in a theoretical context. Hence, it could explain the development of biogas and the enhancement of the anaerobic digestion of nitrogen substrate using MEC.

5.1. Biogas Formation

This chapter will describe the processes implemented to evaluate the biogas formation in the experiment in experiments I and II. As described in chapter 3.2.1, the biogas volume formed in each reactor was graded on the levels of the barrier liquid from the eudiometer, and it was reported in the lab book. In addition, the gas density variates with pressure and temperature, for that reason it is necessary to use a standardised volume when referring to quantities of gases (Instrumentation & Control, n.d.).

The standard or normal conditions are used as reference values in the thermodynamics of gases, which are defined by the temperature and pressure conditions of the volume measurement (Instrumentation & Control, n.d.). Thus, the study implemented the conversion to specify the gas volume measured under the experiment's conditions, as shown in Equation 19.

Equation 18

$$Vnorm. = \frac{(Gas\ produced * (Pambient - PH2) * 273)}{1013 * (273 + Tambient)}$$

V_{norm}= Volume normalised

PH_2 = Hydrogen partial pressure

273,0 = Temperature in Kelvin

P = Ambient pressure

T = Ambient temperature

1013= Standard atmospheric pressure

The hydrogen partial pressure PH_2 is interpolated from values available from literature and the environment temperature conforming to Equation 18.

Equation 19

$$PH2 = PH2{,}1 + \frac{(PH2{,}1 - PH2.2)}{(T - T2{,}1)} * T - T1$$

Figure 13 shows the cumulative and daily biogas production of each reactor in experiment I over a period of 92 days. The cumulative biogas production was used to evaluate AD performance for each reactor since it can provide significant information for the adaptation and growth of anaerobic microorganisms. The graph shows an increase in the volume of biogas production in all reactors since day 6, in special in the control reactor R5 and the reactor R1 with a voltage of 1.0 V. The increase in the volume of biogas could also be related to the feeding of 0.8 g of sugar and 0.4 g of urea that was added to the reactors.

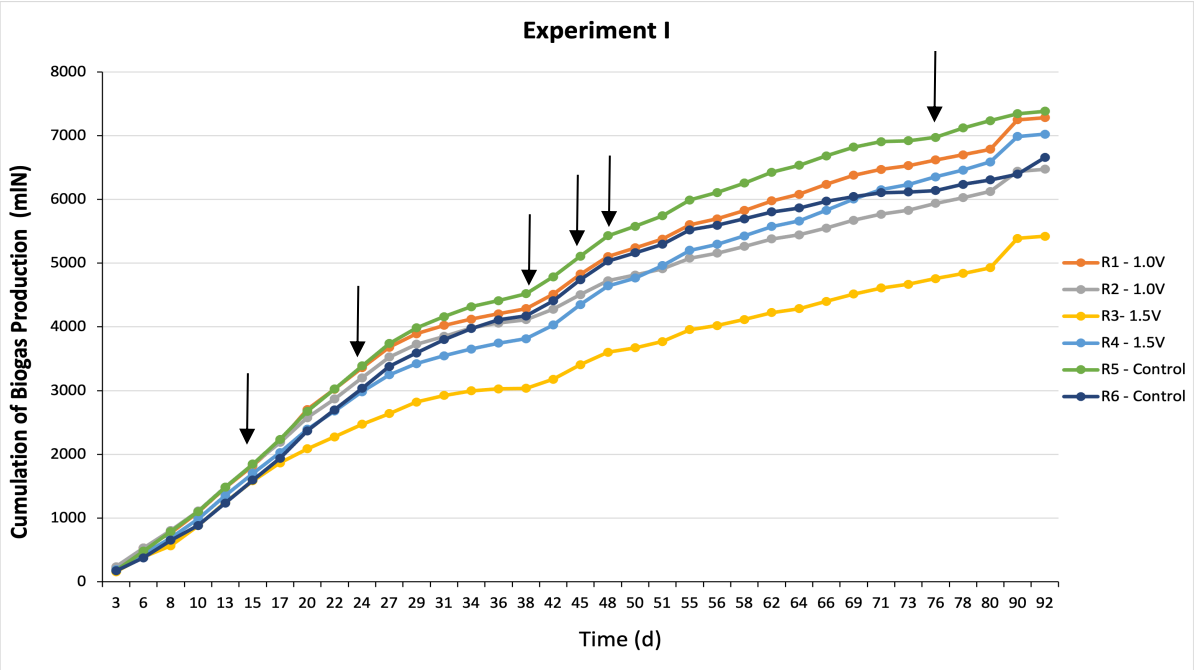

Figure 13 Cumulation of biogas production (ml) of R1-R6 from experiment I, and their perspective voltage. The arrows illustrate the addition of sugar and urea during the test period, own representation.

The R5-control had the highest cumulative biogas volume with a total of 7382 mlN, and R1-1.0 V with the second highest cumulative biogas volume of 7,284 mlN; The respective values were R2–1.0 V with a biogas value of 6,474.35 mlN, 809.65 mlN less than R1 which had the same voltage and initial condition. R4-1.5 V with 7023 mlN and the reactor R6-control with 6657 mlN. The lowest value was from R3-1.5 V with 5,422.78 mlN.

From day 17, reactor R3-1.5 V suffered a reduction in the volume of biogas. The decrease in the volume could be a leakage or high amount of oxygen into the system due to improperly sealing the equipment while taking the gas from the eudiometer. On day 42, 2,0 g of sugar was fed to each reactor, resulting in a significant increase in biogas volume in all reactors. From day 47, a feeding of 1,0 g of sugar and 0.4 g of urea was added to the reactors, and on day 90, the reactors were fed with 0.6 g of sugar and no urea. Consequently, from day 90 is notable slightly growth when urea was not added in the reactors. In conclusion, it is possible to observe constant growth in all reactors, especially in R5-control and R1-1.0 V.

In Figure 14 shows the cumulative and daily biogas production of the reactors R1-R6 with their respective voltage over a period of 34 days in experiment II. The graphs show a stable increase of biogas production in all reactors during the lab-scale experiment. Until day 8, it is notable that there is a constant increase of biogas production in all reactors caused by the feeding of 0.6 g of sugar and 0.4 g of urea. From day 11, the reactor was fed with 0.6 g of sugar and no urea till the end of the lab-scale experiment, and that resulted in a stable increase between almost all reactors. From day 18, the reactor R1-0.8 V has decreased biogas production. Nevertheless, the reactors that had the best performance during the entire experiment were R2-0.8 V, R3-1.0 V and R4-1.0 V, and those reactors were composed of a supply voltage. The control reactors had the lowest performance during the experiment. Although R6-control on day 32 had

reached the same volume of R4-1.0 V, it had the lowest start compared to the reactors compost of a supply voltage.

Among that, the total cumulative biogas production of each reactor was 1114 mlN to reactor R1-0.8 V; 1366 mlN to reactor R2-0.8 V; 1488 mlN to reactor R3-1.0V;1290 mlN to reactor R4-1.0 V; 1215 mlN to reactor R5-control; and 1305 mlN to reactor R6-control. Therefore, the highest volume of biogas production occurred in R3-1.0 V, while the second highest volume was from the R2- 0.8 V, and the lowest volume of biogas production was from reactor R1-0.8 V.

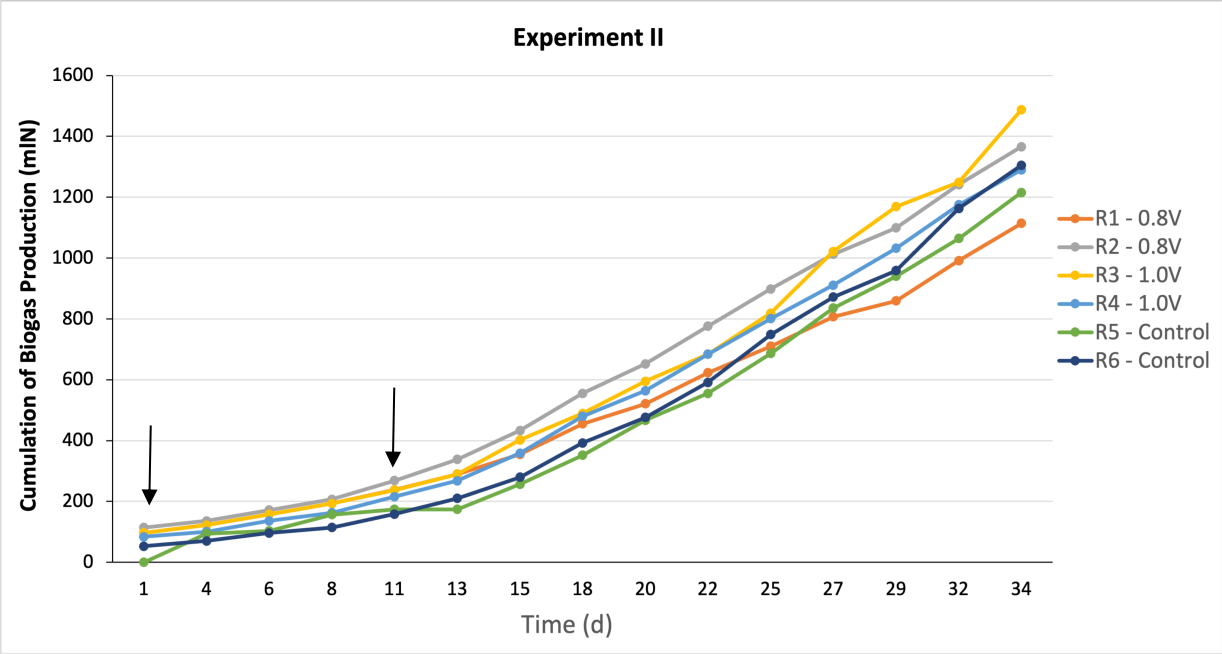

Figure 14 Cumulation of biogas production (ml) of R1-R6 from experiment II, and their perspective voltage. The arrows illustrate the addition of sugar and urea during the test period, own representation.

Although the reactor R2-0.8 V had a good performance, the reactor R1-0.8 V which had the same voltage and initial conditions, had the worst performance. In the beginning, reactor R1-08 V started raising the biogas production as the other reactors, whereas from day 18, it has dropped the production gradually even with no urea feeding. This effect could be due to the oxygen into the system during the operation of the gas measurement device or leakage of the reactor since R2-0.8 V had a good performance.

The reactors with a voltage of 1.0 V have shown a good performance in both reactors during the entire experiment. That could result from a good adaptation of the microbial community to the enriched ammonia concentration sludge. In addition, the control reactors also have shown a constant growth during the entire experiment. At the beginning of the experiment, the reactor R5-control illustrates an unstable growth with some pick and drop down on the biogas production until day 13, where the growth rises again. This effect could be due to the no urea feeding since the amount of sugar addition has not changed. Meanwhile, the reactor R6-control from the first day has grown constantly until day 29, when it has increased and reached the same biogas production as reactor R4-1.0 V. That could also be a result of the adaptation of the microorganism ma present in the digestion of the biodegradation in AD.

In conclusion, it is possible to observe constant growth in all reactors, especially with the reactors that had a voltage of 1.0 V, generating a total biogas production of 2778 mlN, while the control reactors generated a total of 2520 mlN and the reactors with a voltage of 0.8V a total biogas production of 2480 mlN. Nevertheless, the lower production of the reactor R1-0.8 V could be related to the facts mentioned before such as leakage, and oxygen into the system.

Furthermore, by the composition of the gas it is possible to understand the quality and health of biogas. Therefore, Figure 15 represents the gas composition (%) of the reactor R1-1.0 V and Figure 16 from reactor R5-control. Both figures represent the development of the gas composition (%) from reactor R5-control over a lab-scale experiment of 92 days of experiment I. By contrast, the Figure 15 has been chosen because R1-1.0 V had the second-best performance of biogas composition over the experiment period. Likewise, the reactor R5-control which had the highest biogas cumulation over the experiment period of the experiment I.

The gases (CH_4, CO_2, and H_2) and O_2 were detected in the gas analysis device from all reactors. However, CH_4 and CO_2 are the main gas produced, allowing conclusions about the quality of the biogas process. In an efficient biogas process, the stoichiometric ratio of CH_4 and CO_2 is 1:1; implementing MEC helps stimulate the biogas process, and it could allow CH_4 to obtain higher proportions than CO_2. The proportions of O_2 and H_2 were shown graphically but were not found in quantities relevant to the biogas composition. Thus, the sum of CO_2 and CH_4 was considered 100% of the biogas.

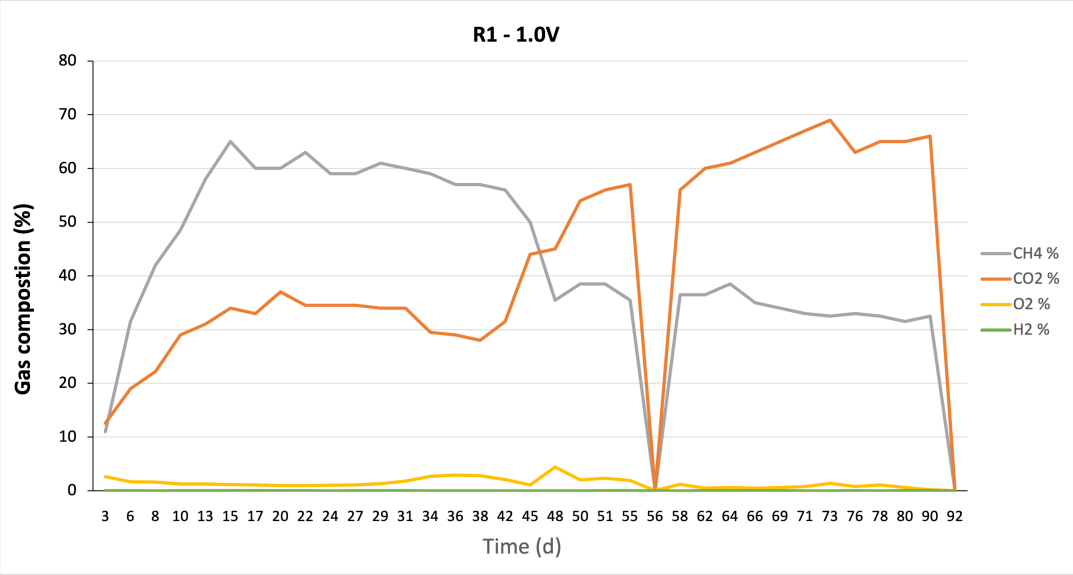

Figure 15 Gas composition in (%) from reactor R1-1.0V over 92 days experiment I, own representation.

The development of methane composition provides information for the adaptation and growth of anaerobic microorganic, mainly the archaeal communities (Lee et al., 2022). A lower methane production indicated that the inhibitory effects were generally stronger for the aceto-lactic than for the methanogens (Y. Chen, et al. 2008). Given this context, as shown in Figure 15 the reactor R1-1.0 V containing graphite as an electrode has increased the CH_4 composition over time but decreased with the increase of CO_2 composition on the reactor. On day 56, is visible a decrease of gas composition from both gases, which could be an effect of the urea feeding in the reactor from day 47.

A lower amount of oxygen is notable from day 31, increasing between day 45 to 48 and dropping from day 50. That could be due to the manipulation of the measurements to analyse the gas composition. Thus, probably letting some air getting inside the reactor or into the gas bag that was used to store the gas while taking from the eudiometer to be further analysed by the gas measurement device.

Figure 16, represents the reactor R5-control which had the highest gas production from the experiment I. A lower oxygen is present into the reactor which shows a good set up and measurement without leaking or interfering with the process. Moreover, it shows that since day 3 the CH_4 composition in the reactor than CO_2. Nevertheless, with the increase of CO_2, it has decreased the CH_4 composition over time.

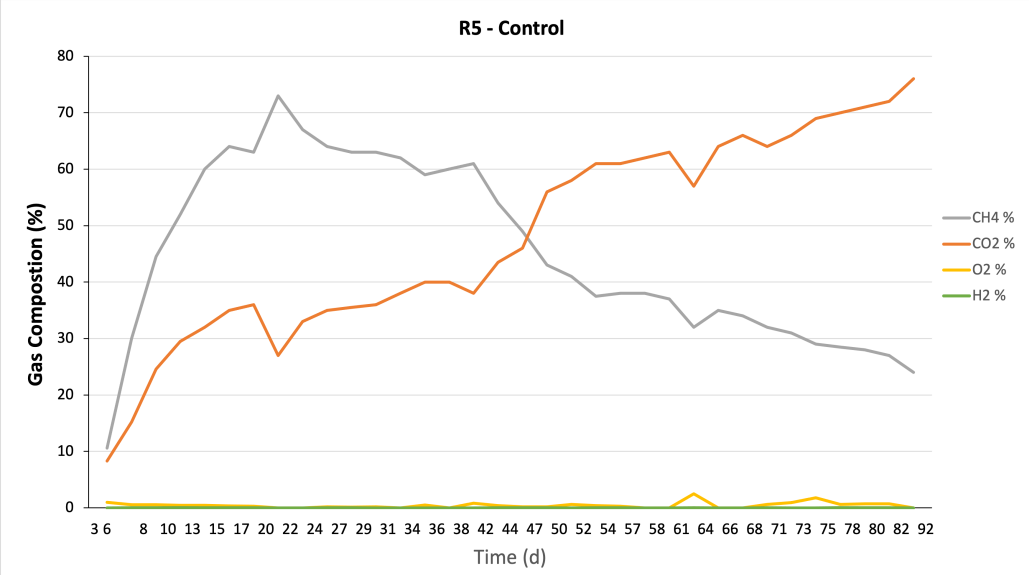

Figure 16 Gas composition in (%) from reactor R5-control over 92 days of experiment I, own representation.

Table 6 illustrates the cumulative CH_4 and CO_2 volumes of reactors R1-R6 with their perspective voltage and the percentage over the entire experiment. It's notable that the reactors with voltage 1.0 V had a slight improvement in biogas production. However, the control reactors R5-R6 have better performance than those with an energy supply. Thus, no improvement in CH_4 was achieved using a voltage in the reactors.

Table 6 Cumulative CH_4 and CO_2 volumes of reactors R1-R6 with their perspective voltage and percentage over the entire experiment in experiment I, own representation

Cumulated Volume in mlN	**R1-1.0 V**	**R2-1.0 V**	**R3-1.5 V**	**R4-1.5 V**	**R5-Control**	**R6-Control**
CH_4 (mlN)	3402	2952	2316	3211	3692	3337
(%)	47%	46%	43%	46%	50%	50%
CO_2 (mlN)	2915	2356	2402	3213	3125	2260
(%)	40%	36%	44%	46%	42%	34%
Cumulatived Biogas Volume(mlN)	7284	6474.5	5422.8	7023	7382	6657

Figure 17 represents the composition (%) of the reactor R-0.8 V from experiment II. The reactor clearly shows the effect of oxygen in AD process, where the gas composition drops drastically with the presence of oxygen from day 22 to 27. Therefore, it could explain the drop in cumulation of biogas production from Figure 14, in which R1- 0.8 V has decreased the performance of the biogas production compared to the other reactors. Additionally, the reactor R1-0.8 V shows a lack of gas composition from day 8 till day 13, which could be related to the absence of sugar feeding during this period.

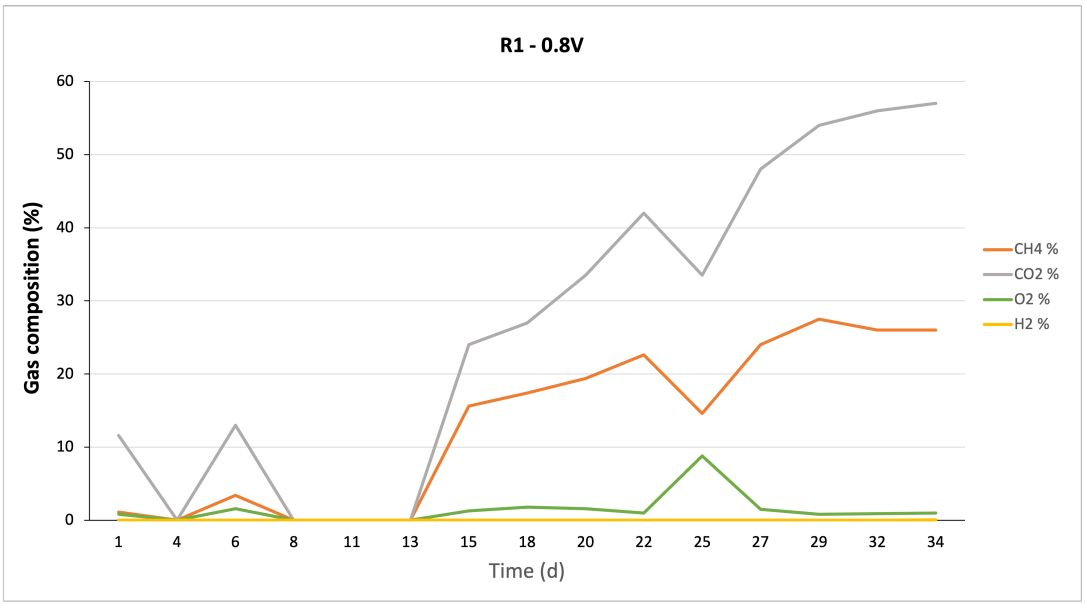

Figure 17 Gas composition in (%) from reactor R1-0.8 V for 34 days of the experiment II, own representation.

Figure 18 represents the gas composition (%) of the reactor R5- control. At the beginning of the experiment, the control reactor had an inconstant gas composition, dropping and up, which could be related to the stage of adaptability of the microorganism with the environment.

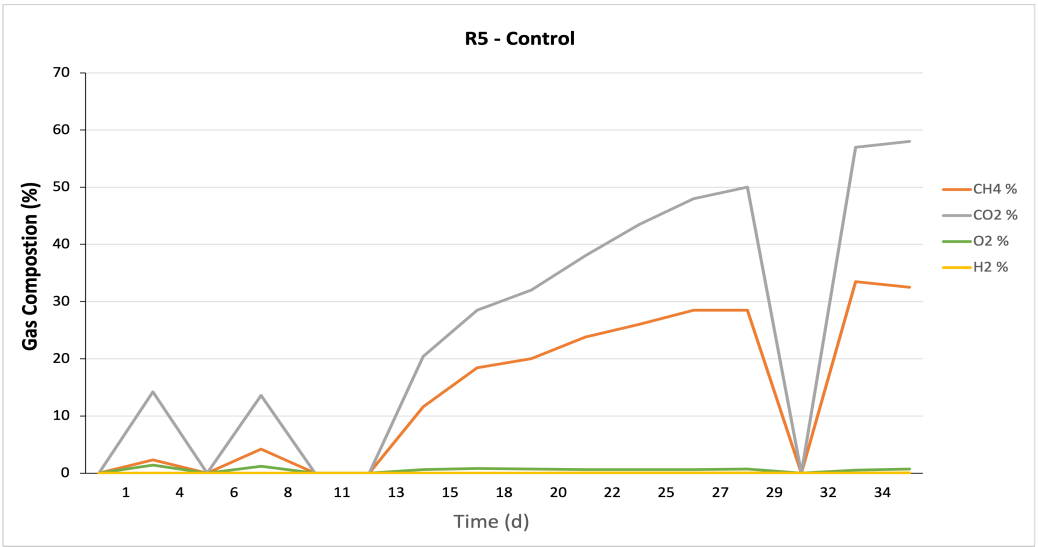

Figure 18 Gas composition in (%) from reactor R5-control for 34 days of the experiment II, own representation.

From day 9 to 11 there was no gas composition, and this effect is seen due to the feeding of 0.4 g of urea to the reactor. Thus, from day 11 when started with no feeding sugar and no urea, the gas composition has arisen again until day 29 when it dropped and increased again. This short drop and increase could be due to a leakage of the system.

Table 7 illustrates the cumulative CH_4 and CO_2 volumes of reactors R1-R6 with their perspective voltage as well as the percentage over the entire experiment in experiment II. The reactor with a voltage of 1.0 V slightly improved biogas production compared to the reactors with a voltage of 0.8 V. However, the control reactors R5-R6 have shown better performance compared to the reactors with an energy supply. Thus, no improvement in CH_4 production was achieved using a voltage in the reactors.

Table 7 Cumulative CH_4 and CO_2 volumes of reactors R1-R6 with their perspective voltage and percentage over the entire experiment II, own representation.

Cumulatedulated Volume in mlN	**R1-0.8 V**	**R2-0.8 V**	**R3-1.0 V**	**R4-1.0 V**	**R5-Control**	**R6-Control**
$CH_{4\ (mlN)}$	182	272	340	283	256	326
(%)	16%	20%	23%	22%	21%	25%
$CO_{2\ (mlN)}$	372	500	599	505	433	492
(%)	33%	37%	40%	39%	36%	38%
Cumulatived Biogas Volume(mlN)	1114	1366	1488	1290	1215	1305

5.2. Development of ammonium nitrogen concentration

This chapter will represent the analytical values of ammonium nitrogen concentration over the entire experiment and the possible increase of ammonium nitrogen effect on methane. The samples were taken in the form of composite samples over a period of one week, which represent an average value of the actual concentration over a period of one week.

Figure 19 shows the NH_4-N analytical concentrations values from reactors R1-R6 in experiment I. To obtain a higher ammonia concentration in the reactors, the system was fed with urea conforming described in chapter 4.2.1, illustrating the operation process and the amount of urea and sugar added during each experiment I. Each reactor was fed with a total of 13.8 g of urea during the experiment I, resulting in a constant increase of NH_4-N over time, as shown in Figure 19.

The measured point 3 represented when each reactor was fed with 0.8 g of urea, causing a slight change in the NH_4-N concentration. In point 4, urea was not added to the reactors, and the concentrations remained constant without much change. From point 6, when the reactors were fed with 0.4 g of urea, the concentration of NH_4-N slightly increased again. In point 7, the reactors have reached a total NH_4-N concentration of 3926.4 mg/l in R1-1.0 V, 4000.87 mg/l in R2-1.0 V, 4075.67 mg/l in R3-1.5 V, 3988.83 mg/l in R4-1.0 V, 4096.12 mg/l to R5-control and 3968.38 mg/l to R6-control.

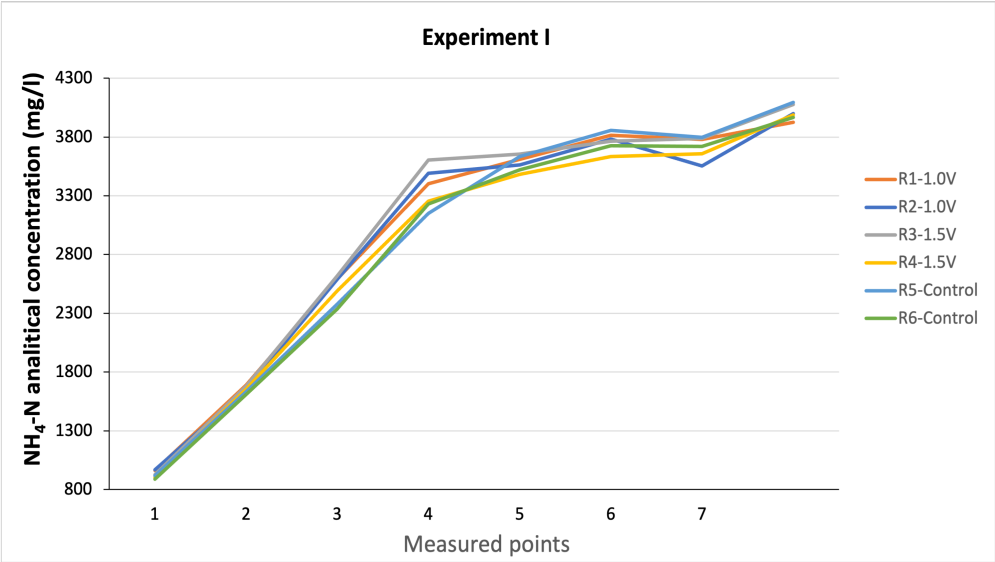

Figure 19 Development of NH_4-N concentrations determined from the analytical values of the composite samples of reactors R1-R6 in experiment I, own representation.

Therefore, summing up the NH_4-N concentration by the reactors with the same voltage and without, the lowest value was 7927,27 mg/l from the reactors with 1.0 V. The reactors with 1.5 V and the controls had the same NH_4-N concentration, equal to 8064.5 mg/l. The lowest NH_4-N concentration in reactors with 1.0 V could result from the implementation of MEC and DIET systems which use an energy supply and an electrode to enhance the AD process. Hence, helping the microorganisms, mainly archaea to degrade the substrate under higher concentrations of ammonia. However, the implementation of the reactors with 1.5 V has shown no improvement in the AD digester.

In Figure 20 represents the development of the analytical concentration values of NH_4-N composite samples from reactors R1-R6 in experiment II. The reactors had an enriched sludge with a higher ammonia concentration as described in chapter 4.2.2 the start-up of experiment II.

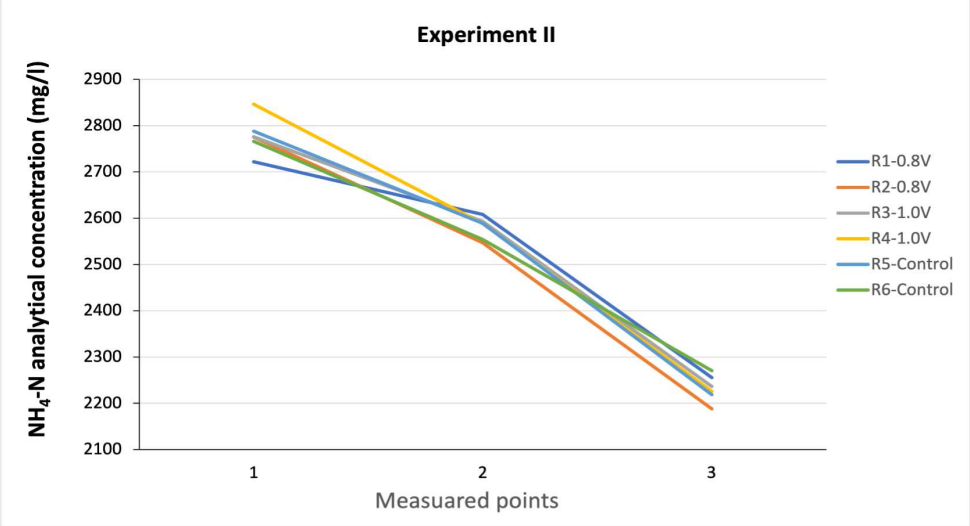

Figure 20 Development of NH_4-N concentrations determined from the analytical values of the composite samples of reactors R1-R6 in experiment II, own representation.

Among that, the reactors were still fed with a total of 1.6 g of urea, and 8.4 g of sugar during experiment II. From measured point 2, the reactors were provided with no urea, which resulted in a constant decrease of NH_4 concentration during experiment time. The reactors started with a substrate with a higher ammonia concentration and conforming the experiment occurred the values were reduced. The reactor R1-0.8 V started with the NH_4-N concentration of 2776 mg/l to 2188 mg/l, R3-1.0 V with 2776 mg/l to 2236 mg/l, R4- 1.0 V with 2846 mg/l to 2225 mg/l, R5- control 2788 mg/l to 2218 mg/l and R6- control with 2766 mg/l and ended with 2271 mg/l.

By contrast, sum up the NH_4-N concentration by the reactors with the same voltage and without, the lowest value was 4444 mg/l from the reactors with an energy supply that of 0.8 V. The second lowest NH_4-N concentration was 4461 mg/l from the reactor with 1.0 V, and the control reactors had the highest value with 4489 mg/l. These results suggest the implementation of MEC and DIET systems with the use of an energy supply and an electrode to enhance AD process work. Thus, helping the microorganism, mainly archaea to degrade the substrate under higher concentrations of ammonia.

In order to show the development of NH_3, which is formed based on the equilibrium of temperature and pH value. It was considered the average of a week of pH and temperature to obtain the values as a composite sample. Figure 21 is a representation of how NH_3 affects methane production and the correlation to the pH values.

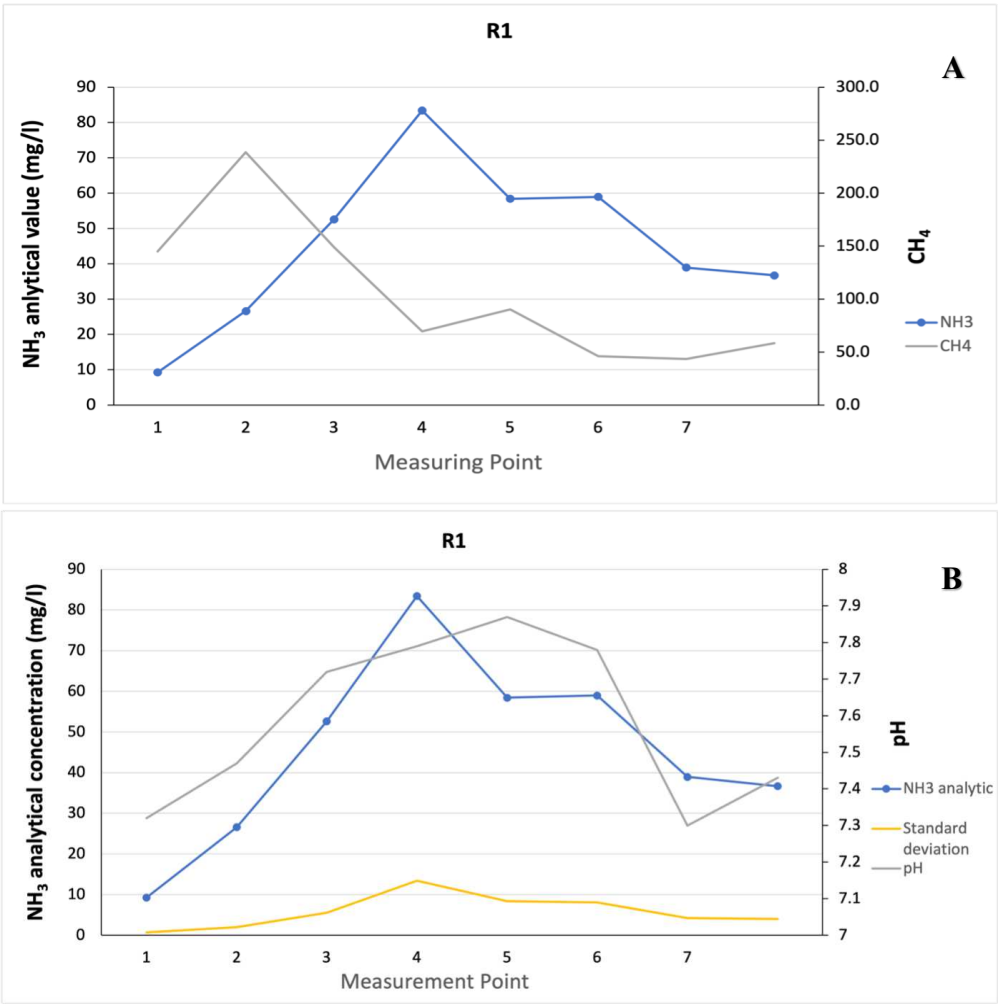

Figure 21 Representation of the interference of NH_3 to CH_4 (A), and the relation between pH and NH_3 analytical concentration (B), own representation.

In contrast, Figure 22 represents the development of NH_4-N and NH_3 analytical concentration over the entire experiment I. It shows that all the reactors have reached the maximum concentration of NH_3 on experiment day 36. The reactor R1-R2 with a voltage of 1.0 V and R3-1.5 V have reached the maximum value near each other, as R4- 1.5 V and the control.

The R2-1.0 V had an average concentration of 89.7 mg/l, R4-1.5 V as 79,6 mg/l and R5-control with 61,1 mg/l. From day 15, the reactors were fed with 1.0 g of sugar and 0.6 g of urea, which could be the result of the continuous increase of NH_3 concentration. However, on day 27 the pH values ranged between 7.2 to 7.8, the amount of urea increased 0.8 g, and the continual addition of 1.0 g of sugar could be the cause of the decrease in the concentration of NH_3.

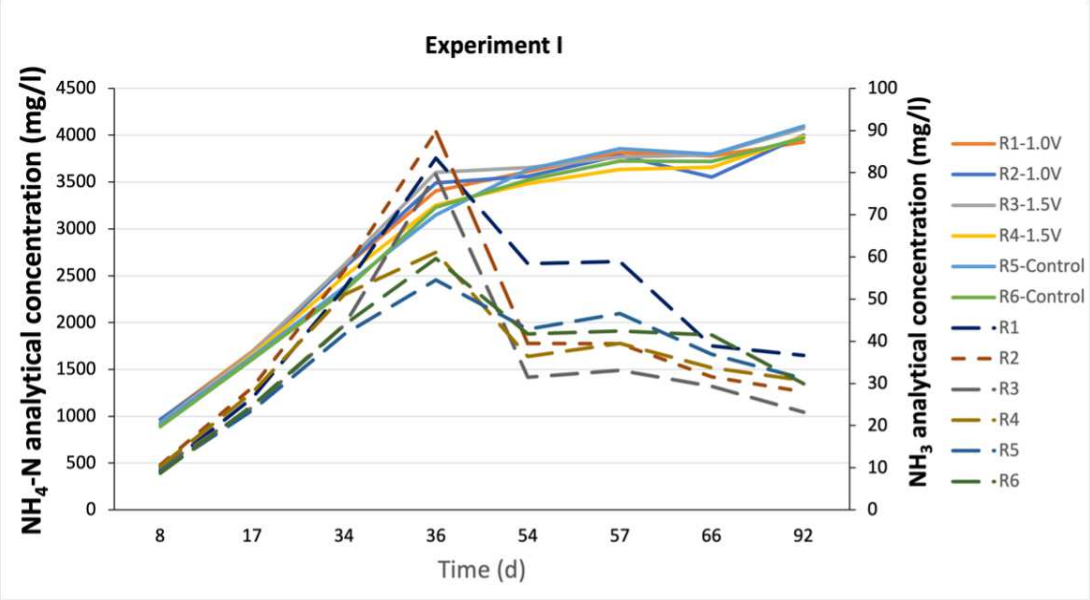

Figure 22 Development of NH_4-N and NH_3 analytical concentrations over the entire experiment I, own representation.

The flat stationary state occurred from day 54 to 57 when no urea was added, them when started to feed in the reactors with 1.0 g and 0.5 g of urea, the values returned to drop. On those days the pH values ranged between 7.2 to 7.4. Likewise, Figure 23 illustrates the development of NH_4-N and NH_3 analytical concertation from the composite samples over experiment II. The graph initiated with a higher NH_3 concentration because the substrate used in experiment II was enriched with ammonia. At the beginning of the experiment, the reactors were fed with 0.6 g of sugar and 0.4 g of urea. As a result, the reactors R4-1.0 V and reactor R5-control maintained a stationary state. Then, with the addition of 0.6 g of sugar and no urea, the concentration dropped. Among that, the reactor R1-0.8 V, R3-1.0 V and R5-control have decreased the NH_3 concentration over the entire experiment time. In addition, on those days the pH value of the reactors in a range of 7.1 to 7.7.

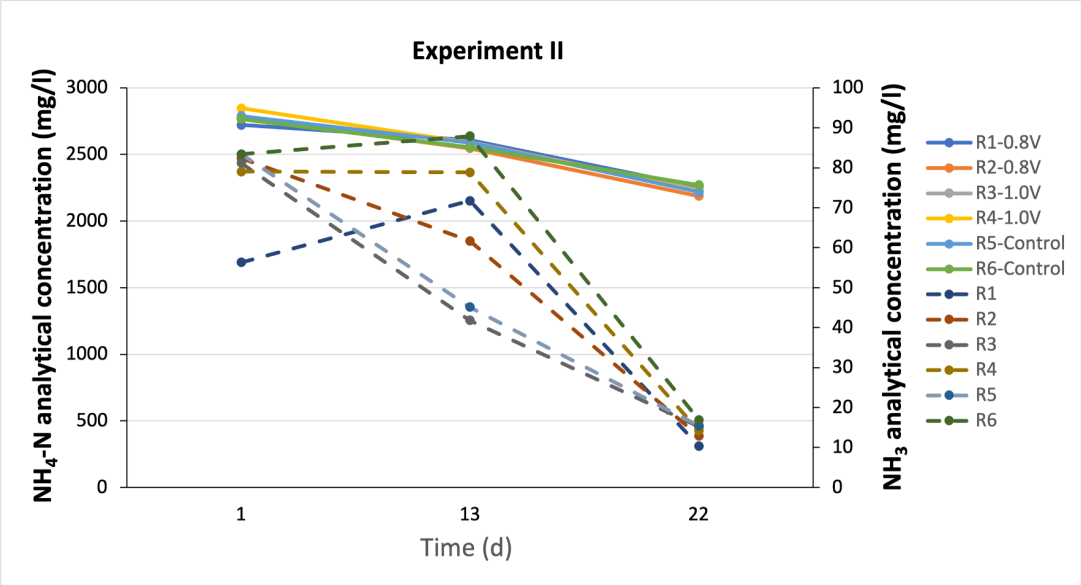

Figure 23 Development of NH_4-N and NH_3 analytical concentrations over the entire experiment II, own representation.

5.3. Analysis values

This chapter will evaluate the samples collected during feeding from both experiment phase. The samples have been analysed by the TU Dresden laboratory at the Institute for Waste Management and Circular Economy. Evaluating the chemical analysis, such as COD degradation and organic acids, makes it possible to conclude the reactors degradation and biogas production and how successfully the methods implemented have accelerated the anaerobic process.

5.3.1. Organic acids

The volatile fatty acids (VFAs) are important intermediate metabolites of anaerobic sludge digestion. When they accumulate as end products in reactors, they are degraded to produce methane (Cai et al., 2016). The analysed VFAs included acetate acid, propionic acid, butyric acid, isobutyric acid, n-valeric acid and iso-valeric, and VFAs were their sum. The changes in VFAs from the experiment I were reproduced in Figure 24 and from experiment in Figure 25.

As show in experiment I, it is notable that the total concentration of organic acids increases with the time due to the addition of sugar. At the beginning of the test, the samples showed 0.10 g/l of total organic acids, and the last samples showed concentrations ranging from 1,4 to 1,53 g/l. The highest values are from the reactor R3-1.5 V, but the reactor R4 which had the same voltage has not increased in the same proportion. Additionally, the lowest value is from reactor R2-1.0 V, while reactor R1 with the same voltage supply has reached a higher value than reactor R2.

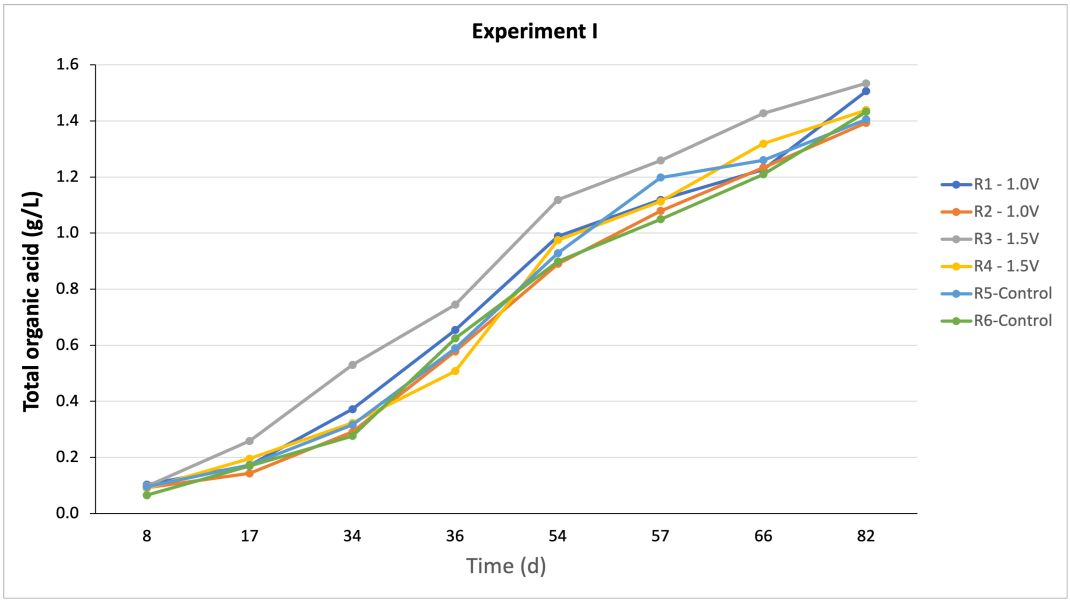

Figure 24 Development of the total organic acids in reactors R1-R6 with their respective voltage in experiment I, own representation.

When sum up the total organic acids' values by reactors with the same voltage and those without. The reactors with 1.0 V had the total organic acids as 2.9 g/L, which was the same value as the reactors with an energy supply of 1.5 V. The control reactors total organic acids values were equal to 2.8 g/L, which was only 0.10 g/L less than the reactors with an energy supply. Hence, in this study the result could suggest that there is not a big difference in values related to the accumulation of total organic acids with the implementation of MEC and DIET in AD digester.

By contrast, Figure 25 represents experiment II, where all the reactors increased the total concentration of organic acids during the experiment test. At the beginning of the test, the concentrations of the samples ranged from 0.8 to 0.98 g/l, and the last sample ranged from 0.91 to 1.02 g/l.

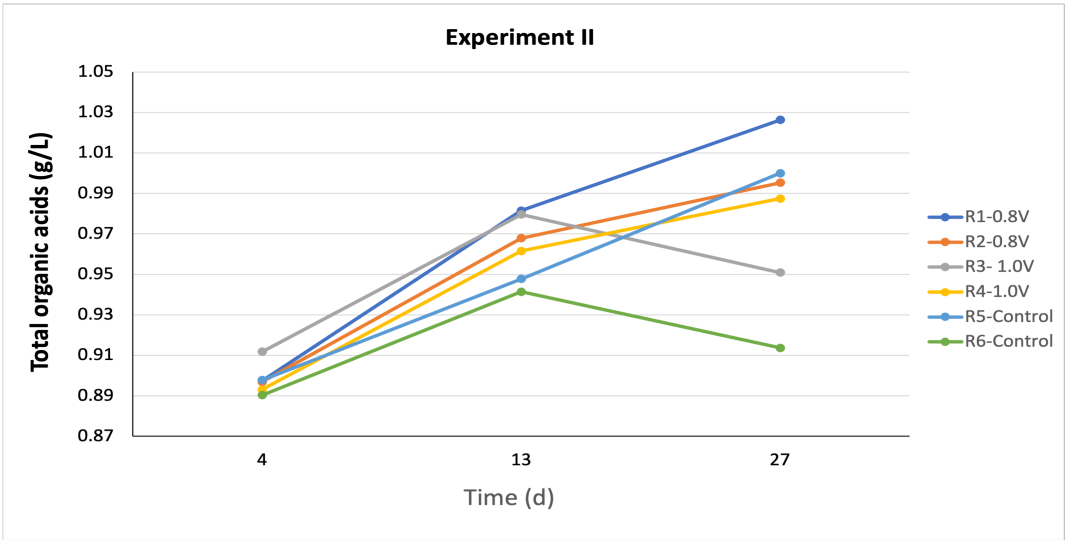

Figure 25 Development of the total organic acids in reactors R1-R6 with their respective voltage in experiment II, own representation.

However, from day 13, the values started to variate, which could be due to the continually fed of 0.6 g sugar and no urea. The reactor R3-1.0 V and R6-control have decreased the load value proportion compared to the other reactors. The highest concentration value was R1-0.8 V, and the lowest was R6-control. However, when analysing the sum up of the total organic acids based on the voltage and those without, the highest concentration value was from the reactors with 0.8 V and the lowest from the control reactors. This result could suggest that in this study there are not a big difference in values related to the accumulation of total organic acids with the implementation of MEC and DIET in AD digester.

Moreover, the total organic acids are used to show their development over time, as described in chapter 3.3. Nevertheless, to obtain precise information about each acid concentration gas chromatography was used because it can provide information from the sum parameter of the organic acids. Therefore, Figure 26 represents the organic acids development from experiment I which is given in the form of acetic acid from reactor R1-1.0 V, while Figure 27 represents the reactor R6-control. Those reactors were chosen because they had higher proportions of some components and the presence of almost all the organic acids. Thus, they could give an overall of what kind of organic acids were found in this study.

Reactor R1-1.0 V shows a constant increase in the concentration of organic acids over time. The most significant increase was from acetic acid, which started at 58.1 mg/l and the end of the experiment, the value measured was 5,301.1 mg/l. It is also notable an increase in propionic acid. Additionally, the total value of organic acids was equal to 1,506 mg/l measured at the end of the test experiment.

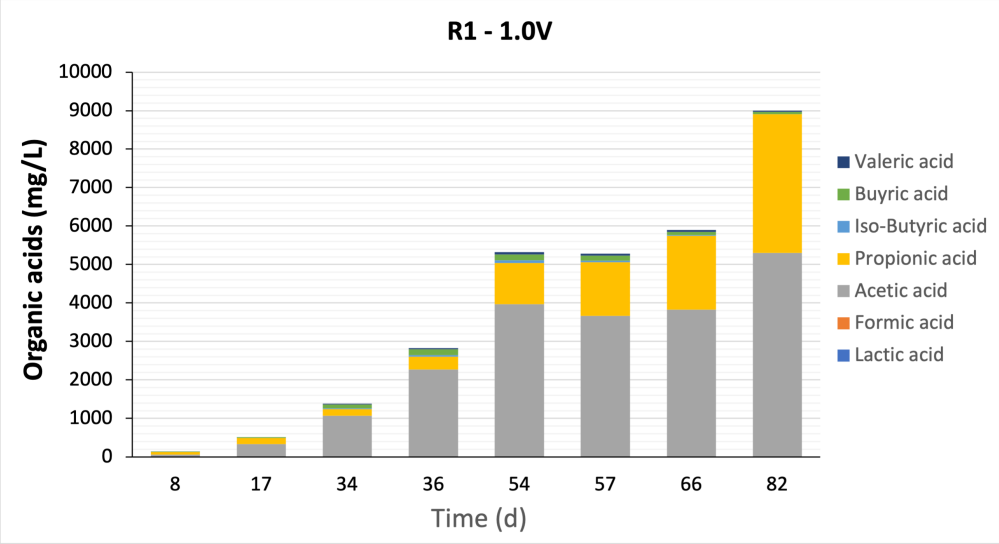

Figure 26 Development of the organic acids in reactor R1-1.0 V during experiment I, own representation.

Comparing the R6-control, acetic acid increased with an initial 40.1 mg/l and ended with 4,732 mg/l. The second highest acid content in the composite sample was also propionic acid, with an initial value of 12.8 mg/l and finishing with 4,829 mg/l. Moreover, on day 36 it was notable the presence of 184.2 mg/l of formic acid with 184.2 mg/l in the reactor. In addition, a total of 1,4328 mg/l of organic acids were measured at the end of the test experiment. In general, comparable acid content concentrations could be observed in all reactors. The only exception is the appearance of formic acid in R4-1.5 V with 57.1 mg/l and in R6-Control. The reactor with the lowest organic acid concentration was R2-1.0 V with 1,3935 mg/l.

Evaluating the acids compounds with the highest concentration in the reactors such as acetic acids and propionic acid from the experiment I, by the sum of the concentration and their respective voltage and those without it.

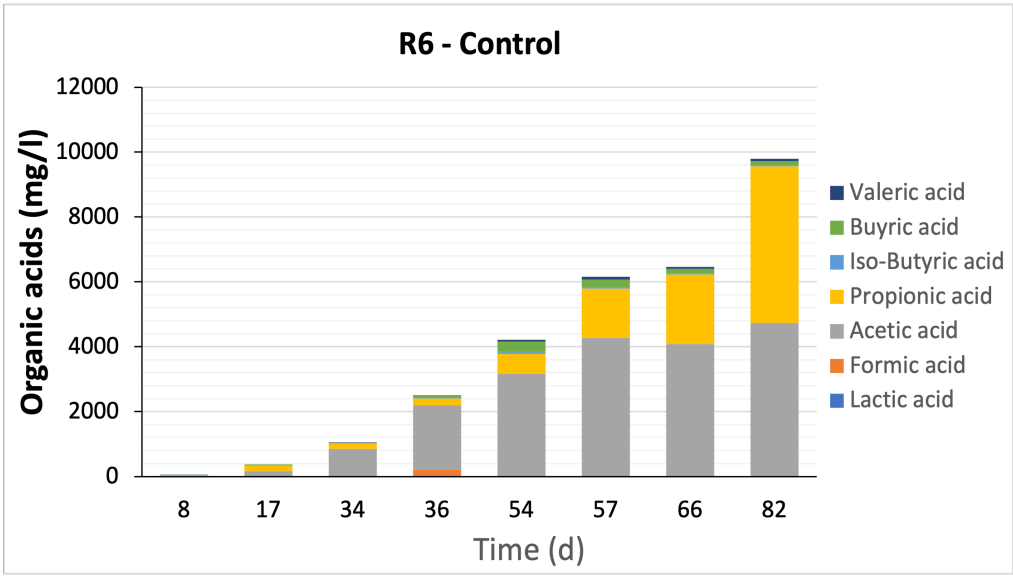

Figure 27 Development of the organic acids in reactor R6-control during the experiment I, own representation.

The acetic acids in the reactors with 1.5 V had a total sum of 10.705 mg/l, the controls reactors with a total of 9.777 mg/l, while the reactors with 1.0 V had an accumulation of 9.650 mg/l. Whereas, the total concentration of propionic acetic was 12.606 mg/l in reactors with 1.5 V, 8724 mg/l in the control reactors and 8493 mg/l to reactors with 1.0 V. Hence, it shows that both compounds were higher in the reactor with 1.5 V, and the lowest values were from the reactors with 1.0 V which could suggest that the implementation of MEC and DIET system have helped the reduction of accumulation of those acids compared to the control reactors. It also suggests that the reactors with 1.5 V have not suffered any interference with the MEC and DIET implementation.

Likewise, in Figure 28 shows the development of the organic acids from the reactors R2-0.8 V and Figure 29 shows the organic acids development of the R5-control in experiment II. Those reactors were chosen because one had an energy supply and the other without, giving an overall of both setups. Also, because their have the presence of all the organic acids. Thereby, it would give a better illustration of the acids present in this study.

At the reactor R2-0.8 V, the most significant concentration present over time is from propionic acid, starting with 2,894 mg/l and ending the experiment with 3,640 mg/l. The second most significant value is 1,990 mg/l of acetic acid at the end of the period with 2,909 mg/l. In addition, a lower concentration of 132 mg/l of lactic acid was present on experiment day 13 and 97 mg/l on day 27. The total organic acid present at the end of the test in R2-0.8 V was equivalent to 995 mg/l.

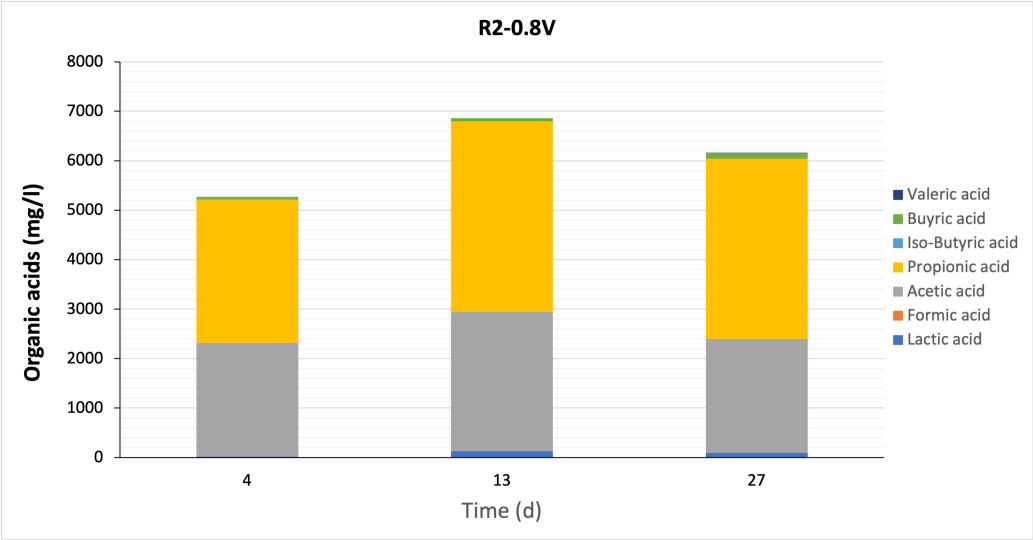

Figure 28 Development of the organic acids in R2-0.8 V during the experiment II, own representation.

Evaluating the reactor R5-control, lactic acid is present during the entire experiment starting with a concentration of 37 mg/l and ended with 116 mg/l. Despite that, acetic acid shows higher quantity during the entire experiment II, initiating with 2280 mg/l and ending the experiment with 2099 mg/l and the second highest values was the propionic acid which started with a concentration of 2853 mg/l and on the last sample test ended with 3664 mg/l. The iso-butyric acid also had appeared have appeared only on day 4 with 12.6 mg/l, butyric acid was present with 21.4 mg/l and ended with 62.4 mg/l. In addition, valeric acid had a concentration of 3.6 mg/l on day 27 and there was no presence of formic acid.

The difference between the reactor R2-08 V with an energy supply and the R5-control is that when analyzing the values separated, the reactors with voltage had lower concentrations of lactic acid, acetic acid, propionic acid. However, R2-08 V ended with higher value of butyric acid than reactor R5-control.

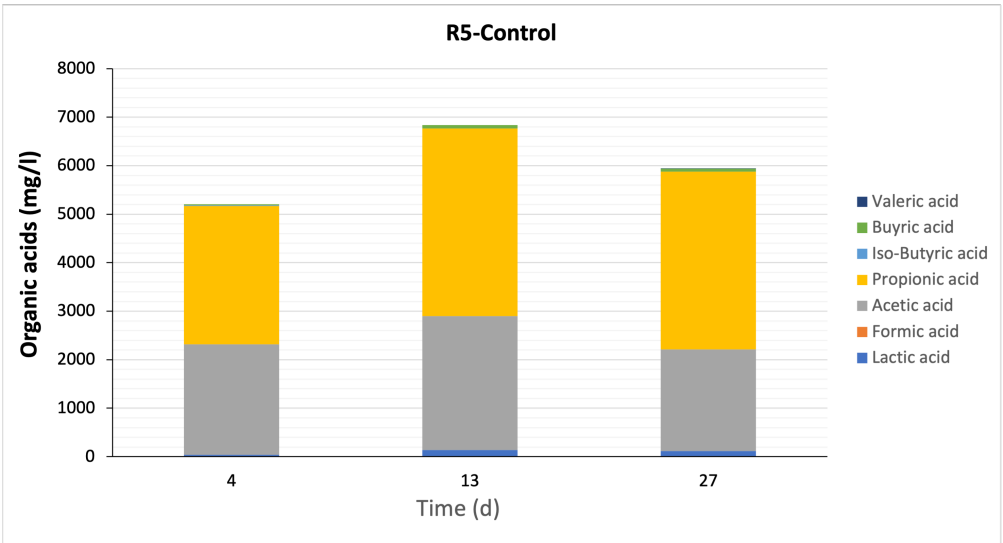

Figure 29 Development of the organic acids in R5-control during experiment II, own representation.

As in experiment I, by evaluating the acids compounds with the highest concentration in the reactors such as acetic acids and propionic acid from experiment II, based on the sum of the concentration and their respective voltage and those without. The acetic acids in the reactors with 1.5 V had a total sum of 5.394 mg/l, the controls reactors with a total of 4.395 mg/l, while the reactors with 1.0 V had an accumulation 5.212 mg/l. Whereas, the total concentration of propionic acetic were 8.395 mg/l to reactors with 1.5 V, 7.260 mg/l to the control reactors and 7.518 mg/l to reactors with 1.0 V. Hence, it shows that both compounds were higher in reactor with 1.0 V, and the lowest values were from the control reactors which could suggest that the implementation of MEC and DIET system were not effective to reduce the accumulation of those acids.

Comparing to the other acids present in the reactors, propionic acid was significantly represented with a large space load over all reactors in experiments I, as shown in Figure 30. From day 36, the space load is visible in all reactors, mainly in R3-1.5 V and R2-1.0 V. On day 66, the reactor R4-1.5 V increased the space loading, while reactors R5-R6-control had a moderate increase in concentration which continued until the end. The lowest propionic acid concentration was from reactor R1-1.0 V.

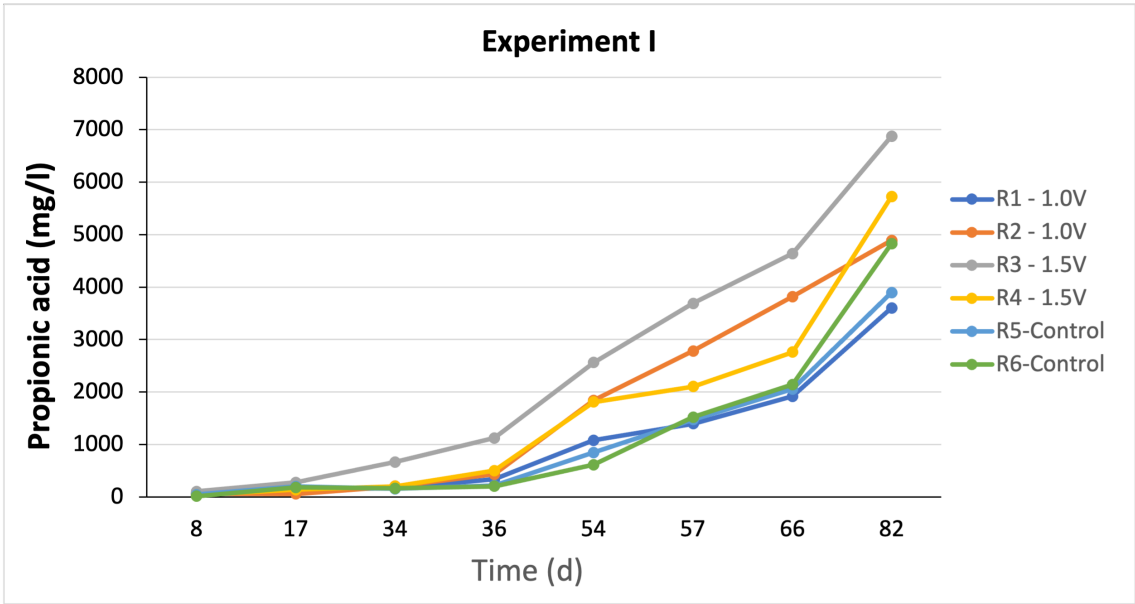

Figure 30 Development of propionic acid concentration in reactors R1-R6 over the entire experiment I, own representation.

As in experiment I, propionic acid was significantly represented with a large space load over all reactors compared to the other acids present in the reactors, as shown in figure 31. In experiment II, all the reactors increased the propionic acid concentration until day 13. A constant increase only occurred in R6-control, which continued until the end of the test experiment and the highest propionic acid concentration was from R3-1.0 V. However, the reactors R4-1.0 V and R5-control decreased the concentration after experiment test day 13.

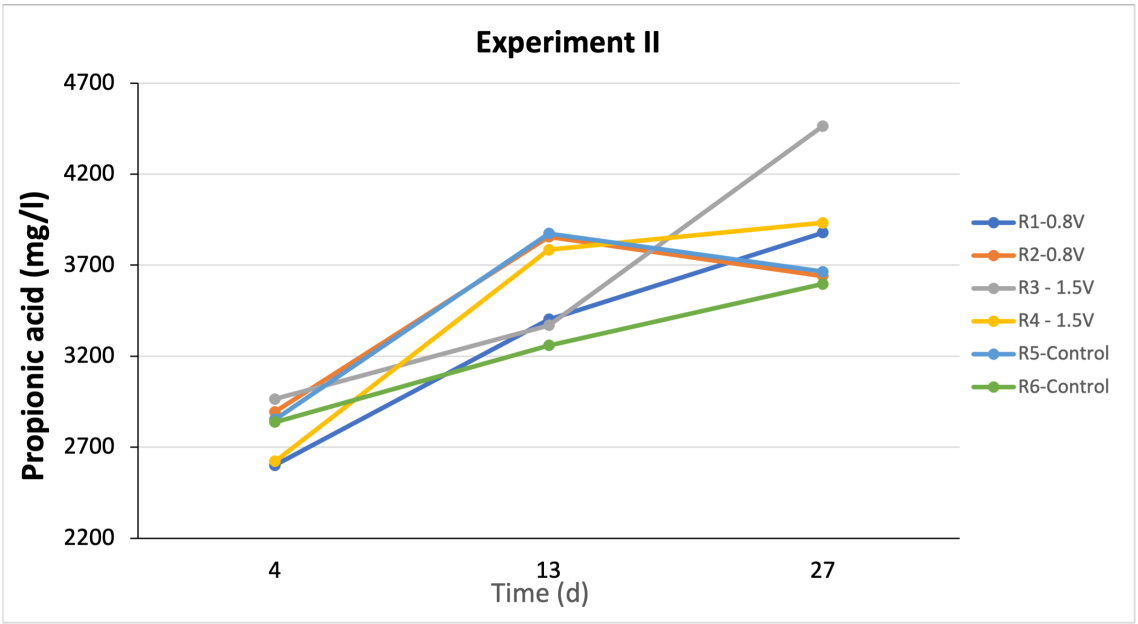

Figure 31 Development of propionic acid concentration from reactors R1-R6 over the entire experiment II, own representation.

Analysing the content of acids present in the reactors, a small fraction of butyric acid is noticed in almost all of them. For that reason, Figure 32 illustrates the development of the butyric acid concentration over time. Therefore, in experiment I is observed that the control reactors R5 and R6 have reached the highest concentrations value over time.

By contrast, reactors R3-1.5 V and R4-1.5 V followed a similar trend, while reactors R2-1.0 V obtained the lowest concentration. In addition, reactor R1-1.0 V started with an increase in space loading and decreased after day 57, reaching the same level as R2-1.0 V.

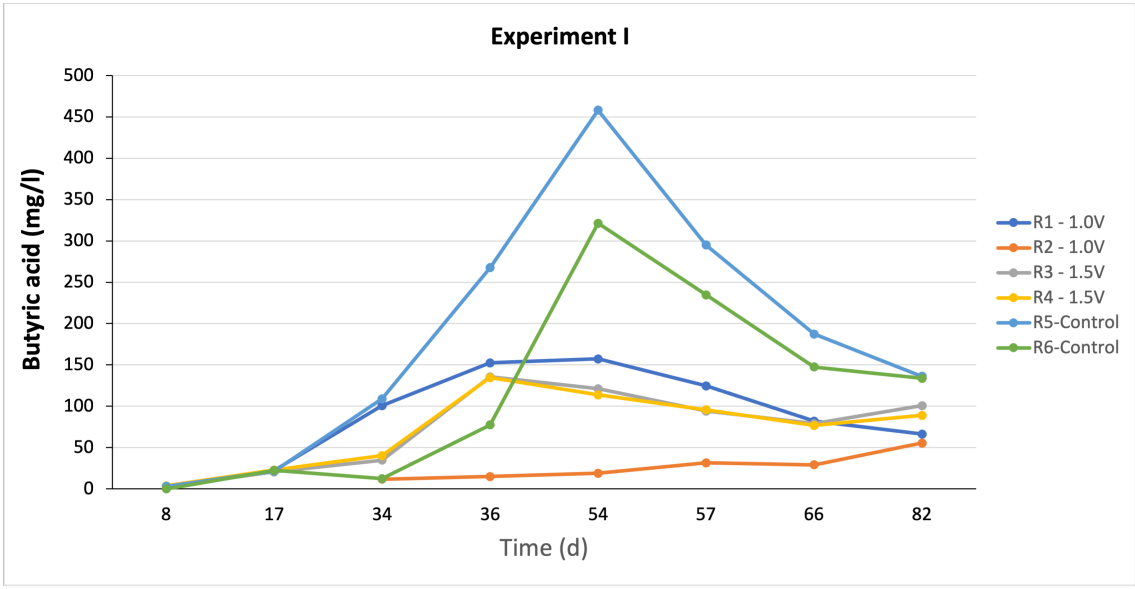

Figure 32 Development of butyric acid concentration from reactors R1-R6 over the entire experiment I, own representation.

In Figure 33 represent the butyric acid from experiment II, the reactors R1-R2 with 0.8 V followed an increase of the concentration until the end of the test experiment. After day 13, the reactor with the highest concentration was R2-0.8 V, and the lowest concentration of butyric acid was from reactor R3-R4 with a supply voltage of 1.0V. Among that, R3-1.0 V followed a constant concentration till day 13 and then increased, while R4-1.0 V had a concentration value of 32.8 mg/l and ended the experiment with 48.7 mg/l. By contrast, reactor controls R5-R6 followed the same trend until day 13, and then R5-control dropped the concentration.

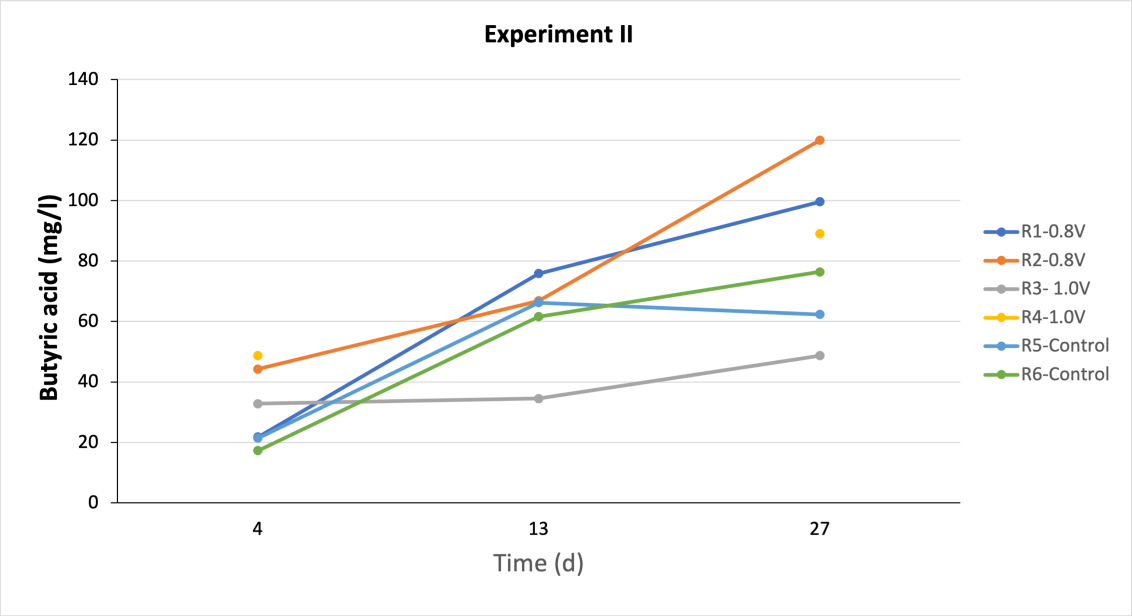

Figure 33 Development of butyric acid concentration from reactors R1-R6 over the entire experiment II, own representation.

5.3.2. COD-Degradation

COD measures the amount of oxygen consumed by reactions in a measured solution (D. Li & Liu, 2019). Therefore, it would be possible to know how many grams of oxygen are required to oxide all the compounds present in a certain volume. In this experiment in phase I, the COD content of the zero samples was given as 4045 mg/l (unfiltered) and was determined for the 1:1 diluted to the digested substrate with H_20 and the added urea and sugar. The determination of the proportion of sugar was done by the reaction equation below, where for the oxidation of one more sucrose, 12 moles of oxygen are required.

Equation 20 $$C_{12}H_{22}O_{11} + 12O_2 \rightarrow 12CO_2 + 11H_2O$$

Nevertheless, for a reactor with a volume of 1L, the addition of 1,0 g of sucrose affects the COD as follows:

Equation 21 $$COD_{sucrose} \frac{15{,}999 * 2 * 12}{12{,}017*12 + 22 * 1{,}008 + 15{,}999 * 11}$$

$$COD_{sucrose} = 1{,}12\ gO_2/l \approx 1121\ mg\ O_2/l$$

From that it was possible to calculate OLR, which is one of the factors that can influence biogas production in AD (Periyasamy et al., 2022). OLR is a measurement of the biological capacity of an anaerobic digestion system. It can show the quantity of raw material of volatile solids used to feed the digester for a unit volume in a day (Periyasamy et al., 2022). Excessive loading could influence digestion because the accumulation of acids in the digester could affect the microorganism, which could not survive, thus reducing biogas production. The unfed reactor might cause an alkaline effect that would also reduce the biogas production (Periyasamy et al., 2022). Therefore, the proper assessments must be done to load the digester with the optimum organic loading matter. For that reason, the calculation of OLR was follow by equation 23.

Equation 22

$$OLR = \frac{(\text{amount of sugar}^* \text{CODsucrose}) + (\text{amount of urea} * \text{CODurea}) + \left(\frac{\text{total amount of substrate added}}{\text{n° of reactors}} * \text{COD zero sample}\right)}{\text{reactor volume} * \text{interval days between measurement}}$$

As a result, the Figure 34 shows the development of OLR over the experiment I by the measurement time. Since the begging of the experiment I, the feeding of urea and sugar occurred in all reactors, increasing the feeding of urea from 0,6 g/l to 0.8 g/l and maintained same values of sugar. From point 13, no urea was being added to the reactors and consequently occurred a drop of OLR concentration in that period. However, as soon as urea started being fed with 0,4 g/l in the reactors and sugar maintained same values a drop of OLR concentration happened. Then, no urea was being fed to the reactors and the OLR concentration dropped again. In addition, since all the reactors had the same amount of sugar and urea addition, they have same trend during the experiment test.

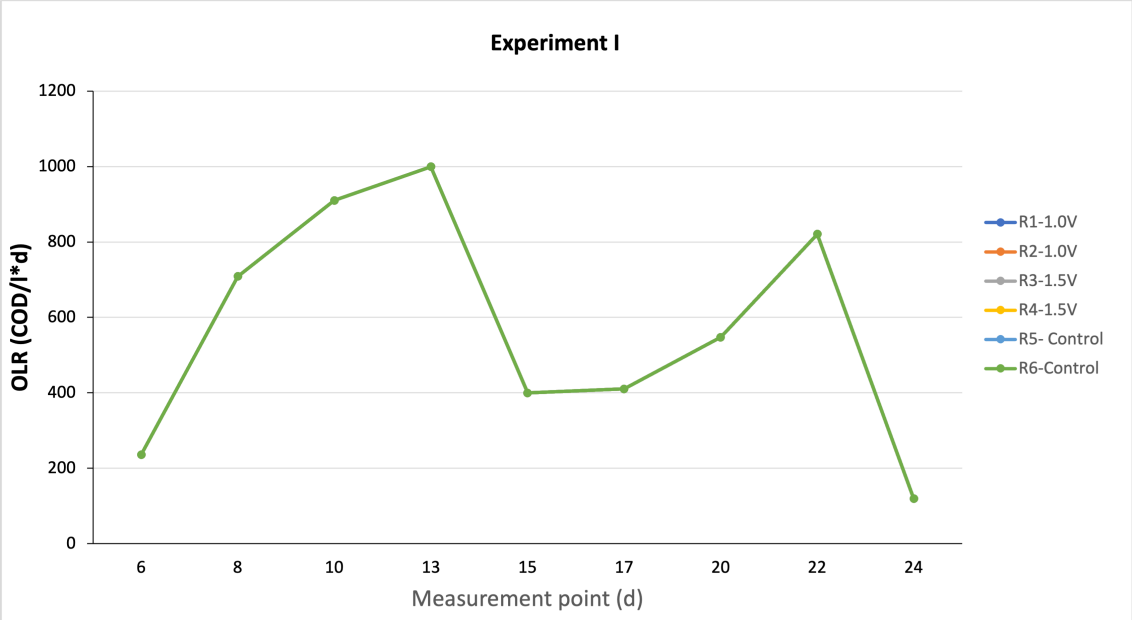

Figure 34 Development of OLR (COD/l*d) of all reactors during experiment I, own representation.

Among that, Figure 35 shows the OLR development in experiment II, that contain a substrate enriched with ammonia, for that reason OLR concentration starts with high concentration. However, with the feeding of 0.6 g of sugar since the beginning of the experiment, the OLR values have decreased even with the feeding of 0.4 g of urea. From point 8, only 0.6 g of sugar was added to the system and no urea, resulting in a stabilization of OLR over the experiment. In addition, OLR values have ranged initially between 1430 to 1487.0 COD/l*d and ended with a rage between 536 to 564.0 COD/l*d. In general, the values kept almost same range since the reactors were fed with the same amount of urea and sugar.

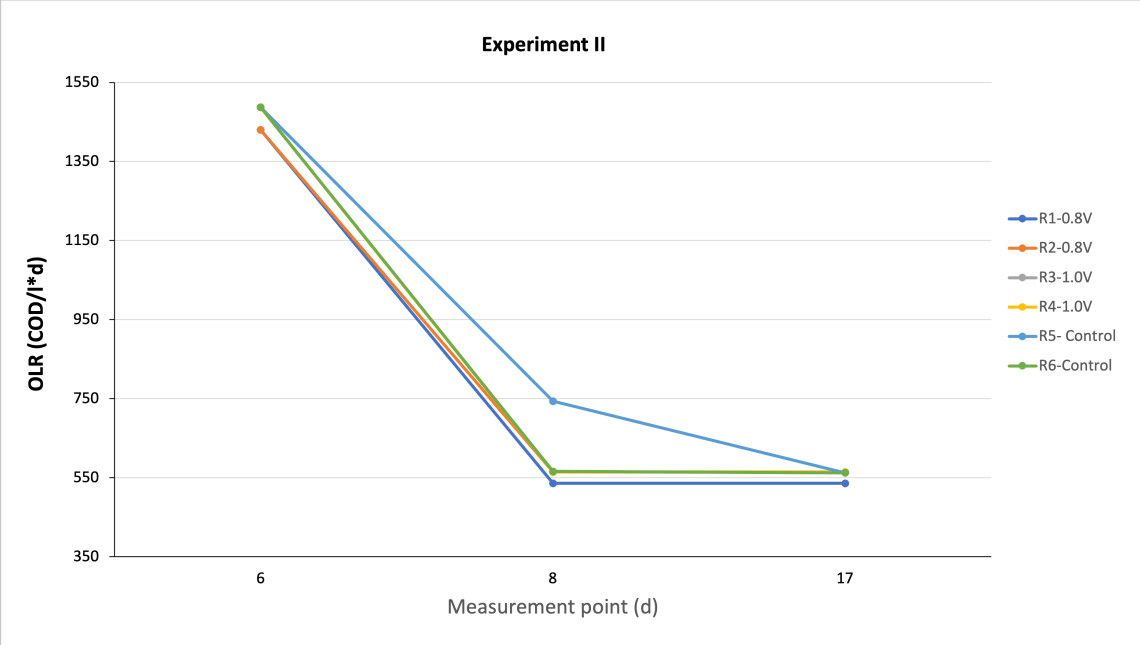

Figure 35 Development of OLR (COD/l*d) of all reactors during experiment II, own representation.

6. Discussion

This chapter will evaluate the results from experiments I and II. The evaluation is based on comparing the biogas and analysis values obtained from the reactors with MEC and DIET stimulation and the control reactors working without any stimulation process. From that, it would be possible to understand if the implementation systems have improved the reactor's stability by reducing the inhibition effect and increasing methane formation.

6.1. Experiment I

The biogas production results represented in chapter 5.1 suggests there is no significant stimulation of the anaerobic microbiome in almost all reactors from experiment I, mainly reactor R3-1.5 V. Comparing the reactors with an energy supply, those with 1.0 V have produced more biogas than those with a voltage of 1.5 V. However, the most successful biogas production was from the reactors without energy supply R5-R6. That effect was seen due to the inhibition of methanogenesis and reduced CH_4 production caused by the higher ammonia and accumulation of organic acids in the reactors.

Evaluating the performance of the reactors with energy supply to produce biogas in experiment I, those with a voltage of 1.0 V produced 1312 mlN more than the reactors with a voltage of 1.5 V. That difference represents 827 mlN of CH_4 in higher quantity than the reactors with a voltage of 1.5 V. However, the composition of CO_2 in the biogas was 675 mlN less than in the reactor with a higher voltage. The higher amount of CH_4 in the reactors with a voltage of 1.0 V was also reported by Choin et al. (2017), as mentioned in chapter 3.4.4. They observed a decrease in methane production when raising the voltage to 1.5 V in a glucose substrate. Thus, they have suggested that a higher voltage could have destroyed the archae microorganism and syntrophic relations because the acids present in the substrate are rising, and the methane value is going down.

Comparing in percentage the total amount of CH_4 and CO_2 present in the reactors with voltage and those without energy supply. The control reactors produced 7 % more CH_4 than those with a voltage of 1.0 V, but there was no difference in CO_2 composition between them. In contrast, when increased the voltage to 1.5 V, the composition of the reactors had 11 % less CH_4 than the control reactors and 14 % more CO_2 than the controls. Additionally, the average percentage of the reactors with 1.0 V was 47 % of CH_4 and 38 % of CO_2, but the sum of rates of CH_4 and CO_2 does not represent 100 % in all reactors. That occurred because some cases, the quantitative determination of the biogas was possible, but the volumes were not sufficient for a determination of the composition; The reactors with 1.5 V had an average of 45 % of both composts, while the control reactors had 50 % of CH_4 and 38 % of CO_2.

However, Andriani et al. (2014) mention that the levels of CH_4 from the decomposition of different substrates might range from 50-70 %, and the level of CO_2 around 30-50 %, which fits with the control reactors in this experiment. It is also mentioned that a large amount of CO_2 could lower biogas' heating value, reducing the combustion efficiency, effectiveness and economic value of biogas equipment generated. Therefore, the results suggest that there is no significate stimulation on the reactors using voltage to improve nitrogen inhibition and increase CH_4 production in the AD system in psychrophile systems.

Furthermore, analysing experiment I, which shows the gas composition of the reactors, reinforces the instability of CO_2 and CH_4 content from the reactors R3-1.5 V and R6-control over the experiment time. At the same time, the R1-R2 with 1.0 V offers better stability of biogas

content over the experiment time without many drops in the gas content. On day 42, most reactors have increased biogas production, which could be related to adding 2,0 g of sugar and no urea into the system. Subsequently, from day 47, 0,4 g of urea was added to each reactor, and that could have been the cause of the decrease in CH_4 in all the reactors.

Among that, evaluating the increase of acids in the reactors and CH_4 values going down is explained by the concentration of NH_4-N in the reactors starting with a minimum value of 888 mg/l and achieving a maximum of 4075 mg/l. From day 17, the reactors reached NH_4-N concentrations around 1607 - 1691 mg/l, which might have occurred due to the constant feeding of urea into each reactor that aims to achieve the inhibition effect in the AD system. Rajagopal et al., (2013) mention that the range of up to 1500-7000 mg/l of (TAN) levels can cause inhibition. By contrast, the studies conducted by Sung & Liu, (2003) and Lauterböck et al., (2012) have reported that TAN concentrations higher than 4000 mg/l could cause inhibition of methanogenesis. Lauterböck et al., (2012) also observed ammonia inhibition while digesting slaughterhouse waste, mainly with FAN concentrations higher than 100-2000 mg/L, 6000 mg NH4-N at 38 °C and pH of 8.1. When the study reached NH_4-N concentration near 1500-7000 mg/l of (TAN) and NH_3 concentrations around 80 mg/l, it was notable an inhibition process with the CH_4 production decreasing disproportionally. On day 42 of the experiment, NH_4 reached 3403mg/L and NH_3 with 83 mg/l at a pH of 7.62. It's seen the effect of decreasing CH_4 production in all reactors.

In addition, the inhibition effect of ammonium nitrogen is higher affected by parameters such as pH and temperature, which determine the formation of the equilibrium between NH_4 and NH_3. A change in pH from 7 to 8 could increase the free ammonia levels in mesophilic conditions and at thermophilic temperatures because the temperature affects the dissociation of constant ammonia nitrogen (Rajagopal et al., 2013). This effect can be seen in Figure 21, where shows the analytical concentration of NH_3 correlated to pH and the effects on methane illustrating that the increase of NH_3 in the digester caused a drop in the pH values and, consequently, CH_4 production. Additionally, NH_3 toxicity increases with temperature and could generate the washout of microbial population, especially from the undissociated form of ammonia which is responsible for inhibition at concentrations above 80 mg/l (Weiland, 2010). The inhibition by ammonia could generate an increase in the concentration of VFA, leading to a decrease in pH, which could partly counteract the effect of the ammonia (Weiland, 2010).

Nevertheless, to achieve pH values for optimal methane-forming archae, as described in chapter 3.2. The pH should be neutral to slightly alkaline, which is a probable reason for the acidification of the digester that leads to the inhibition effect of CH_4 formation. As observed in Figure 16 which illustrate the gas composition in (%) from the reactor R5-control in experiment I. Around day 38, there is a shift between CH_4 content to CO_2 where CH_4 decreases, while CO_2 increases the range. That could be explained by the change in equilibrium between liquid to gas phases of CO_2 when the pH value decreases. The AD process can be inhibited if the pH drops below 6.0 or rises above 8.6; the increase in pH value is due to the ammonia accumulation during the degradation of proteins, while accumulated VFA decreases the pH value. However, the accumulation of VFA will often not always results in a pH drop because of the buffer capacity of the substrate (Weiland, 2010).

In contrast, the increase in space load could lead to higher forms of organic acids due to lower pH values and the inhibition effect of ammonium nitrogen concentration. VFA is considered a key intermediate in the process and can inhibit methanogenesis in high concentrations (Weiland, 2010). Propionic and butyric acids are more inhibitory and effective in methanogenesis, related to the association with the undissociated form. However, propionic acid has been

reported to have a higher inhibition effect at concentrations lower than those reported for acetic or butyric acids, with values around 900 mg/l of propionic acid for observing negative effects versus values of 24000 mg/l for acetic and 1800 mg/l for butyric acid when dealing the cellulolytic activity, whereas glucose degradation (González et al., 2018). Kalamaras et al., (2021) also reported inhibition of methanogens growth in AD at a similar propionic acid concentration of 950 mg/l.

In this study, it is notable that the higher proportion and increase of organic compounds such as propionic and butyric acids, as demonstrated in Figures 25 and 27, represent the development of the organic acids from the experiment I. Based on the analyse of acetic acid and propionic acid from the reactors with the same voltage and those without, it shows that the reactors with 1.5 V had the highest accumulation of both acids. The lowest values were from the reactors containing an energy supply of 1.0 V. This result could suggest that MEC and DIET implementation have helped a better performance over the reactors with 1.0 V, while the reactors with 1.5 V have not suffered any interference. However, the difference between reactors with energy supply and without was only 127 mg/l. Those results might be justified by the study of J. Park et al. (2018), where the results reported that COD removal and methane production efficiency in the final steady state was almost the same as AD. Still, it has sped up the methane production rate without a drop in pH or accumulation of VFAs by MEC. That has occurred because the bioelectrochemical reactions prevented the factors that cause inhibition during start-up.

Nevertheless, in the main phase of experiment I, there is an increase in organic acids from 372 mg/l to 530 mg/l, ending the experiment with a range of 1405 mg/l to 1534 mg/l. At the same time, the propionic range on day 34 from 153-662 mg/l and ended the test with 3895 – 6878 mg/l, while butyric acid was initially with 3-101 mg/l and ended the test with 25-72 mg/l. Such an effect could also be observed in all reactors with increased of NH_4-N. Thus, the reduction in CH_4 production and accumulation of VFAs could be caused by toxicity factors and probably by the inhibition of the action of ammonia.

Due to the fact that biofilm plays an important role in enriching the high density of methanogens to improve biogas and methane production because they can potentially increase productivity by retaining microorganisms in the reactors, thus enriching the methanogens (Liu et al., 2017). Therefore, the reactors with a voltage of 1.0 V could suggest that the community of microorganisms present in the biofilm helped to obtain a better performance than the reactors with a voltage of 1.5 V as it seems higher cumulate biogas production and CH_4 production in reactors with 1.0 V. Thus, it could support the statement from Andriani et al. (2014) that higher voltages could have destroyed the archae microorganism and syntrophic relations because the acids present in the substrate are rising, and the methane value is going down.

However, the implementation of DIET with graphite electrodes as a conductive material has not stimulated the AD process to overcome the ammonium nitrogen inhibition and increase CH_4 formation. Therefore, it might suggest that ammonium nitrogen has affected the microorganism on the of biofilm in the anode and cathode over the other reactors, as shown in Figures 36 and 37. conversion of CH_4 even though the graphite electrodes from all reactors have demonstrated the presence of biofilm in the anode and cathode electrode surface, expected from reactor R1-1.0 V which appears to have biofilm only in the anode electrode. At the same time, reactor R2-1.0 V is the electrode with the highest presence.

Figure 36 Presence of biofilm on the graphite electrode from reactor R1-1.0 V in experiment I, own representation.

Figure 37 Presence of biofilm on the graphite electrode from reactor R2-1.0 V in experiment I, own representation.

6.2. Experiment II

The biogas production results represented in chapter 5.1 suggests there is a slightly significant stimulation of the anaerobic microbiome in almost all reactors from experiment II, mainly the reactor with an energy supply of 1.0 V. However, the most successful biogas production was from the reactors without energy supply R5-R6 which had almost the same amount of CH_4 and CO_2 composition from the reactors with an energy supply of 1.0 V. The unstimulated reactors also had the lowest accumulation of acetic acid and propionic acid during the experiment test. Thus, it could suggest that the implementation of MEC and DIET had no significant efficiency to a substrate with higher ammonia concentration in AD digestor from this study.

In experiment II, the anaerobic microbiome in the reactors with an energy supply of 1.0 V produced 259 mlN more biogas production than the reactors control R5-R6. This value represents an amount of 41 mlN of CH_4 and 179 mlN of CO_2 more than in the unstimulated reactors. Comparing the reactors with an energy supply of 0.8 V suggests no stimulation with the energy supply. They have produced 55 mlN less biogas than the control reactors, representing 114 mlN less CH_4 and 128 mlN less CO_2 than the control reactors.

Among that, Figures 17 and 18 which illustrate the gas composition in (%) from the reactors R1-0.8 V and R5-control over experiment II could potentially explain the lower cumulative production of CH_4 and CO_2 in the reactors with an energy supply of 0.8 V. During the gas measurement of the reactor R1-0.8 V on days 22 to 25, the presence of oxygen in the reactor directly affected the content and production of CH_4 and CO_2, as shown in the graph, a drop in

production of those compost. Nevertheless, the pre-requirement of substrate degradability to biogas in an anaerobic digestion system is an environment without oxygen. Thus, the decrease of CH_4 and CO_2 is notable with the presence of oxygen. Thus, the drop could be a result of a mistake during the operation of gas measurements allowing oxygen into the system or manipulating the gas measurement device.

By analysing the total percentage of cumulative biogas over CH_4 and CO_2 from the reactors, there was a slight stimulation with the voltage of 1.0 V. They generated an average of 23 % of CH_4 and 40 % of CO_2, whereas reactors with 0.8 V generated an average of 18 % of CH_4 and 35% of CO_2, being the lowest percentage of the experiment. Furthermore, the unstimulated reactors have generated 23 % of CH_4 and 37 % of CO_2, which are almost the same values as the reactors with a voltage of 1.0 V. As mentioned before, the biogas composition based on CH_4 and CO_2 from the decomposition of different substrates ranges from 50-70 % to CH_4 and 40-50 % to CO_2 (Andriani et al., 2014). Consequently, the rage values of biogas composition are higher than those found in this study that had the implementation of MEC and DIET. Hence, the results suggest there is no higher stimulation achieved with the energy supply in the reactors with high ammonium nitrogen concentrations.

Evaluating the concentration values of NH_4-N, NH_3 and OLR from experiment II, the lowest concentration was from reactors with an energy supply of 0.8 V. The reactors with an energy supply of 1.0 V started with an NH_4-N concentration of 5622 mg/l, and ended with 4461 mg/l, while the control reactors initially had a concentration of 5554 mg/l and ended with 4489 mg/L. The same has occurred with the NH_3, and OLR concentration in the reactors, those with an energy supply of 0.8 V had obtained a lower value at the end of the experiment compared to those without it. Therefore, the reactors with energy supply had better performance during the experiment than those without the implementation of MEC and DIET. However, the difference in the NH_4-N and NH_3 concentrations between the control and those with MEC and DIET implementation was not that higher. That could be because when the values of (TAN) concentration reached near 1500-2200 mg/l, it was notable an inhibition process with the CH_4 production decreasing in all the reactors because TAN values range up to 1500-7000 mg/l it can cause inhibition effects to the methane production (Rajagopal et al., 2013).

When analysing the total organic acids concentration, the reactors without an energy supply had the lowest concentration than those with an energy supply. The highest concentration was from the reactors with a voltage of 0.8 V, but the difference between the reactor with 0.8 V and those without was only 0.50 mg/l. The same trend was observed with propionic and butyric, where the lowest concentration values were observed in the control reactors with a sum of 7260 mg/l and the highest values from the reactors with 0.8 V with a sum up of 8395 mg/l. The difference between the lowest value and those with 0.8 V were only 82.0 mg/l. The concentration values of acetic acid around 900 mg/l or 1800 mg/l of butyric acid, can cause an inhibition process in methanogens growth in an AD digester, which causes the reduction of biogas production (González et al., 2018). Thereby, the results reported that OLR removal and methane production efficiency in the final steady state was almost the same as the control reactors.

During DIET, extracellular electron exchange between different microorganisms via electrically conductive pili, cytochromes and conductive materials (L. Li et al., 2021). It's reported that bacteria and archaea connect electrically to produce CH_4, which is involved in the stages of acetogenesis, acetate oxidation and methanogenesis in the AD process (L. Li et al., 2021). That relation of the conductive material could accelerate the interaction with the microorganic and increase methane production. This study shows an initial methane production, lowering down during the experiment. This could be related to the inhibition of syntrophic between acetogenic and methanogenic microorganisms because of ammonium nitrogen inhibition. In addition, DIET-half reactions occur at the anode exergonic, as it is shown in Figure 38, the electrode from reactor R3-1.0 V had the presence of biofilm in the graphite, as shown in the red arrow. Nevertheless, it was the only reactor with a visible biofilm present.

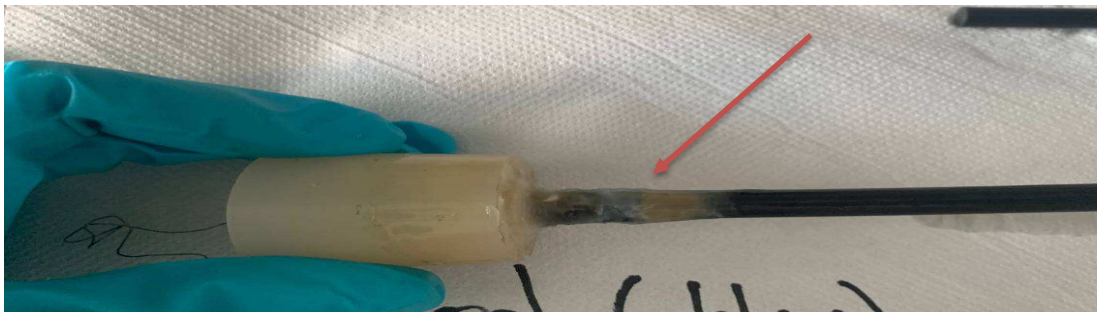

Figure 38 Presence of biofilm on the graphite electrode from reactor R3-1.0 V in phase II, own representation.

The results suggest a lower stimulation from the implementation of DIET and MEC in this study. This effect could be seen due to the inhibition of ammonium nitrogen, which has caused a lower biogas composition, higher concentration of NH_4-N, NH_3, total organic acids, the propionic and butyric acids than the control reactors. Still, at the sped up the methane production rate without a drop in pH or accumulation of VFAs by MEC, and that could have occurred due to the bioelectrochemical reactions preventing the factors that cause inhibition during start-up (J. Park et al., 2018). However, during the test accumulation of the acids and lower production of methane were noted and that could suggest that the implementation of MEC and DIET in a substrate with higher ammonia concentration was not capable of improving the AD of those substrates compared to the results of the control reactors of this study.

6.3. Possible Errors

During the experiments test, some mistakes were identified, which could have influenced the results. Those mistakes were taken in place in the construction and during the operation of the experiment test.

One of the mistakes noted was due to the higher percentage of oxygen in the gas analysis. This effect could suggest that during the operation of the experiment, oxygen was being introduced into the anaerobic process, which inhibits the anaerobic microorganisms. These could have occurred during the gas sampling when the eudiometer was opened to attach to the gas bag and then measured in the gas detector device. For that reason, during this procedure, it could have occurred of air had been introduced to the bag while sampling. In addition, the method of sampling could also be considered a possible error. The reactors were open for some

minutes for sampling, but that might have disturbed the environment, allowing CH_4 that was formed to escape and introduce oxygen into the system.

Another source of error is that it was not possible to ensure the level of equality between the level vessel and the eudiometer tube in the experiment test. Consequently, the quantitative gas detection could deviate a bit from the actual gas quantity. Besides that, the *x-am 8000* multi-gas measurement detectors from the manufacturer *Dräger* was used to determine the gas composition. However, this measuring device is used mainly for limit value monitoring with the purpose of personal protection. This means that the device displays the percentage of gas components of a gas stream, which changes with continuous measurement. Thus, it recorded the measurement percentage values that appeared most time. Nevertheless, there is a more reliable way to measure the real composition related to the total volume measured by gas chromatography or Infrared spectroscopy.

7. Conclusions and Future Prospects

This study was based on the performance of the fermentation of two individual experiments under the influence of a current feed and high NH_4-N concentrations. The first experiment had a duration of 92 days and the second 34 days. The aim is to improve the implementation of bioelectrochemical stimulation of the microbiome and the efficiency of the reactor stability in the anaerobic degradation of a substrate with a nitrogen inhibition fermentation process. It also evaluated the implementation of the stimulation of DIET to increase the production of CH_4 compared to the control reactors.

To evaluate the performance of the fermentation of the individual experiments, a setup was chosen where 4 reactors were subjected to a current energy supply, and two reactors were used as control, with no energy supply. Furthermore, the difference between the experiments was that one had an old substrate and reactors R1-R2 with an energy supply of 1.0 V, whereas R3-R4 had an energy supply of 1.5 V and reactors R5-R6 were the control. In experiment II, the substrate was prepared with higher ammonia concentration before starting the test; reactors R1-R2 had an energy supply of 0.8 V, R3-R4 had an energy supply of 1.0 V, and reactors R5-R6 were the control.

The inhibition of the anaerobic microbiome due to the high ammonium nitrogen concentration was achieved by feeding during the experiment test. Based on the data presented, the increase of NH_4-N concentration and the compounds NH_4+ and NH_3 were identified with higher concentrations capable of inhibition process and causing the accumulation of organic acids, the shift between the spectrum, the inhibition of CH_4 and biogas formations. In experiment I, the lowest NH_4-N sum-up concentration was from reactors with an energy supply of 1.0 V with 7927.27 mg/l and a pH average of 7.24, the reactors with an energy supply of 1.5 V had values equal to 8064.95 mg/l, with pH of 7.16 and the control reactors 80645 mg/l, with pH as 7.23. Meanwhile, in experiment II the reactors with a voltage of 0.8 V had the lowest sum-up concentration of NH_4-N at 4444 mg/l and a pH average of 7.07. In contrast, reactors with 1.5 V had a concentration of 4461 mg/l with a pH of 7.16, and the control reactors had concentrations of 4489 mg/l with a pH average of 7.19. In conclusion, both experiment values have a higher inhibition effect.

During experiment I, the reactors with a voltage of 1.0 V suggested a better performance than those reactors with 1.5 V, which could be seen by the biogas production, CH_4 composition and the VFAs. By contrast, the reactors with 1.0 V had a slightly better performance than the control reactors, the acetic acid from the reactors with 1.0 V had the lowest sum value of 9650 mg/l and the control reactors with 9777 mg/l. The total organic acids, propionic acid and NH_4-N also had the lowest value from the reactors with an energy supply of 1.0 V. However, the difference between them was not big, with no higher significance. Therefore, that could suggest that the implementation of MEC had a slightly better performance in the reactors with an energy supply of 1.0 V but a lower improvement than the control reactors. Thus, not achieved a high increase in the degradation of ammonia with the electrodes.

By analysing the CH_4 production of experiment I, the control reactors had the highest sum up of CH_4 composition with 7029 mlN, while the second highest CH_4 composition was from the reactors with an energy supply of 1.0 V with a total sum up of 6354 mlN. These results suggest that the DIET implementation has not increased CH_4 production compared to the control reactors. That effect was seen due to the inhibition of methanogenesis and reduced CH_4 production caused by the higher ammonia and the higher accumulation of organic acids in the reactors.

In experiment II, the control reactors had the lowest accumulation of total organic acids, acetic acid, and propionic acid during the experiment test. The anaerobic microbiome was slightly stimulated in the reactors with 1.0 V compared to those with 0.8 V, which had not produced a significant effect. The lowest NH_4-N concentration, NH_3 and OLR were from the reactors with an energy supply of 1.0 V. Nevertheless, the difference between the acetic acid, propionic acid NH_4-N, and NH_3 concentrations from the control reactors and those with MEC implementation was not that higher. The sum up of NH_4-N from the reactors with a voltage of 1.0 V was 45 mg/l lower than the control reactors, whereas the acetic acid concentration was 127 mg/l lower than the control reactors' sum. This effect could have been seen due to the values of (TAN) concentration reaching near 1500-2200 mg/l, generating an inhibition effect, and consequently, CH_4 production decreased in all the reactors. Hence, it could suggest that the implementation of MEC had not generated a higher increase in the degradation of ammonia.

Among that, the sum of the CH_4 composition from the reactors with the same voltage and those without it, the reactors with an energy supply of 1.0 V had a CH_4 composition of 41 mlN and CO_2 of 179 mlN more than the control reactors. That increase was probably caused by the expression of an electro stimulated DIET, causing the oxidation of propionate and acetate oxidation to produce CH_4 even under inhibition of ammonium nitrogen. Thus, the implementation of DIET has caused a slight stimulation in the reactors with an energy supply of 1.0 V. However, the implementation of electrodes has not produced a significant effect.

Among that, during biogas production of both experiments, it was notable the influence of changes in the operating conditions. Such as temperature, pH, the variation in the NH_4+ and NH_3, the formation of the acid under a change in space room loading and CH_4, and biogas formation depending on the space loading and NH_4-N concentration. Although the stimulation of DIET and MEC was not higher in both experiments, the results show a better performance in those reactors with an energy supply of 1.0 V than in 0.8 V and 1.5 V. The reactors with 0.8 V reported a higher percentage of oxygen, which could have caused the lower biogas production and higher VFAs in the reactors. In comparison, the energy supply of 1.5 V might have inhibited the microorganism, mainly the archaea, due to the higher voltage.

Therefore, based on experiment I, the reactor with a voltage of 1.0 V obtained a lower production of acetic acid, propionic, and NH_4-N, and it had the second highest CH_4 production. The reactors with a voltage of 1.0 V also had the best performance in experiment I compared to the reactors with 0.8 V. Thus, it gives a reason to further analyse the influence of an external voltage and conductive material described in the literature as they are described as capable of stimulating AD digester. Further experiments should consider the issues during the performance of the experiments, such as avoiding introducing air into the system while operating the gas measurements and the problems described in the error analysis.

In the end, the study could not increase the degradation of ammonia with the electrodes. However, maybe with a longer time experiment test, working with voltages around 1.0 V and 1.2 V, slightly changing pH or a better electrode surface which could allow biofilm growth, and the experiment could improve AD digestion. Among that, some types of electrodes which could substitute graphite are carbon and metals-based conductive materials like biochar, GAC, magnetite, and many other options that significantly affect methanogenesis in anaerobic digesters. It also could have helped slightly decrease the pH of the AD with $Ca(OH)_2$ to cause a reduction of ammonia and pH adjustment leading to a slight decrease in the ammonia nitrogen. Therefore, the FAN level and proton accumulation are induced in the anode compartment of the MEC due to change and cation transport to the cathode.

Furthermore, another option to further overcome this situation is to study the possibility to implement a hybrid system. This hybrid system could be compost of submersible counteracts ammonia inhibition during the use of a desalination chamber. This is a new method which can interact with MEC on ammonia recovery, avoiding the risk of ammonia toxicity on the anodic biofilm and during biogas production. Furthermore, such technology could be applied to existing biogas facilities and thus save integration costs.

In conclusion, this study and technology require more study to better understand the DIET and MEC under ammonia nitrogen as the reactors with different voltages have reacted differently. The implementation of DIET and MEC are known to help in shortening lag phase time, increasing methanogenesis rate and methane yield, and providing resistance to high organic overloading conditions. Therefore, there are diverse research possibilities for practical applications such as the energy sector as renewable energy generation in the form of climate-neutral CH_4 and to facilitate the digestion of wastes with higher concentrations of NH_4-N.

For that reason, a proper investigation of the requirement of microorganisms for carrying out DIET and the inhibitory conditions for the microbes in AD digestion is needed. In addition, at the end of the experiments, samples were taken to provide insights into the formation of the microbiome, which could explain the trace of degradation, CH_4 formation and how the microbiome responds to the increase of NH_4-N. Thus, understanding the formation of microbiomes could clarify their interaction with NH_4-N, whereas the electron transfer mechanism or conductive material selection could be a promising strategy to stabilize AD against organic and nitrogen overloads while improving the quality of effluent and biogas production.

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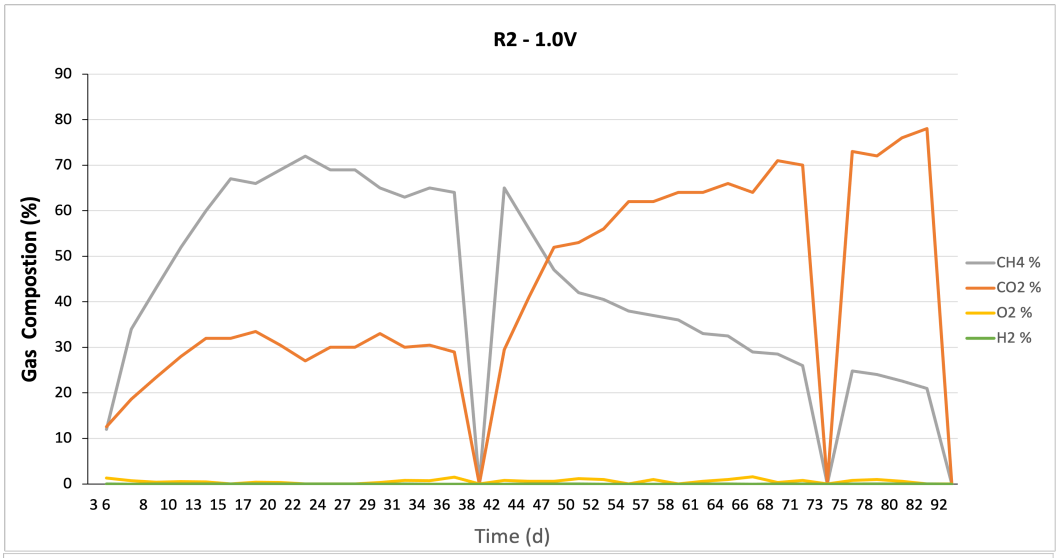
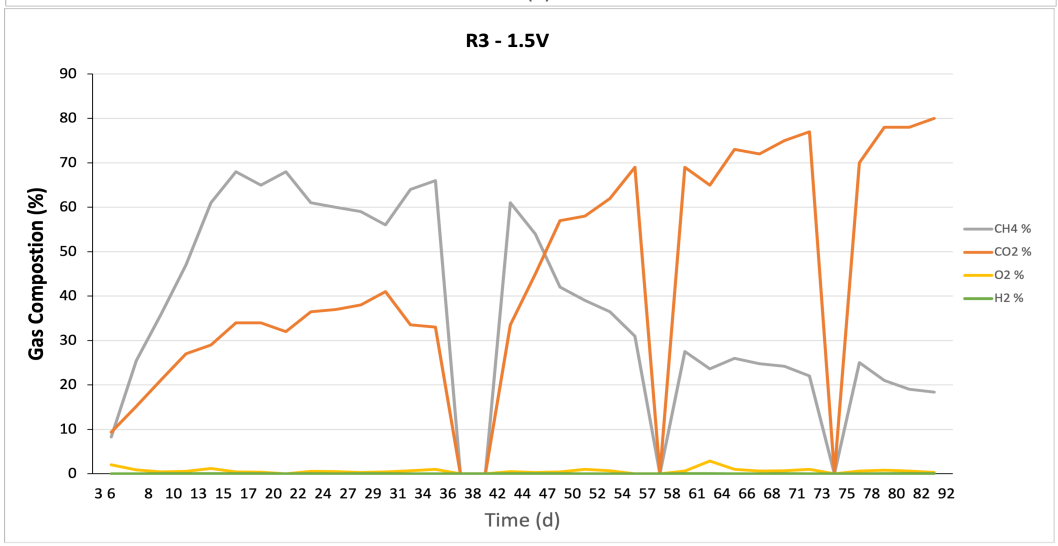
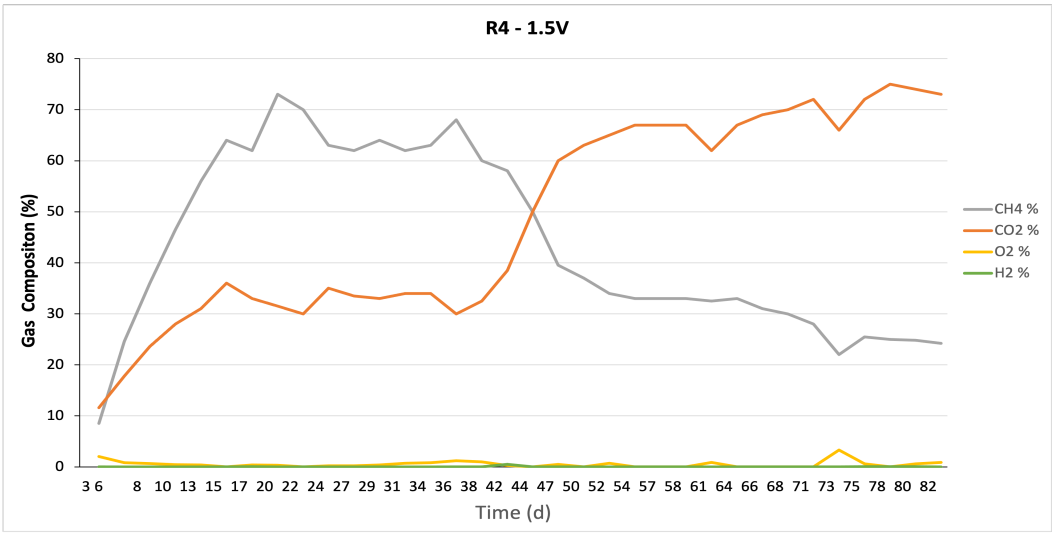
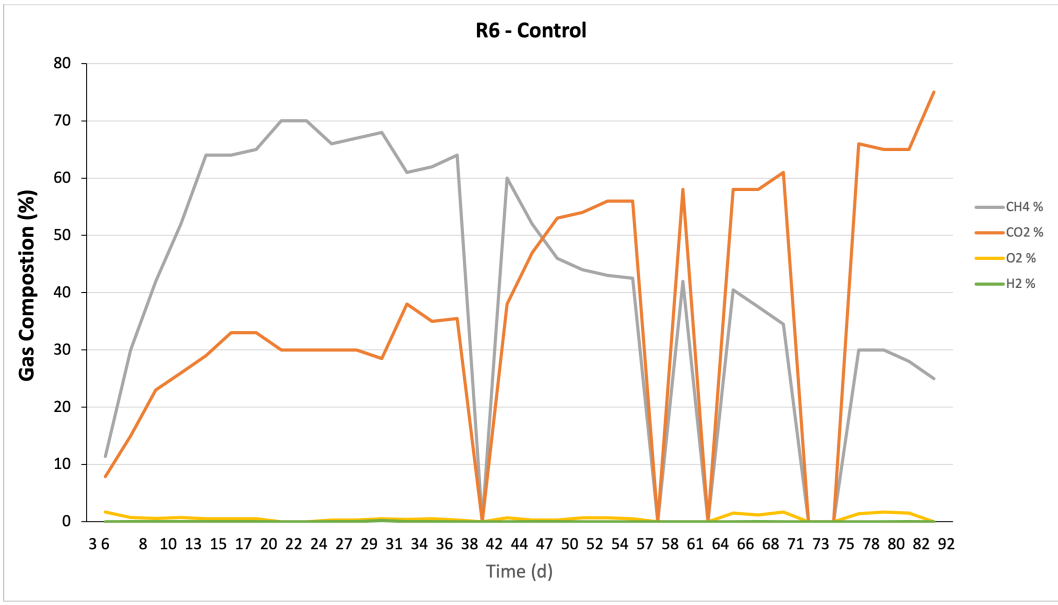
9. Appendix

Appendix 1 Gas composition in (%) from reactors R2-1.0 V, R3-1.5 V, R4- 1.5 V and R6 - control from experiment I, own representation.

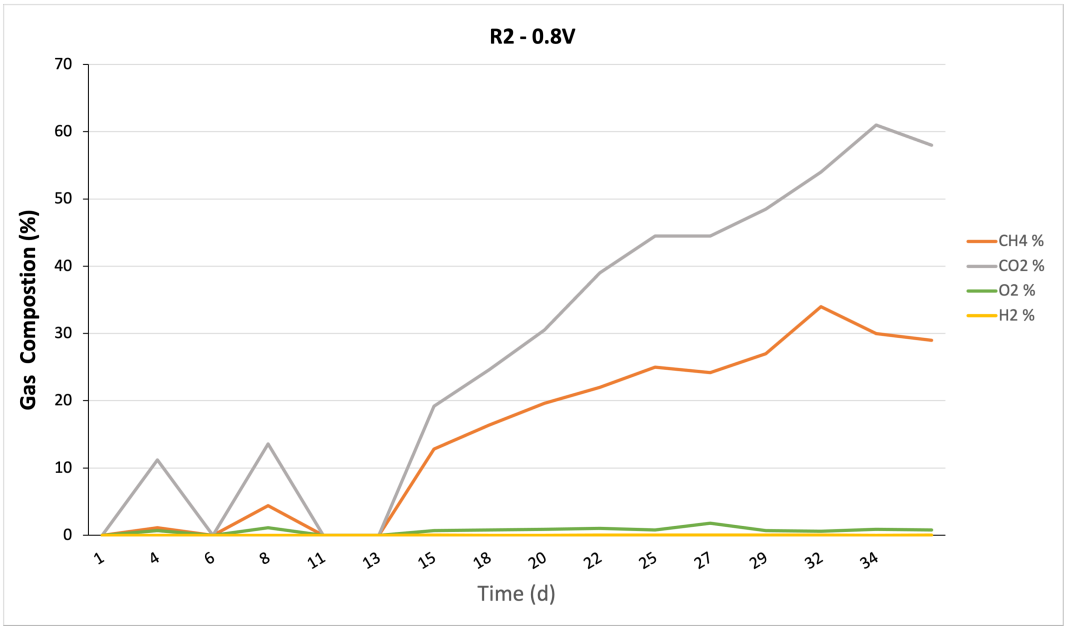


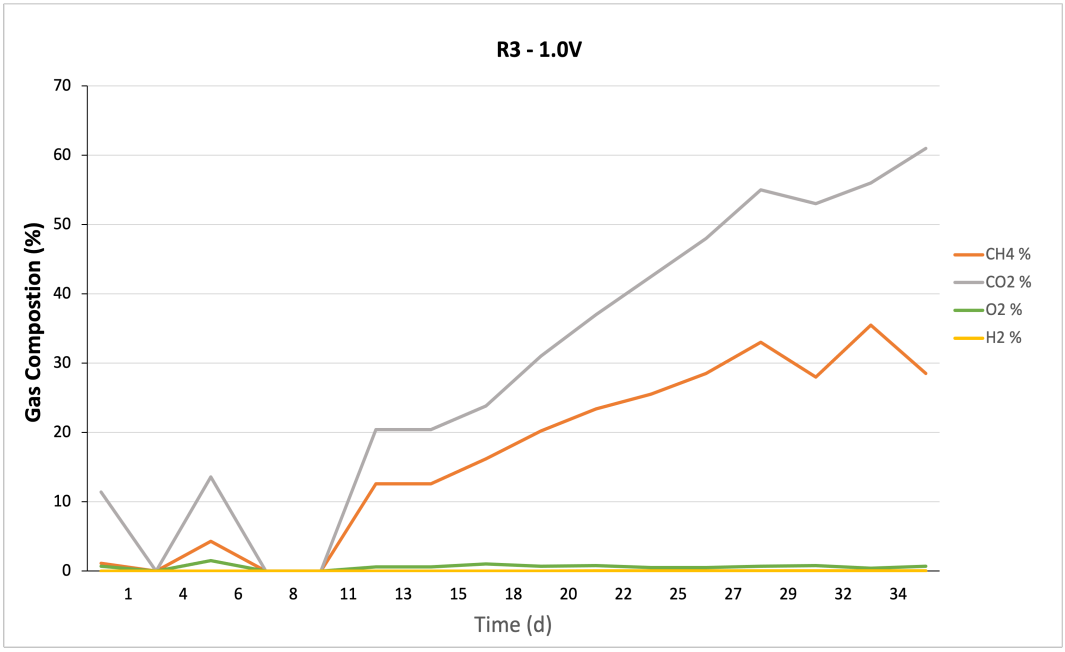


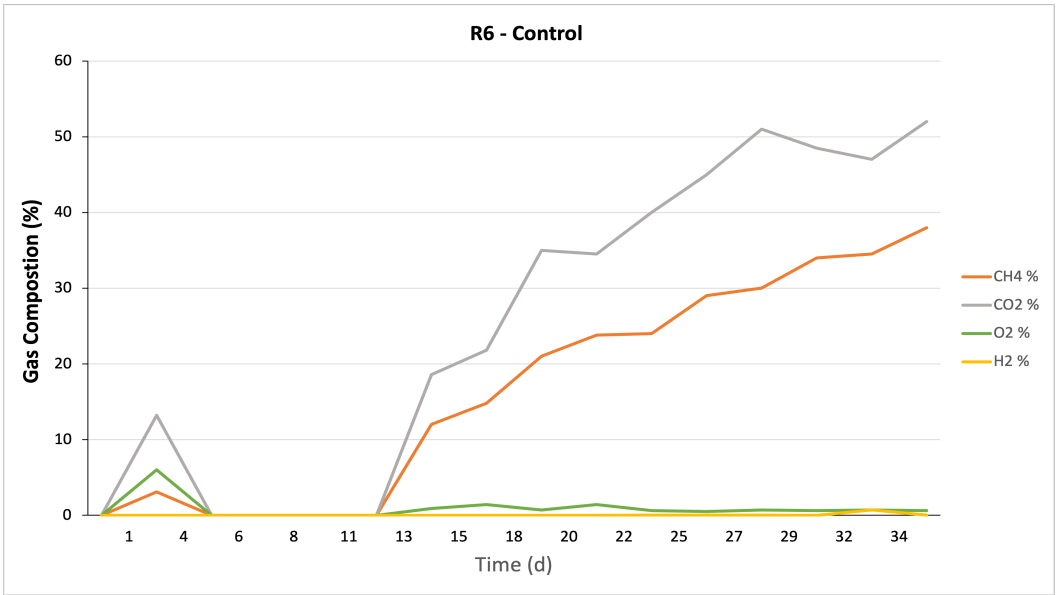




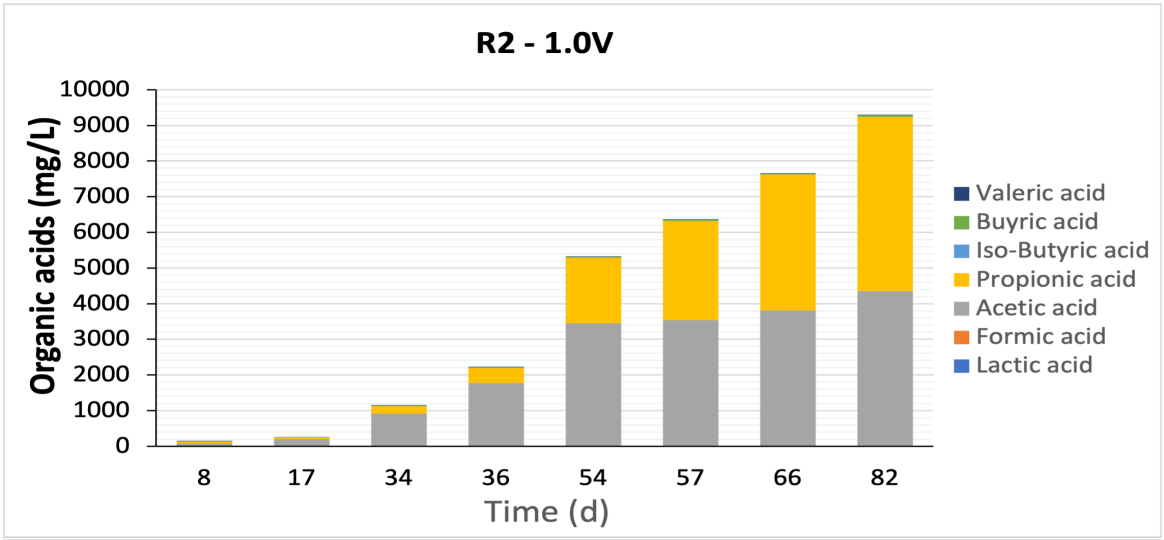
Appendix 2 Gas composition in (%) from reactors R2-0.8 V, R3- 1.0 V, R4- 1.0 V and R6 - control from experiment II, own representation.

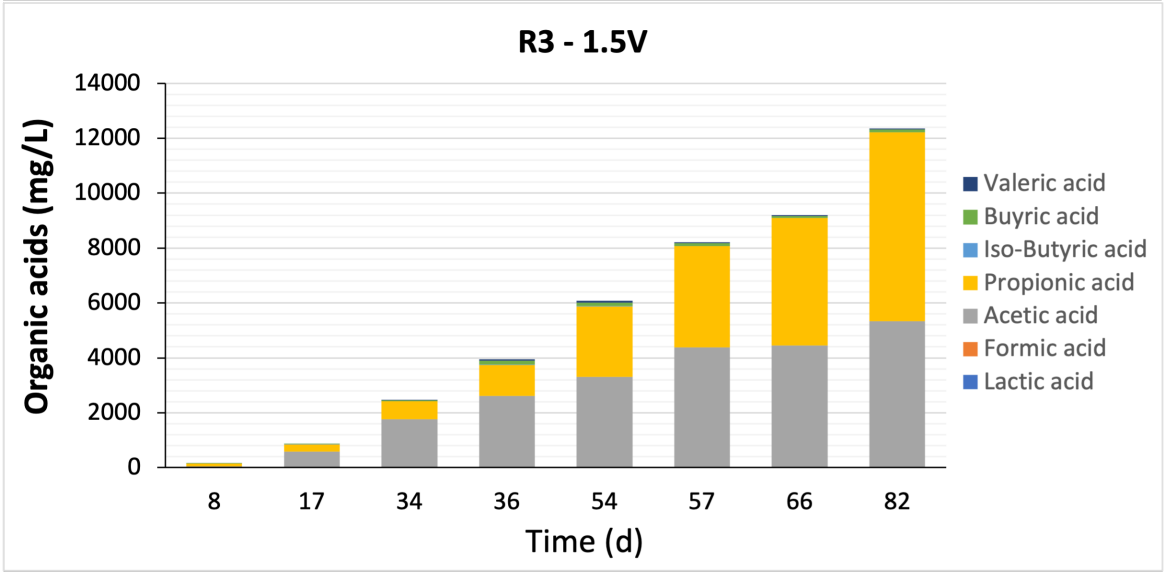


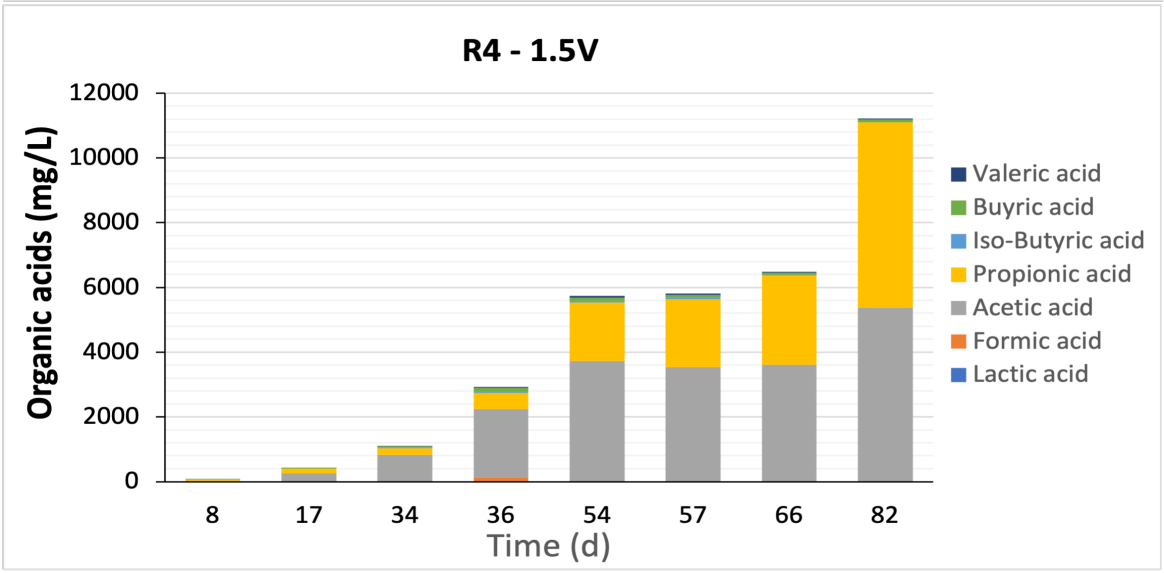

R3 - 1.0V
Gas Compostion (%)
70
60
50
40
30
20
10
0
1
4
6
8
11
13
15
18
20
22
25
27
29
32
34
Time (d)
CH4 %
CO2 %
O2 %
H2 %

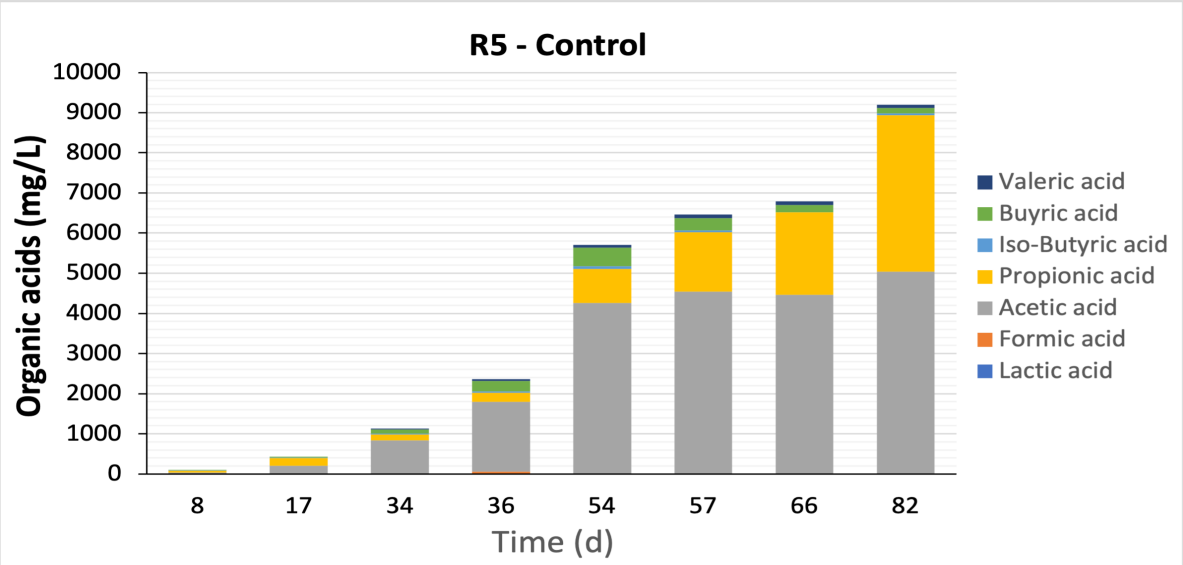

R6 - Control
Gas Compostion (%)
60
50
40
30
20
10
0
1
4
6
8
11
13
15
18
20
22
25
27
29
32
34
Time (d)
CH4 %
CO2 %
O2 %
H2 %

Appendix 3 Development of organic acids in R2-1.0V, R3-1.5V, R4-1.5V and R5-control from experiment I, own representation

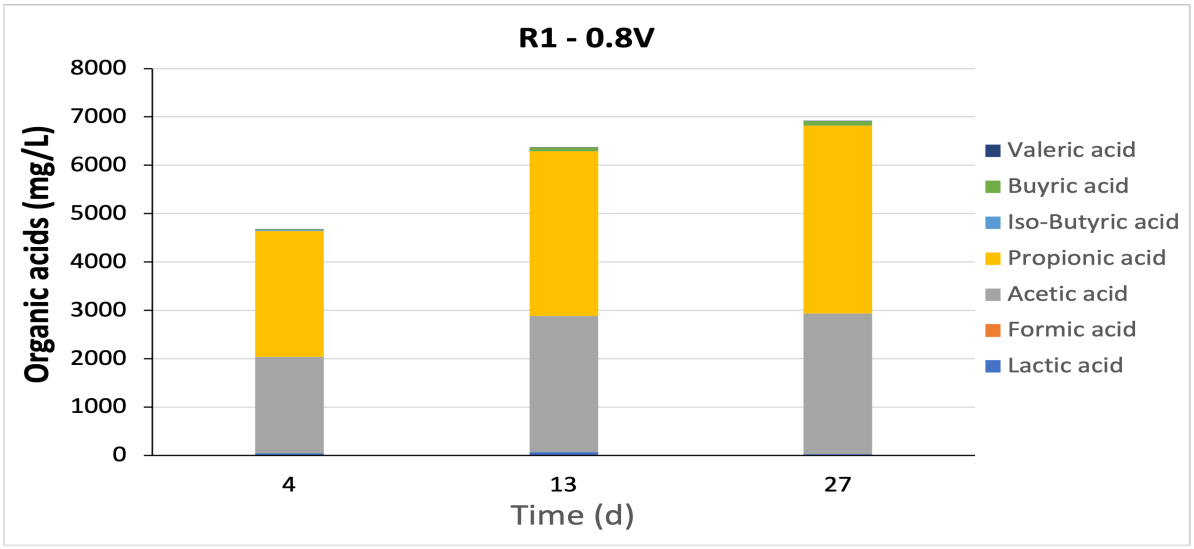


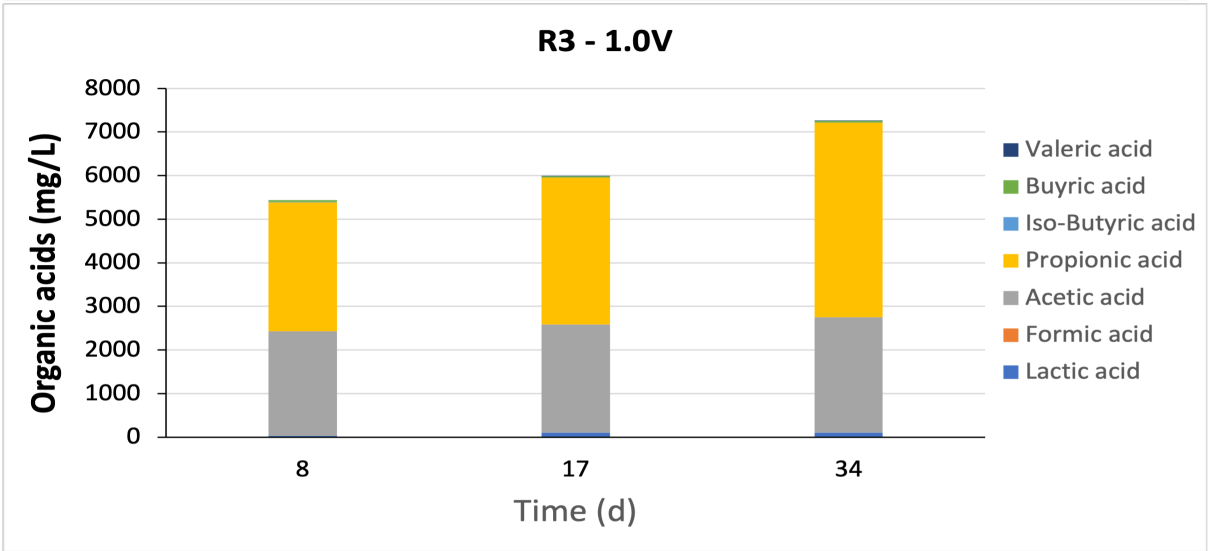


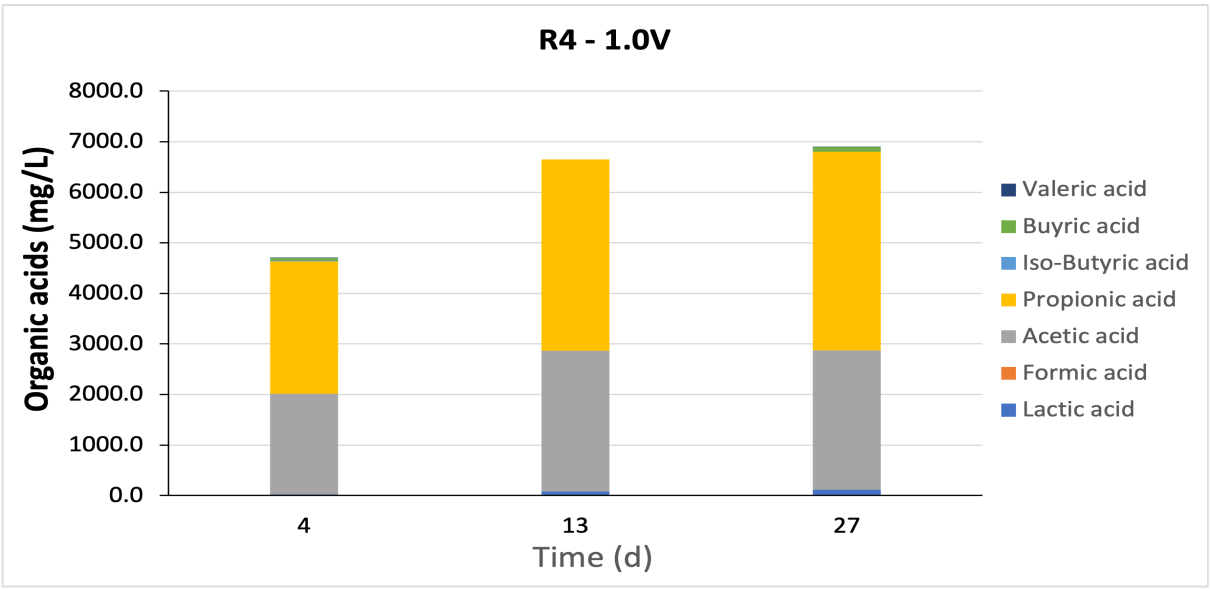


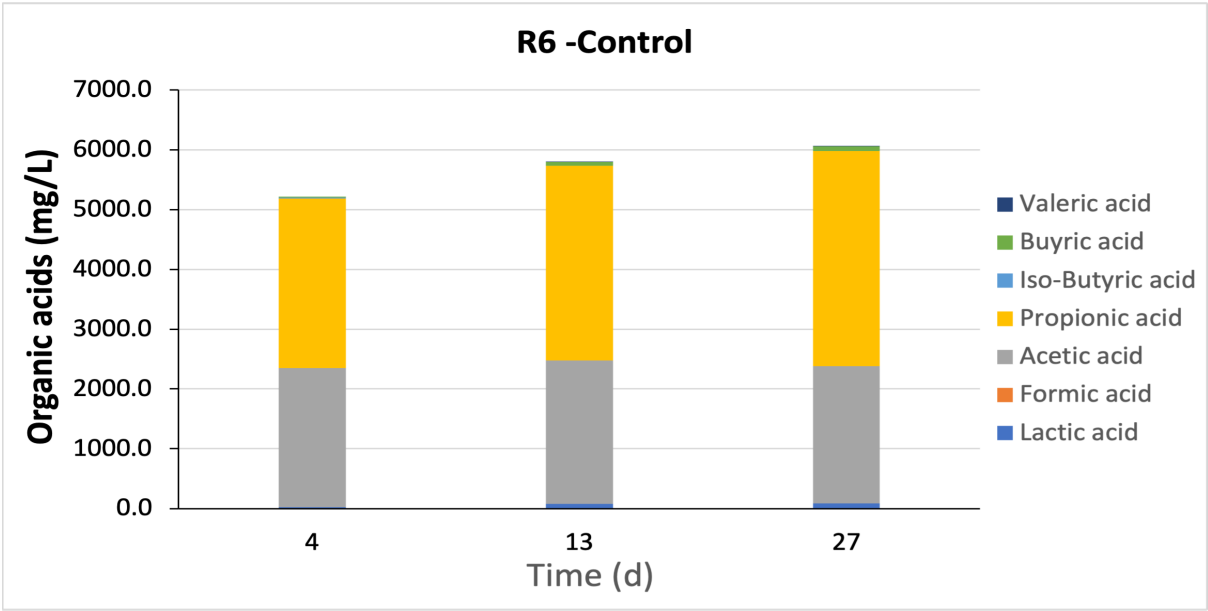


Appendix 4 Development of organic acids in R1-0.8 V, R3-1.0 V, R4-1.0 V and R5-control from experiment II, own representation.






R4 - 1.0V
Organic acids (mg/L)
8000.0
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
4
13
27
Time (d)
Valeric acid
Buyric acid
Iso-Butyric acid
Propionic acid
Acetic acid
Formic acid
Lactic acid


R6 -Control
Organic acids (mg/L)
7000.0
6000.0
5000.0
4000.0
3000.0
2000.0
1000.0
0.0
4
13
27
Time (d)
Valeric acid
Buyric acid
Iso-Butyric acid
Propionic acid
Acetic acid
Formic acid
Lactic acid