UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS
Departamento de Engenharia de Alimentos

Beatriz Egerland Bueno

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"VERSÃO CORRIGIDA"

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Área de Concentração: Biotecnologia Ambiental

Orientadora: Profa. Dra. Giovana Tommaso

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Beatriz Egerland Bueno

Tratamento contínuo da fase aquosa da liquefação hidrotermal de <u>Spirulina</u> em reator anaeróbio horizontal de leito fixo utilizando lodo bioestimulado.

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RESUMO

BUENO, B. E. ***Tratamento contínuo da fase aquosa da liquefação hidrotermal de Spirulina em reator anaeróbio horizontal de leito fixo utilizando lodo bioestimulado.*** 2020. 133 f. Tese (Doutorado) – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

A liquefação hidrotérmica é um processo que converte a biomassa úmida em biocombustíveis, mais especificamente o bio-óleo bruto. No entanto, juntamente com esse processo, é gerada uma fase aquosa (PHWW) rica em nutrientes e conteúdo de matéria orgânica, porém com grande quantidade de compostos tóxicos. A digestão anaeróbia é uma opção promissora para o tratamento da PHWW. Neste estudo, a digestão anaeróbia de PHWW da *Spirulina* foi avaliada usando lodo enriquecido como estratégia para otimizar o processo. O enriquecimento foi realizado em um reator sequencial em batelada alimentada com ácidos orgânicos e metanol, visando a bioestimulação de microrganismos acetogênicos e metanogênicos. Foram realizados ensaios de biodegradabilidade anaeróbia de PHWW, com lodo não-bioestimulado e bioestimulado O lodo bioestimulado foi capaz de atingir valores superior de rendimento de metano em concentrações mais altas de matéria orgânica (11 e 16 g $DQO.L^{-1}$) em relação ao lodo não bioestimulado (7 g $DQO.L^{-1}$) e apresentou menor grau de inibição nas condições inibitórias testadas. Portanto, a bioestimulação foi um processo fundamental para selecionar e favorecer potenciais microrganismos envolvidos na degradação especializada de compostos recalcitrantes, como os gêneros *Mesotoga* e *Methanometethylovorans*, que foram fundamentais para a bioconversão do PHWW. Uma estratégia interessante para o tratamento de compostos tóxicos é o uso de biomassa aderida a suportes inertes formando biofilmes. Neste trabalho, o uso de biomassa imobilizada em espuma de poliuretano foi avaliado para a degradação anaeróbia do PHWW. Maior velocidade de produção de metano e menor acúmulo de ácidos voláteis graxos mostraram as vantagens do uso de biomassa imobilizada em espuma de poliuretano em ensaios em batelada. O tratamento contínuo em um reator anaeróbio horizontal de leito fixo (RAHLF) alcançou a eficiência de remoção de matéria orgânica (DQO), de 40 a 69%, quando operado com carga orgânica de 0,8 e 1,6 g $DQO.L.\ d^{-1}$, respectivamente. Metanol foi avaliado como co-substrato na degradação anaeróbia PHWW da *Spirulina* e sua adição na proporção de 1:1, resultou em maior produção de metano em relação em ensaios em batelada.

Palavras-chave: digestão anaeróbia; efluentes tóxicos; liquefação hidrotermal; microalgas; metano.

ABSTRACT

BUENO, B. E. ***Continuous treatment of aqueous phase of hydrothermal liquefaction of Spirulina in horizontal fixed bed anaerobic reactor using biostimulated sludge.*** 2020. 133 p. PhD. Thesis – Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2020.

Hydrothermal liquefaction is a process that converts wet biomass into biofuels, more specifically bio-crude oil. Nevertheless, along with this process, a wastewater is generated rich in nutrients and organic matter content, however presenting large amount of toxic compounds. Anaerobic digestion is a promising option for post-hydrothermal liquefaction wastewater (PHWW) treatment. In this study, the anaerobic digestion of PHWW from Spirulina, was evaluate using biostimulated sludge as strategy to optimize the process. The biostimulation was conducted in a sequential batch reactor fed with an organic acids solutions and methanol aiming the development of acetogenic and methanogenic microorganisms. Two PHWW anaerobic biodegradability assays were performed, one with biostimulated sludge and another with non-biostimulated sludge. Biostimulated sludge was able to reach higher methane yields at higher organic matter concentrations (11 and 16 g $COD.L^{-1}$) in relation to the non-biostimulated sludge (7 g $COD.L^{-1}$) and presented a lower degree of inhibition under the inhibitory conditions tested. Therefore, the biostimulation was a key process to select and favor potential microorganisms involved in a specialized uptake of recalcitrant compounds, such as *Mesotoga* and *Methanomethylovorans* genera, which were fundamental to the bioconversion of PHWW. An interesting strategy for the treatment of toxic compounds is the use of biomass adhered to inert supports forming biofilms. In this work, the use of immobilized biomass was evaluated for the anaerobic degradation of PHWW in batch assays and continuous treatment. Small lag phase periods and volatile fatty acids balance showed the advantages of the use of biomass immobilized in polyurethane foam. Continuous treatment in a horizontal anaerobic immobilized biomass (HAIB) reactor reached chemical organic demand (COD) removal efficiencies from 40 to 69%, operating with 0,8 e 1,6 g COD.L. d^{-1}. A second HAIB reactor was operated for evaluating the benefits of using methanol as co-substrate in the anaerobic degradation PHWW from *Spirulina*. However, methanol did not present positive contribution for degrading the PHWW.

Key-words: anaerobic digestion; toxic effluent; hydrothermal liquefaction microalgae; methane.

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Abbreviations

B	Biostimulated sludge
BCO	Bio crude Oil
BMP	Bio-Methane Potential
BTEX	Benzene, Toluene, Ethylbenzene and Xylenes
COD	Chemical Oxygen Demand
HAIB	Horizontal Anaerobic with Immobilized Biomass
HAIB-R1	Continuous Reactor treating PHWW
HAIB-R2	Continuous Reactor treating PHWW and methanol
HTL	Hydrothermal Liquefaction
LCFA	Long-Chain Fatty Acid'
MTBE	Methyl Tart-Butyl
NB	Non-biostimulated sludge
OC	Operational Condition
OLR	Organic Load Rates
SOLR	Specific Organic Load Rates
PBC	Polychlorinated Biphenyl-Contaminated
PCE	Tetra-Choro- Ethylene
PHWW	Post Hydrothermal Liquefaction Waste Water
SBR	Sequencing Batch Reactors
SMA	Specific Methanogenic Activity
TVS	Total Volatile Solids
TSS	Total Suspended Solids
UASB	Up flow Anaerobic Sludge Blanket
VFA	Volatile Fatty Acids

Summary

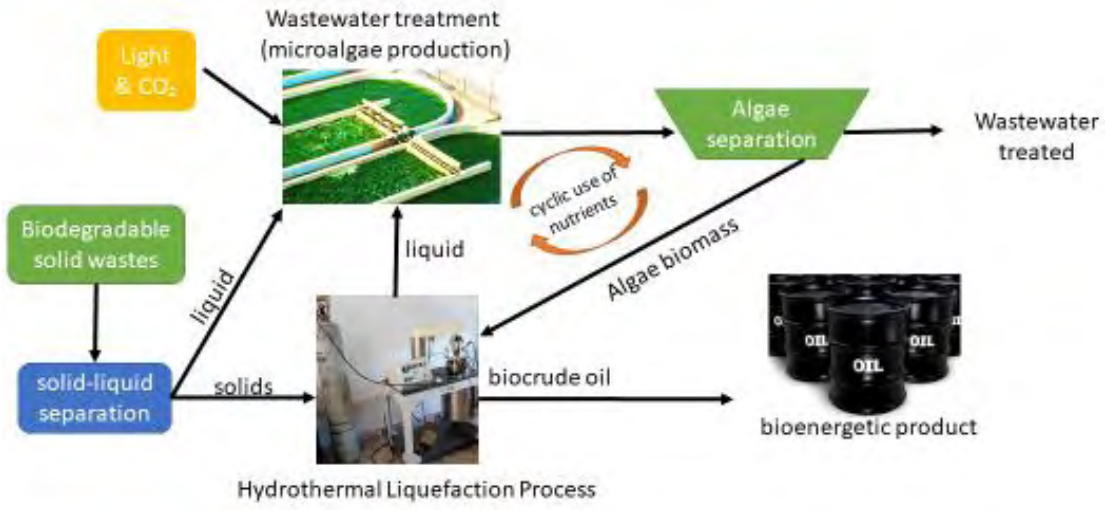
Chapter 1. Introduction

Since the launch of the concept of sustainable development in the late 1980s, industries, taking this into consideration, make efforts to minimize the effect of their waste on the environment by reducing the amount or reducing the toxicity in them. El-Halwagi (2012) defines sustainable design as: "the design activities that lead to economic growth, environmental protection, and social progress for the current generation without compromising the potential of future generations to have an ecosystem which meets their needs". Alternatives that allow industrial production with minimal environmental impact should be objective and the concern of every company, every government and the scientific community. In this context, the efficient treatment of industrial wastewater is fundamental to ensure the sustainability of industrial processes not only because of the removal of pollutant compounds before its disposal in nature, but also because of the generation of biofuels, energy and chemical compounds which are involved in the process.

In this sense that Zhang and Schideman (2012) proposed a process called Environment-Enhancing Energy (E2-Energy), presented in Figure 1, integrating infrastructure for waste treatment and bioenergy production, while also reducing carbon emissions, captured in algae cultivation. In this process, the biodegradable solid fraction of municipal, industrial and agricultural waste is concentrated and converted through the hydrothermal liquefaction (HTL) process into bio crude oil (BCO), also called young oil, as it has characteristics similar to oil and may be used as fuel. The aqueous phase from hydrothermal liquefaction (PHWW), containing most of the nutrients, is sent to a wastewater treatment system, carried out mainly by algae. Algae produced in such a system are also converted to BCO by HTL. The conversion process also releases in the aqueous phase most of the nutrients to another biomass growth cycle. This phenomenon

is called cyclic nutrient reuse, and makes it possible to transform waste management systems into bio refineries, considering algae as the main raw material.

Figure 1. Environment-Enhancing Energy (E2-Energy) process



Source: own authorship

According to Zhou et al. (2011), this approach has the potential to replace all US oil imports. However, one of the obstacles to the scaling-up of the E2-Energy process and the economic viability of hydrothermal liquefaction processes for oil production is directly related to the cyclic nutrient reuse stage. This occurs because, besides containing nutrients, PHWW has a large amount of organic matter and toxic components, limiting the growth of algae and impossible the disposal in the environment.

The present project aims to contribute precisely to the solution of this obstacle, integrating anaerobic digestion into the HTL process in order to decrease the organic matter concentration load, and maximizing energy production through generation of two

biofuels, bio crude oil and biogas, contributing to the generation of alternative fuels and reducing the negative impact of the process on the environment.

Chapter 2. Working hypotheses and proposed objectives

The conduct of this study was guided by the central hypothesis "**anaerobic reactor may be suitable for the continuous treatment of the aqueous phase of the hydrothermal liquefaction of *Spirulina*, decreasing the concentration of organic matter and maximizing the energy production of the process, through the generation of biogas**".

In this context, the main objective defined sought "**to study new alternatives for the treatment of Spirulina hydrothermal liquefaction effluent, contributing to the generation of alternative fuels, reducing negative impacts on the environment**".

Based on the contextualization presented in the introduction and on the theoretical framework, obtained through bibliographic research, which will be aborted in chapter 2, four sub-hypotheses of work related to a specific objective of the research were listed:

Sub-hypotheses 1: Solution consisted by volatile fatty acids (acetic, propionic and valeric) and methanol, would stimulate the development of microorganisms that would facilitate the anaerobic degradation of PHWW from *Spirulina*.

- specific objective 1: to analyze the microbial communities involved in the anaerobic degradation of PHWW from *Spirulina.*

Sub-hypotheses 2: Biomass immobilized on support material is beneficial for anaerobic degradation of PHWW from *Spirulina.*

- specific objective 2: to compare the use of suspended and immobilized biomass in polyurethane foam in the anaerobic degradation of PHWW from *Spirulina.*

Sub-hypotheses 3: Horizontal anaerobic with immobilized biomass reactor would be suitable for anaerobic degradation of PHWW of *Spirulina*.

- specific objective 3: to study the continuous anaerobic treatment of Spirulina PHWW.

Sub-hypotheses 4: The use of methanol as a co-substrate could be beneficial to the anaerobic degradation PHWW from *Spirulina*.

- specific objective 4: to test the use of co-substrate in the anaerobic degradation of PHWW from *Spirulina*.

Chapter 3. Literature Review

This chapter presents information consulted in the reference literature based on pertinent themes to the hypotheses and objectives proposed for the execution of this work. The selected themes were considered relevant to the context of the research, serving as a basis for comparing and discussing the results.

3.1. Petrochemical/Industrial wastewater

Chemical industries convert oil, natural gas, metals, and minerals into chemical products. Chemicals derived from petroleum or natural gas are known as petrochemicals which are typically extracted during the refining process as crude oil and natural gas liquids are cracked or distilled. These products are manufactured from naturally occurring deposits of oil and gas and involves the usage of substantial quantities of natural resources in the form of raw materials, solvents, water, and energy (CLEWS, 2016b).

Moreover, industrial activities development and higher productions have leaded to significant increase in generation of wastewater originated from industries (Lin *et al.* 2013). These wastewaters present a challenge for the conventional biological treatment methods because usually implying characteristics like high organic strength and extreme conditions like high toxicity, high salinity, and extremes pH values (VAN LIER *et al.* 2001).

Wastewater from petrochemicals and coal gasification industries are, specially, high polluter. The compounds present in these kind of wastewaters are mainly refractory, non-biodegradable and environmentally polluter, such as aromatics, hydrocarbons, acids, phenolic compounds, sulfides, etc (MACARIE, 2000; JI et al., 2016). These compounds are a challenge to be degrade for both aerobic and anaerobic treatment due

the benzene ring present in the aromatics compounds which provides stability and recalcitrance. In both biological treatments, bacteria need to destabilize the ring through chemical modifications and a terminal electron acceptor is required (LADINO-ORJUELA *et al.*, 2016).

Transforming these raw materials into useful products involves a highly complex and global industrial organisation, which is subject to a variety of environmental, political, legal and economic pressures. The petroleum industry value chain is the linked series of distinct but inter-related activities that transform crude oil and natural gas into valuable end-user products. As a result of the complex interaction among the different building blocks in the petrochemical industry and the need to reduce the associated usage of mass and energy resources and environmental impact, there is a need to develop and apply an approach to improving the design and operation, enhancing mass and energy efficiency, and mitigating negative impact on the environment (CLEWS, 2016a).

The impact of this industry on the environment is substantial, and for that reason, initiatives aimed the decrease in the use of non-renewable fuels by alternative processes that generate renewable fuels.

The hydrothermal liquefaction process is a promising alternative for reducing the consumption of non-renewable raw materials such as oil and it will be covered in the following topic.

3.2. Hydrothermal liquefaction

Hydrothermal Liquefaction (HTL) is a thermochemical process which a solid biodegradable raw material plus water are subjected to high temperatures (200-380ºC) and pressure (10-20 MPa) and then converted into bio crude-oil (BCO), also called young oil, which can be used as biofuel after refining process. At these conditions,

water becomes a highly reactive medium promoting the breakdown and cleavage of chemical bonds of the feedstock, allowing the rearrange of biological molecules into BCO. These conversion simulates the natural geological processes which produces our current fossil fuel reserves (TOOR, ROSENDAHL and RUDOLF, 2011; CHEN *et al.*, 2014). The BCO produced by HTL is a viscous dark liquid with high energy density and its energy content is equivalent to 70 to 95% of the petroleum crude oil (BROWN, DUAN and SAVAGE, 2010), reaching a higher heating value of 39 MJ/kg (ROBERTS *et al.*, 2013).

The HTL process is an emerging technology that can convert wet biomass to BCO, also generating gaseous, liquid and solid by-products (LÓPEZ BARREIRO *et al.*, 2013). This process allows the conversion of a wide range of feedstock as food waste (POSMANIK *et al.*, 2017), agriculture waste (SHEN *et al.*, 2016; ZHU *et al.*, 2017) swine manure (VARDON *et al.*, 2011; ZHOU *et al.*, 2015), algae and cyanobacteria (GAI *et al.*, 2015; TOMMASO *et al.*, 2015; ZHENG *et al.*, 2017; LI *et al.*, 2018; WANG *et al.*, 2018) etc.

HTL is a rapid reaction process in which wet raw materials can be used, avoiding the drying step. This is one of the main advantages of this process in relation to the other thermochemical processes since drying is a step that is known to be energy consuming and, consequently, increases the process costs (COSTA and DE MORAIS, 2011). Also there are no restrictions on the lipid content of feedstock used in the HTL. These characteristics make low-lipid algae and cyanobacteria raw materials especially suitable for obtaining BCO by HTL adding to their rapid growth and composition (TIAN *et al.*, 2014).

Due to their high water content (80-90%) the traditional thermochemical processes like pyrolysis and gasification are not economically suitable. The high water content of

microalgae adding to the lower energy requirements compared to other technologies turn the HTL to be a very promising technology for microalgae conversion (LÓPEZ BARREIRO *et al.*, 2013).

Low-lipid microalgae species such as *C. fritschii*, S. *obliquus*, *P. purpureum*, *P. cruentum* and *Spirulina* have higher growth rates and are more resistant to unfavorable conditions in comparison with high-lipid species. They also present higher BCO production yields because the HTL technique process the whole algae, thus the oil is produced also from the protein and carbohydrate fraction, not only from the lipid fraction (BILLER and ROSS, 2011; BILLER, RILEY and ROSS, 2011; VARDON *et al.*, 2011; LÓPEZ BARREIRO *et al.*, 2013; GAI, ZHANG, CHEN, ZHOU, *et al.*, 2015). Jena et al. (2011) has shown that up to 39.9% yield of bio crude could be produced from the TCL of microalgae *Spirulina plantensis*, and the bio crude obtained from HTL at 350–380 °C had fuel properties similar to that of petroleum crude and could be further refined to a liquid transportation fuel.

The energy balance for HTL process is still divergent between different authors depending on what is taken into account in the calculations (LÓPEZ BARREIRO *et al.*, 2013; ELLIOTT *et al.*, 2015; COUTO *et al.*, 2018). However, some authors report a positive net energy balance obtained by HTL using microalgae as feedstock (MINOWA *et al.*, 1995; JENA and DAS, 2011; ZHOU *et al.*, 2013). Temperature is one of the main critical points in increasing energy costs however. BILLER and ROSS (2011) reported that decreasing the liquefaction temperature from 350 to 300ºC reduced the energy consumption by 22% but only 3% in the bio crude oil yield. The non-need to dry the raw material and the nutrients recycling are the main factors that contribute to the positive energy balance (LÓPEZ BARREIRO *et al.*, 2013; ZHOU *et al.*, 2013). Thus,

not only the bio crude oil yield should be optimized, but also the possibility of recycling nutrients.

Besides the bio crude oil, the HTL process generate solid and liquid by-products. The solid by-product generated from the HTL of algae and cyanobacteria has a yield of about 10% (m/m) and has a high content of ash, sulfur and nitrogen, and little hydrogen. These solid residues can be used as feedstock in subsequent thermochemical processes, such as pyrolysis or gasification, in the production of other energy sources, while the remaining ashes can be reused as nutrients for microalgae growth. The gaseous by-product yields approximately 20% (m/m) of the organic matter present in the algae and carbon dioxide (CO_2) is the main gas obtained, corresponding to approximately 70% of the gas produced., followed by hydrogen gas (H_2), 30% (LÓPEZ BARREIRO *et al.*, 2013). The liquid by-product, called post hydrothermal liquefaction wastewater (PHWW), will be discussed with more emphasis in the following topic.

3.3. Post Hydrothermal Liquefaction Waste Water (PHWW)

One critical challenge of HTL process is its liquid by-product. PHWW has been proposed as a source of nutrients for the growth of microalgae in the cultivation of this raw material for use in HTL (BILLER *et al.*, 2012; TIAN *et al.*, 2014; ELLIOTT *et al.*, 2015; LENG *et al.*, 2018a). However, during the hydrothermal liquefaction process, the distribution of the organic components occurs between the crude oil produced and the PHWW. Thus, the same components found in crude oil are also observed in the PHWW, but at lower concentrations (ELLIOTT *et al.*, 2015). A wide variety of organic compounds can be found in the PHWW depends on the feedstock used (PHAM *et al.*, 2013). These compounds at high concentrations can cause inhibition of microalgae

growth (BILLER *et al.*, 2012; TIAN *et al.*, 2014; GAI, ZHANG, CHEN, ZHOU, *et al.*, 2015; LENG *et al.*, 2018b).

The treatment of the PHWW is the key to decrease the compounds concentration for obtaining sustainable production of a renewable fuel through HTL (ELLIOTT *et al.*, 2015). Due to the presence of high concentrations of organic matter in the PHWW and the large volumes generated, the treatment and reuse of this effluent are essential to obtain an economically viable HTL process. In Table 1 the composition of PHWW obtained from different feedstock is presented.

Table 1. PHWW Composition

Feedstok	Compounds	%	Reference
Rice straw	Alcohols	0.93	CHEN et al. (2017)
	Furans	24.94	
	Ketones	46.48	
	Acetic acid	2.9	
	Phenols	24.8	
Food waste	Furans	29.64	SI et al. (2018)
	Catechol	0.73	
	Ketones	5.68	
	Hydrocarbon-heterocyclic	6.67	
	N&O-heterocyclic compounds	1.8	
	Phenols	20.53	
	Benzene derivatives	1.3	
	Others	33.65	
Spirulina	N&O-heterocyclic compounds	45.8	ZHENG et al. (2017)
	Straight amides derivatives	13.2	
	Short chain organic acid (C2-C4)	2.37	
	Long chain organic acid (6 >C>4)	2.86	
	Benzene		
	Fatty acids	6.32	
	Amino acids	3.32	
	Phenol	0.4	
	Benzene derivatives	6.37	
	Others	19.36	

In addition to the feedstock, the conditions applied to the hydrothermal liquefaction process also influence the composition of the PHWW. Gai et al. (2015) investigated the characteristics of the PHWW coming from HTL of *Chlorella pyrenoidosa*, comparing different reactor operating conditions. Highest concentrations of organic acids were found in the optimized operating conditions for the production of crude oil (280 °C, 60 min, 35 wt% and 300 °C, 60 min, 25 wt.%), and higher concentrations of cyclic N & O-heterocyclic compounds were verified at the milder condition (260 ° C, 30 min, 35 wt.%).

In this sense, Tommaso et al. (2015) evaluated different retention (30, 60 and 90 minutes) times and temperatures (260, 280, 300 and 320°C) in the hydrothermal liquefaction process of microalgae grown in an effluent treatment plant. The authors verified that the effluent characteristics were directly related to the variations of the conditions applied in the hydrothermal liquefaction process. The auteurs evaluated the anaerobic degradation and, in the Bio-Methane Potential (BMP) tests performed with PHWW obtained at higher temperatures (300 and 320 ° C) and 60 minutes, higher methane production (13 and 16 mol $CH_4.gVSS^{-1}$) and higher periods of lag phase (110 and 175 hours) were obtained. When PHWW obtained at lower temperatures (260 and 280 ° C), lower methane production (8 and 9 mol $CH_4.gVSS^{-1}$) were obtained and no lag phase was observed.

On the other hand, Posmanik et al. (2017) performed anaerobic digestion of the PHWW of the HTL of food waste, rich in proteins, carbohydrates and lipids. Different combinations of these components were tested and it was concluded that the chemical composition of PHWW differs depending on the feedstock used and affects the anaerobic degradation in different ways. High temperatures (300 and 350 ° C) during

the HTL process favored oil production, but PHWW components caused less methane production. This was due to the possible formation of recalcitrant or inhibitory compounds, whose concentration is directly linked to the temperature used in the HTL process, feedstock composition and pH of the medium.

3.4. Anaerobic digestion of toxic/inhibitory compounds

Despite the reluctance against the biological treatment of industrial wastewater in the past (VAN LIER *et al.* 2015), this process presents an excellent opportunity to treat these waste streams (CHAN *et al.* 2009; SPEECE 1983). Anaerobic treatment, particularly in the high rate anaerobic process, has proven to be a technology suitable for the industrial wastewater treatment (DERELI *et al.* 2012).

Anaerobic digestion is considered the best option for the treatment of effluents with elevated concentrations of biodegradable organic matter. This is a biological process in which a microbial consortium degrades organic matter in the absence of molecular oxygen. In addition of being efficient in the removal of organic matter from effluents, one of the main benefits of anaerobic digesters is the biogas production.

Although it is a complex biological process in which many variables have significant effects on its performance, anaerobic biodigestion has been considered as the most energy efficient process of bioenergy production (HAMAWAND, 2015). About 70 to 90% of the biodegradable organic material present in an anaerobic system can be converted to biogas, which can be used as an alternative energy source in the facility where treatment of the waste is carried out (KAPDI *et al.*, 2004).

The anaerobic treatment is commonly applied for the treatment of sewage and wastewaters from the food and related industries. Only in the beginning of the 1970s the first studies on the anaerobic treatment of chemical and petrochemical industries wastewater started. This postulation was maybe because of the anaerobes, particularly

methanogens, were said too sensitive to deal with these type of wastewaters, supposedly highly toxic (MACARIE, 2000).

Speece (1996) defined toxicity, as an adverse effect (not necessarily lethal) on bacterial metabolism, and inhibition, as an impairment of bacterial function. Latter on, IWA (2005) defined biocidal inhibition as a reactive toxicity, normally irreversible e.g. LCFA, detergents, aldehydes, nitro-compounds, cyanide, azides, antibiotics and electrophiles; defined by Speece (1996) as toxicity, and biostatic inhibition as a nonreactive toxicity, normally reversible e.g. product inhibition, weak acid/base (including VFA, NH_3 and H_2S) inhibition, pH inhibition, cation inhibition, and anything else that disrupts homeostasis; which was defined by Speece (1996) as inhibition. Non-biological aromatics hydrocarbons are generated in effluents from fuel, chemical, plastic, pharmaceutical and others industries. The presence of benzene ring in aromatic compounds provides structural and chemical stabilities and also recalcitrance for some of these compounds (VOGT, KLEINSTEUBER and RICHNOW, 2011).

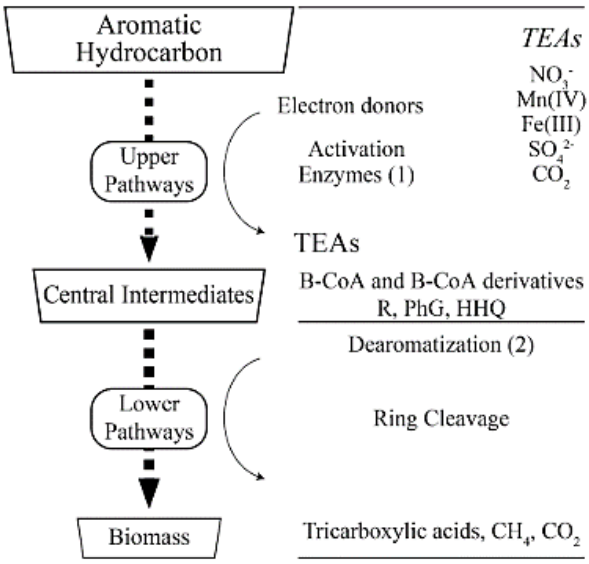
Organics compounds like phenol, cyanide and long chain alkenes, presented in industrial wastewaters, are the most difficult compounds for the anaerobic treatment because they are inhibitors of methanogenic archaea that are not able to break the benzene ring (CHAKRABORTY and VEERAMANI, 2006; HERNANDEZ and EDYVEAN, 2008). Besides that, while aromatic-degrading strains are specialized, like *Geobacte*r and *Syntrohorhabdus* that can degrade aromatics compounds like phenol (FUCHS *et al*., 2011; CHEN *et al*., 2017), the dominant bacterial strains are generalists and can use other compounds stand of aromatic hydrocarbon as carbon and energy sources (STAATS *et al*., 2011).

Microorganisms need to destabilize the benzene ring through reversible and irreversible chemical modifications to break this compounds down (DÍAZ *et al.*, 2013).

The initial conversion of aromatic compounds is done by cleavage of carbon-carbon from the benzene ring, decarboxylation, oxidation or reduction of substituent groups. Most of these reactions lead to lower aromatic compounds, like benzoate. Further reduction of benzoate requires high energy which can be overcome by ATP hydrolysis (BOLL, FUCHS and HEIDER, 2002). Aromatic rings without any functional groups face another problem. They have large resonance energy (0.0454 eV per *pi* electron for benzene ring), which provides great stability to the compounds. Anaerobic bacteria usually insert a carboxyl group from carbon dioxide or succinic acid to the poly-chain aromatic hydrocarbons (SEO, KEUM and LI, 2009).

In the anaerobic pathways of aromatic hydrocarbon biodegradation, a terminal electron acceptor (TEA) is required and it determines the energy balance and metabolic reaction used by the microorganisms (Figure 2). In the absence of oxygen, oxidized inorganic compounds, such as nitrate (NO_3^{-1}), manganese (Mn (IV)), iron (Fe (III)), sulfate (SO_4^{-2}) and carbon dioxide (CO_2)) act as electron terminal receptors (PHILIPP and SCHINK, 2012).

Figure 2. Anaerobic pathways of aromatic hydrocarbon biodegradation.



Source: Ladino-orjuela et al. (2016).

Under anaerobic conditions, the activation of the upper pathways is a reduction reaction catalyzed by enzymes: synthases, dehydrogenases and carboxylases. Currently, five ways of activating the upper pathways in anaerobic conditions are established: phosphorylation, fumarate insertion,) O_2-independent hydroxylation, carboxylation and methylation. In the case of phenol degradation, for example, it can be phosphorylated or carboxylated. The upper pathways are characterized by different metabolic pathways with several central intermediates, the most common being benzoyl-CoA. Phenol, for example, is carboxylated in the para-position yielding 4-hydroxybenoate and benzoyl-CoA is the intermediate of the degradation. The lower pathways begin with the disarmatisation of the central intermediates by reducing or oxidative reactions. The dearomatization of benzoyl-CoA is catalyzed by benzoyl-CoA reductase, and the cleavage of the ring is a reductive step of the lower pathways (GIBSON and HARWOOD, 2002; PHILIPP and SCHINK, 2012; LADINO-ORJUELA *et al.*, 2016).

According to Razo-Flores *et al.*(2003) major phenolic components in petrochemical effluents can be biodegraded simultaneously during anaerobic treatment.

3.5. Anaerobic digestion of PHWW

PHWW has a high concentration of organic matter and however, potential several fermentation inhibitors, such as cyclic furfural compounds, phenolic and nitrogen derivates, which makes it to be classified together with the wastewater from petrochemical refineries (APPLEFORD, 2005).

Anaerobic digestion of PHWW has been studied since 2011 (ZHOU, 2011), but results regarding the process are still scarce in the literature. Different feedstock has been tested and the main published studies regarding the anaerobic degradation of PHWW and the COD removal efficiencies as well, are presented in Table 2.

Table 2. Feedstock used for the HLT process, COD values of the PHWW obtained and the removal of COD by anaerobic digestion.

Feedstock	**PHWW COD (**$g.L^{-1}$)	**COD Removal (%)**	**Reference**
mixture of algae	30-50	44-61	TOMMASO et al. 2015
	75,2-87,3	-	FERNANDEZ et al. 2018
swine manure	104	45-55	ZHOU et al. 2016
Spirulina	90	31-43	ZHENG et al. 2017
	144	49-59	QUISPE-ARPASI et al. 2016
rice straw	20	-	CHEN et al. 2016
cornstalk	40-74	-	POSMANIK et al. 2017
food waste	76	67	SI et al. 2018

According to these existing studies, the PHWW anaerobic digestion can be conducted, however, low efficiencies of conversion of organic matter to methane have been obtained, added to the need for high rates of dilution and high reaction times. All studies pointed to the possibility of methane production digesting such effluent, however, it was verified high toxic potential of the material under anaerobic consortium.

Zheng et al. (2017) performed anaerobic toxicity test with effluents from the hydrothermal liquefaction of Spirulina, using acetate as reference. When PHWW additions ranged from 2 to 4%, the toxic effect was considered non-existent. The percentage of inhibition ranged from 13.7 to 99.1% when inclusion rates ranged from 5 to 24%. Complete inhibition occurred with an inclusion rate of 24 to 33%. It was verified, such as by Tommaso et al. (2015), the accumulation of valeric acid in the anaerobic digestion of PHWW from microalgae, indicating that the conversion of this acid may be a rate-limiting step. According to Pind et al. (2003), the accumulation of valeric acid is an indication of inhibition, because in the acetogenesis stage it can be

degraded into propionate and acetate, so if the valeric acid accumulation occurs, acetate may not be being generated.

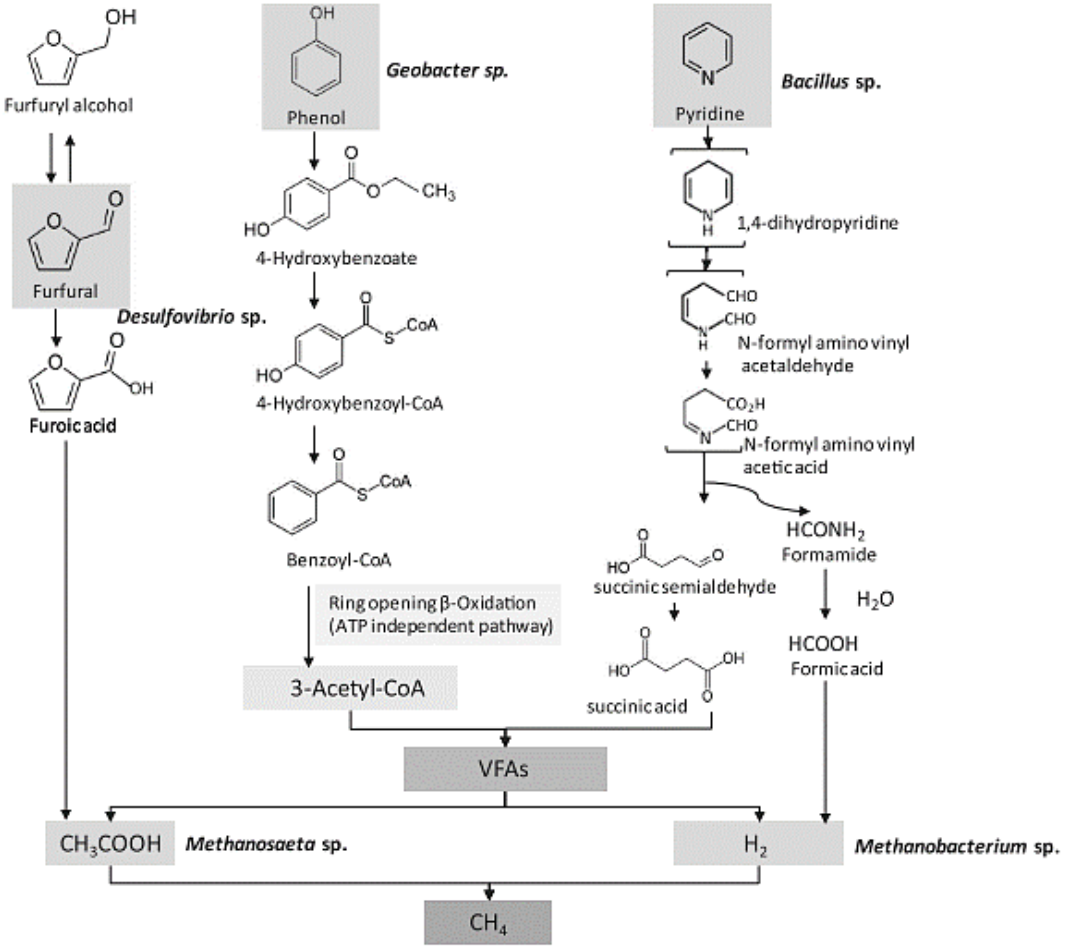
Phenols, N-heterocyclic compounds and furans are considered the main potential fermentation inhibitors in the PHWW. Si et al. (2018) studied the effect of the PHWW from cornstalk on the microbial communities. They found that the relative abundance of the family *Peptococcaceae* (VAN DER ZAAN *et al.*, 2012), which was reported as having a role in benzene degradation under denitrifying conditions, increase as well as the family *Pseudomonadaceae* (KIM *et al.*,2006; PADOLEY *et al.*, 2008), which has been reported to degrade N-heterocyclic compounds and phenols, and the family *Nitrospiraceae* (rhee et al. 1997), responsible for nitrification. Figure 3 shows the pathways and the involved microorganisms of anaerobic metabolism of these main fermentation inhibitors in the anaerobic degradation of PHWW.

According to Figure 3, *Desulfovibrio* sp. are capable of degrading furfural, which is first converted to furoic acid and then to acetate. In the anaerobic degradation of phenol, by *Geobacter* sp., benzoyl-coenzyme A, the central intermediate, is firstly produced, and the β-oxidation-like ring opening, and the reduction of the aromatic ring is performed in an ATP-independent pathway. Finally, three molecules of acetyl-CoA are produced and can be use by the acetogens and methanogens in sequence (FUCHS, BOLL and HEIDER, 2011). *Bacillus* sp., facultative anaerobes, can degrade N-heterocyclic compounds, like pyridine, by cleaving ring between C-2 and C-3 via a hypothetical 1,4-dihydropyridine intermediate releasing formamide and succinic semialdehyde. The formic and succinic acid were then produced, and the methane can be produced from succinic acid by acetogens and methanogens in sequent, whereas formic acid can be directly converted to methane (PADOLEY et al. 2008). Pyridine can

be also degraded by anaerobic N-heterocyclic-degraders like *Eubacterium hallii* and *Azoarcus* sp (Rhee *et al.*, 1997; Fekry *et al.*, 2016).

Regarding the archaea communities, *Methanosaetaceae* (acetoclastic methanogens) and *Methanobacteriaceae* (hydrogenotrophic methanogens) families were predominant (SI *et al.* 2018). The methanogenic archaea also play a critical syntrophic role for the degradation of inhibitors once the fermentation of such compounds it is always accompanies the production of hydrogen, which needs to be consumed to avoid the inhibition of the products (AQUINO and CHERNICHARO, 2005).

Figure 3. Pathways and the involved microorganisms of anaerobic metabolism of these main fermentation inhibitors in the anaerobic degradation of PHWW.



Source: Si *et al.* (2018).

3.6. Strategies to minimize toxic and inhibitory effects in the anaerobic microbial consortium

The toxic effect of a compound is directly related to the biological process conditions. It is also known that the microbial consortium can undergo changes in its population dynamics, adapting to the toxic compounds present in the environment. The decrease of the compounds in relation to the exposed sludge can significantly increase the efficiency of the anaerobic treatment (CHEN; CHENG; CREAMER, 2008).

Another interesting strategy for the treatment of toxic compounds is the use of sludge adhered to inert substrates forming microbial films or biofilms. When microorganisms are organized in biofilms, their interaction intensifies due to the optimization of their functions, which often complement each other. This situation makes the resistance of such organisms to toxic compounds increase greatly, which also occurs as a function of the protection afforded by extracellular polymers excreted for microbial adhesion (MAH and O'TOOLE, 2001; SUTHERLAND, 2001; KARADAG *et al.*, 2015).

Aiming improvement of the degradation process of specific compound, a co-substrate can be added due to nutrient balance and the synergistic effects of microorganisms (Siddique and Wahid, 2018). Some studies in the field of anaerobic digestion of recalcitrant wastewater described that the addition of an easily biodegradable compound as co-substrate can be positive for the process. (SIDDIQUE, SAKINAH ABD MUNAIM and ZULARISAM, 2014; YAJIE LI *et al.*, 2018).

The concepts of adaptation and enrichment of the microbial consortium as well as biofilm and co-degradation will be briefly discussed in the following topics.

3.6.1. Enrichment and adaptation of the microbial consortium

The effective functioning of biological treatment systems depends on highly active microorganisms that perform the process, and the relationship between resistant

microorganisms and sensitive microorganisms to the contaminant to be treated. According to Herrero and Stuckey (2015), microorganisms can degrade a wide variety of organic contaminants and can adapt to different hostile environments. The adaptation of the microbial consortium to anaerobic digestion is a factor that can influence the degree of inhibition of toxic compounds (CHEN, CHENG and CREAMER, 2008).

Adaptation is the result of internal changes in the predominant species or a change in the population of the consortium. The microbial consortium can undergo changes in its population dynamics, adapting to the toxic compound. Once adapted, microorganisms can maintain viability at concentrations well above the initial inhibitory concentrations (ANGELIDAKI and AHRING, 1992).

After a long- term adaptation the lag phase of biogas production can be reduced, and the anaerobic microbes should be able to break down many toxic compounds including a variety of polycyclic aromatic hydrocarbons and nitrogen heterocyclic compounds (CHAMPION, ZENGLER and RABUS, 1999; CARMONA *et al.*, 2009).

Zheng et al. (2017) treated PHWW from Spirulina by anaerobic digestion in batch assays and observed satisfactory results when adapting the microbial consortium to PHWW. The reactor flasks were first fed with 6.5 mL, representing 6.5% of the total useful volume of the reactor, for 90 days. After this period the organic load was increased to 13 mL of effluent and feed was carried out for 130 days, and the methane production increased without any sign of inhibition or stability.

However, selecting an adequate sludge as inoculum containing an adapted microbial consortium is a relevant factor to improve the removal of pollutants, such as phenolic compounds (FRANCHI *et al.*, 2018). On the other hand, poor performance of the bioreactor can occur due to the lack of a sufficient number of a specific

microorganism harboring a key metabolic pathway to degrade the toxic compound. Although different strains may perform the same functions or similar functions, it is more likely that toxic compounds may be degraded by a specific mixture of microorganisms cooperating synergistically. The importance of microbial consortia is such that, without their combination, many biodegradation processes could not be explained thermodynamically (HERRERO and STUCKEY, 2015).

Microbial degradation of contaminants can also be enhanced by biomass enrichment that is, by adding nutrients or electron receptors to activate the microbiota present in the reactor. The enrichment is carried out in order to stimulate the degradation of metabolite steps that can be limiting in the process. This strategy stimulates the biodegradation potential of the present microbiota however, it requires a natural potential of such microbial community to degrade the pollutant (RAVATN *et al.*, 1998; SALANITRO *et al*., 2000).

Enrichment is a process commonly used in bioremediation of contaminated soils, in the most of the cases, by petroleum derivates. Diesel oil bioremediation in soil, for example, can be promoted by stimulating of the indigenous microorganisms, by introducing nutrients and oxygen into the soil. The composition of the microbial population is affected by the environmental conditions and the composition of the hydrocarbons (BENTO *et al.*, 2005).

Haleyur et al. (2019) investigated the bioremediation of soils obtained from a former gas manufacturing site and contaminated with polycyclic aromatic hydrocarbons that were subjected to enrichment through the maintenance of soil aeration, temperature and moisture. The enrichment n is proposed as the best strategy to remediate polycyclic

aromatic hydrocarbons in aged and contaminated soils since a significant reduction in these compounds concentration was observed in all the treatments tested.

Yu et al. (2018) evaluated the effects of enrichment using different nitrogen sources on petroleum biodegradation of anaerobic consortium from marine sediments. They concluded that addition of nitrogen can promote growth of indigenous petroleum degradation-related bacteria and be helpful to the rapid degradation of petroleum.

Cervantes-González et al. (2019) evaluate the enrichment of microorganisms presented in two polychlorinated biphenyl-contaminated (PBC) aged soils using glucose, urea, and/or KH_2PO_4 until an optimum nutrient ratio of 100/10/1 for C/N/P. The biostimulation process was beneficial for its proliferation, achieving to 60% of PCB biodegradation.

Wenderoth et al. (2003) studied the effect of biostimulation in bacterial communities present in groundwater contaminated with dichlorobenzene and chlorobenzene using two different electron receptors, nitrate and oxygen. Biodegradation potential of microorganisms was observed both under aerobic conditions and under anoxic conditions. Biodegradation was accompanied by rapid and specific changes in the natural composition of the bacterial community. There was an increase in the bacterial growth rate and greater degradability of the pollutants after the addition of the electron receptors in relation to the control condition.

Although the mentioned works are not from the wastewater treatment field, the idea of enrichment can be applied in anaerobic digestion process. The anaerobic consortium can be stimulated by adding specifics substances that stimulate the growth of indigenous microorganisms capable of degrading a particular pollutant.

3.6.2. Use of immobilized biomass

Biofilm is a dynamic environment where microbial cells optimally are organized to use all available nutrients in the environment (SUTHERLAND, 2001). The microorganisms present in the films have more effective mechanisms of resistance to adverse situations than the free cells, being even more resistant to the exposure of toxic components, due to the presence of a wide microbial variety in several places within the matrix. These organisms may degrade different organic substrates simultaneously, promoting the stability of microorganisms that would not survive in suspended growth systems (STEWART *et al.*, 2001).

The structure of biofilms varies according to intrinsic factors, such as available microbial cells and their physiological activities, and environmental factors such as the pH of the medium and the available nutrients. Many physical properties of the biofilms may be related to their extracellular polymer matrices (SUTHERLAND, 2001). Wimpenny et al. (2000) related biofilms to different concentrations of available substrate. In anaerobic reactors for wastewater treatment, the substrate is in intermediate concentrations and the formed biofilms have channels in their upper part through which the liquid phase can pass, thus promoting a molecular flow with the nutrients present in the substrate. In addition, the cellular structure of the biofilm gives the microorganisms protection to the toxic components, keeping them separate from these substances due to their heterogeneity (MAH and O'TOOLE, 2001).

There are several possibilities of support materials for biofilm formation and the roughness, porosity and pore size affect the microbial colonization rate of the support. Polyurethanes foams have interesting characteristics such as density, particle size, high internal porosity, stability to hydrolysis and non-biodegradability (PASCIK, 1990).

Zheng et al. (2017) observed satisfactory results using immobilized biomass in polyurethane foams treating PHWW from *Spirulina*, compared to suspended biomass. In that work, when the effluent was degraded in batch tests, lower values of latency phase, higher value of methane production velocity and higher accumulated production were verified when these values were compared to those obtained using suspended sludge.

3.6.3. Co-substrate addition

The anaerobic co-digestion can be considered as the digestion of two or more substrate or co-substrate mixtures. Anaerobic digestion processes of a variety of substrates the can improve the process stabilisation due to nutrient balance, the synergistic effects of microorganisms and, consequently, improves the methane generation (SIDDIQUE and WAHID, 2018).

Co-metabolism is defined as the transformation of a non-growth substrate in the obligatory presence of a growth substrate or another biodegradable compound (DALTON and STIRLING, 1982). The energy derived from the biodegradation of recalcitrant is insufficient to support microbial growth and induce enzymes/cofactors involved in the biodegradation process. Therefore, the obligatory presence of a growth substrate is critically needed for the biodegradation of this kind of compounds. It can allow the initiating of a reaction to convert recalcitrant compounds to their intermediates (some of them can be toxic to some microbe, but are mineralized by others) that may be more biodegradable and would participate in the central metabolic pathways for the further biotransformation (ARP, YEAGER and HYMAN, 2001; TRAN *et al.*, 2013; OLIVEIRA, ZAIAT and OLIVEIRA, 2019).

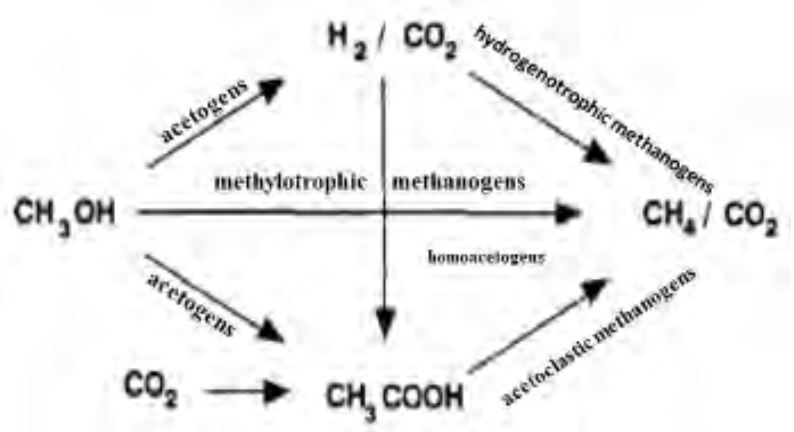
Some studies in the field of anaerobic digestion of recalcitrant wastewater described the addition of an easily biodegradable compound as co-substrate. Youngster

et al. (2008) studied the anaerobic methyl tert-butyl ether (MTBE) degradation using some different compounds, including toluene, benzene, ethanol and methanol. They concluded that the addition of these compounds as co-substrates increased the rate of MTBE degradation.

Qi Yang et al. (2008) investigated the biodegradation of tetrachloroethylene (PCE) using methanol as co-substrate. They found in this study that methanol is an electron donor suitable for PCE degradation and the co-metabolic electron donors are not limiting factors for PCE degradation, indeed when methanol was used as co-substrate most PCE was removed. Methanol was also used by Wang et al. (2010) to improve the biodegradability of coal gasification wastewater. Results indicated that anaerobic biodegradability of the coal gasification wastewater studied improved greatly upon the addition of 500 mg COD.L^{-1} methanol with the influent total COD 3500 mg. L^{-1} and phenol concentration 600 mg. L^{-1}.

Methanol is an easily biodegradable substrate that can be used by methanogens and acetogens. In the anaerobic degradation, methanol can be converted in methane by several pathways (FLORENCIO, FIELD and LETTINGA, 1995; PAULO *et al.*, 2003) (Figure 4).

Figure 4. Methanol conversion to methane pathways



Source: adapted from Florencio et al. (1993).

Methanol can be directly converted into methane by methylotrophic methanogens, like *Methanomethylovorans* and *Methanomassiliicoccus* (De BOK et al., 2006; CHA et al., 2013; KRÖNINGER, GOTTSCHLING and DEPPENMEIER, 2017) or be converted first into acetate by acetogens, and this acetate can be converted to methane by acetoclastic methanogens. Another possibility is the conversion of methanol to H_2 and CO_2 that can be converted to acetate by the homoacetogens, like *Sporomusa acidovorans* (CORD-RUWISCH and OLLIVIER, 1986), or to methane by the hydrogenotrophic methanogens, like *Methanolinea (*SAKAI et al., 2012; LI et al., 2018). According to Paulo et al. (2003), the anaerobic environmental conditions and history of the anaerobic consortium will determine the methanol degradation route and its final fate.

The co-substrate also can be only used during the start-up for sludge acclimation. Donlon et al., (1996) investigated the continuous anaerobic treatment of nitrophenols. The scale-lab reactors were initially started-up after a 15-day adaptation period to VFA. VFA was used as a substrate to provide the electrons for the reduction of nitrophenols. After this adaptation time period, the reactors were initially fed with sub-toxic concentrations of the nitrophenolic compounds added in the VFA substrate. They concluded that the addition organic compounds which more readily provide interspecies hydrogen (propionate, butyrate, ethanol) enhanced the production of reduced metabolites and led to a faster conversion of the nitroaromatic compound. In this sense, Razo-Flores et al., (2003) used only acetate during the star-up UASB reactor for mix phenolic treatment. After the phenolic adding, the acetate dosage was gradually reduced and afterward completely eliminated.

3.7. PHWW Continuous Treatment

Even though the anaerobic degradation of PHWW has been studied since 2011 (ZHOU, 2011), results regarding the continuous treatment of the PHWW are scarce in the literature. When this presented project was proposed, in 2016, there was still no published study reporting continuous anaerobic treatment. In 2018, Fernandez et al. (2018) evaluated the semi-continuous anaerobic digestion to degrade the organic fraction of wastewater streams from HTL of the algae (*Tetraselmis* and *Chlorella*) using clarified manure as co-substrate. Results indicated high methane yields at 20–30% (v/v) microalgae PHWW together with clarified manure. At the same year, an article was published reporting the continuous anaerobic digestion of PHWW from corn stalk via an up flow anaerobic sludge bed reactor (UASB) and packed bed reactor (PBR) fed with 8g. $(L.d)^{-1}$ of PHWW (SI *et al.*, (2018)). They obtained COD removal efficiency of 67.9% and 67.4% from UASB and PBR, respectively.

Normally, reactors with fixed biofilm have higher potential than sludge reactors because they can retain higher concentrations of sludge with higher metabolic activity, and are more resistant to toxicity (COHEN, 2001). Several reactor configurations with microbial consortia in biofilm form can be used in biological waste treatment systems, such as biological filters, fluidized and expanded bed reactors, and horizontal anaerobic with immobilized biomass reactor.

Studies have pointed to the horizontal anaerobic with immobilized biomass (HAIB) reactor as an excellent option for treating of wastewater containing toxic compounds, and this system was patented for the decontamination of gasoline-containing effluents (ZAIAT, 2003). Table 3 show some studies using the HAIB reactor that obtained positive results treating toxic compounds.

Aromatic compounds such as benzene, toluene, ethylbenzene and xylenes, collectively known as BTEX, are important contaminants present in petroleum products (SHIM and YANG, 1999; PRUDEN *et al.*, 2003) and can also be found in wastewater from hydrothermal liquefaction (PHAM *et al.*, 2013; CHEN *et al.*, 2014). The HAIB reactor was also successfully applied to treat water containing BTEX (DE NARDI *et al.*, 2002; RIBEIRO *et al.*, 2013) and as well as gasoline (DE NARDI *et al.*, 2005) (Table 3).

Table 3. Studies using the HAIB reactor for treating toxic compounds and the results obtained.

Substrate	**Results**	**Reference**
gasoline	99% of COD removal	DE NARDI *et al.* (2005)
ethanol and aromatic compounds in the presence of sulphate	90% organic matter removal	CATTONY *et al.* (2005)
pentachlorophenol	98% COD removal and 97% of pentachlorophenol convertion	DAMIANOVIC *et al.* (2009)
BTEX	99% hydrocarbonates conversion	RIBEIRO *et al.* (2013)

In this sense, in the present study, the HAIB reactor is proposed for the continuous treatment of PHWW. This reactor configuration has not yet been tested to treat such effluent, since studies of the continuous treatment of PHWW is still a recent topic and published results are scarce in the literature, which makes this work one of the pioneers in the subject.

3.8. Final considerations

Studies regarding the anaerobic treatment of compounds found in industrial waste water will help to better understand the degradation process and the degrading performance of the anaerobic reactors. Thus, breakthroughs for the industrial wastewater treatment can be delivered presenting more viable options and reducing human negative impact in the environment. In addition, contributing to the treatment of algae HTL effluent using anaerobic digestion will facilitate the future implementation of this process for obtaining renewable and alternative fuels.

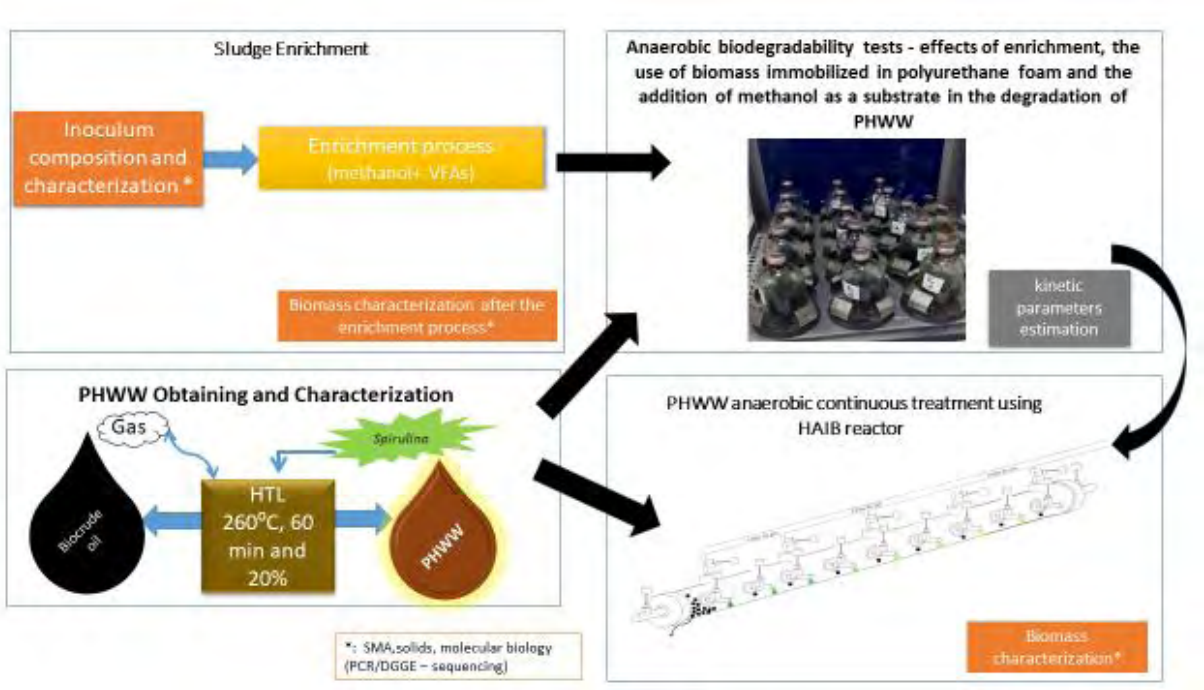
Chapter 4. Material and Methods

4.1. Experimental design

In this chapter, the material and methods used in this study will be described. Figure 5 presents the experimental design of this study.

First, the sludge enrichment, called in the study as biostimulation process, was carried out and its effect on the microbial population was analyzed. At the same time, the PHWW was being produced and characterized. Batch anaerobic biodegradability tests were performed to assess the effect of the enrichment, the use of immobilized biomass and the use of methanol as a co-substrate in the anaerobic degradation of PHWW. Finally, the study of continuous treatment in RAHLF was carried out.

Figure 5. Experimental design



Source: own authorship

4.2. Materials and methods

4.2.1. Inoculum and characterization

The inoculum used in the biostimulation reactor was composed of anaerobic granular sludge from three UASB reactors treating corn processing wastewater (Ingredion Brasil – Ingredients Industrials Ltda – Mogi Mirim, SP), wastewater from a poultry slaughterhouse (Dacar, Ltda – Mogi Mirim, SP) and domestic sewage (ETE Laranja Azeda- Pirassununga, SP), in a ratio of 2:2:1 in terms of volume (v/v), respectively. The tree different sludge was mixed in order to obtain a greater diversity of microorganisms' population. Specific Methanogenic Activity (SMA) assays were carried out according to Aquino et al. (2007) and solid analysis were performed based on the method 2540 from Standard Methods for Examination of Water and Wastewater (APHA, 2017) (Table 4). The sludge had a similar concentration of solids and specific methanogenic activity. The mixture of the three microbial consortia decreased the speed of specific methanogenic production, which may be related to the period of adaptation in which the three populations could be going through together.

Table 4. Total volatile solids and specific methanogenic activity for each sludge used

Sludge	**TVS ($g.L^{-1}$)**	**SMA (mmol CH4. (g TVS.h)$^{-1}$)**
Dacar	55	0.13
ETE	64	0.11
Ingredion	69	0.13
Mixed	66	0.07

In the continuous reactor and batch testes using immobilized biomass, the biostimulated biomass was immobilized in polyurethane foams according to the procedure described in Zaiat et al. (1994) after the biostimulation process.

4.2.2. Analysis of the microbial community

Optical microscopy analysis was performed to evaluate each sludge used and

the sludge mixture. The sludge samples were observed under an optical microscope at, under fluorescence contrast, for the visualization of methanogenic cultures that have the coenzyme F420. For these analyses, the sludge was fixed in slides with 1% agar. To analyze the diversity of the microbial community of the sludge before and after the biostimulation process, the technique of polymerase chain reaction and denaturant gradient gel electrophoresis (PCR/DGGE) was used.

The non-biostimulated and biostimulated sludge samples were washed in a phosphate buffer, and the total DNA was extracted using acid-washed glass beads (Sigma), followed by washing consecutively with phenol and chloroform (Griffiths et al., 2000). The extracted total DNA was purified using Ilustra GFX PCR DNA e Gel Band Purification (GE Healthcare) kit. In order to evaluate the DNA purity, DNA degradation and potential contamination as well as to quantify the DNA concentration, Nanodrop, agarose gel electrophoresis were performed. The indices of diversity, Shannon, and Domminance of the genomic libraries were analyzed.

For the in- depth microbiome characterization, the V3/V4 region of 16S rRNA gene of Bacteria and V4 region of 16S rRNA gene of Archaea were amplified using the 341F/806R (YU *et al.*, 2005) and U519F/806R (TURNER *et al.*, 1999) primers, respectively. The libraries were sequenced at the Genome dx (Rio de Janeiro, RJ, Brazil) by Illumina high-throughput sequencer with paired-end sequencing strategy, following the Illumina manufacturer's guidelines. Paired-end reads were merged using FLASH software (MAGOČ and SALZBERG, 2011), high-quality clean tags were obtained using QIIME (version 1.7.0) (CAPORASO *et al.*, 2010) and chimera sequences were identified and removed sing UCHIME algorithm (EDGAR *et al.*, 2011). The sequences with similarity higher or equal to 97% were assigned to the same OTUs using the Uparse software (EDGAR, 2013) and taxonomically classified using Green

Gene Database based on RDP (WANG *et al.*, 2007). The Chao1, Shannon, and Good-coverage indexes were calculated with QIIME (Version 1.7.0) and displayed with R software (Version 2.15.3).

4.2.3. Biostimulation process

The biostimulation was conducted in a 5L flask reactor operated in sequential batch in anaerobic conditions The reactor had 5L of total volume in which 3L corresponded of sludge and 2L corresponded of the substrate, the head space volume can be considered negligible, as shown in the Figure 6.

Figure 6. Biostimulation reactor

Source: Own authorship

The reactor was equipped with pipes to supply substrate and to exhaust biogas, and it sealed with rubber lid and silicon and the biogas pipe was kept submerged in a

water seal to ensure anaerobiosis. Two silicone tubes were installed at the top of the reactor one intended for the purge of gas and another intended for the feeding and removal of the system effluent. The tube destined for the gas outlet has been submerged in water to prevent oxygen inlet into the system. The tube from de left was the gas outlet and it was connected to a beaker with water to keep the system in anerobiosis. The tube from de right was used for the reactor substrate exchange. After the sludge sedimentation, the liquid phase was removed through the tube, with the aid of a syringe, by pressure difference. And the reverse way was made for feeding the reactor with new substrate. The process was conducted in a way so that no or little biomass was washed out of the system. When biomass loss occurred, it was reintroduced into the system along with the new substrate. The reactor was kept in room temperature which varied from 26 to 30 ºC, and 48h batches were used. The parameters applied in the biostimualtion reactor are presented in Table 5.

Table 5. Parameters applied in the biostimulation reactor

Parameters	Values
Substrate (g COD.L^{-1})	7
g COD added	21
Reactor (g COD.L^{-1})	4.2
Sludge (g TVS. L^{-1})	66
g TVS added	200
Reactor (g TVS.L^{-1})	40
gCOD.g^{-1}TVS	0.1
gCOD. (g TVS. d)$^{-1}$	0.05

Substrate composition is presented in Table 6. The substrate was exchanged every 48 hours by drainaging. In the first 80 days the substrate carbon source was composed by a solution of acetate, propionate, valerate and methanol (40:20:20:20) resulting in 5 g COD.L^{-1}. During this phase the applied organic loading rate was 0.025 g

COD. $(gTVS.d)^{-1}$.

Zheng et al. (2017), verified the accumulation of valeric acid in the anaerobic digestion of PHWW, indicating that the conversion of this acid may be a rate-limiting step. According to Pind et al. (2003), the accumulation of valeric is indicative of inhibition since it is degraded to propionate and acetate in the acetogenesis. For that reason, propionate and valerate were used to favor the growth of acetogenic microorganisms, while acetate and methanol were used with the intention of favor the propagation of methanogenic microorganisms.

Table 6. Substrate composition used in the biosmulation process.

Reagent		**Amount**
Acetic acid ($g.L^{-1}$)		1.5
Propionic acid ($g.L^{-1}$)		0.75
Valeric acid ($g.L^{-1}$)		0.75
Methanol ($g.L^{-1}$)		0.75
Whey powder ($g.L^{-1}$)		0.75
Sodium bicarbonate ($g.L^{-1}$)		0.75
Macronutrients solution ($mL.L^{-1}$)		1.8
NH_4Cl	17.0 g	
KH_2PO_4	3.7 g	
$CaCl_2.2H_2O$	8.0 g	
$MgSO_4.2H_2O$	9.0 g	
Ultrapure water	1000 mL	
Micronutrients solution ($mL.L^{-1}$)		1
$FeCl_2.6H_2O$	2.00 g	
H_3BO_3	0.05 g	
$ZnCl_2$	0.05 g	
$CuCl_2.2H_2O$	0.038 g	
$MnCl_2.4H_2O$	0.5 g	
$(NH_4)6Mo_7O_{24}.4H_2O$	0.05 g	
$AlC_{l3}.6H_2O$	0.09 g	
$CoCl_2.6H_2O$	2.00 g	
$NiCl_2.6H_2O$	0.092 g	
$Na_2SeO_3.5H_2O$	0.164 g	
EDTA	1 g	
Resazurina	0.2 $g.L^{-1}$	
HCl 37%	1 mL	
Ultrapure water	q.s.p 1000mL	

From the 81th day on, in order to provide a better C/N rate, dehydrated whey were added to the subtract, resulting in an addition of 2 g.L^{-1} of COD to the substrate. Therefore, the organic loading rate was increased to 0.043 g COD. (g TVS.d)$^{-1}$, and maintained at this value until the end of the experiment. Macro and micronutrients solutions were added according to Zehnder et al. (1980) and sodium bicarbonate was used to adjust pH value in 7.

Samples for the reactor monitoring were taken from the bulk liquid just after the feeding process and from the drained liquid. With those samples, COD and bicarbonate alkalinity were measured weekly. Specific methanogenic activity (SMA) assays (Aquino et al., 2007) were performed before and after the biostimulation process.

4.2.4. PHWW Generation

Spirulina was chosen as feedstock for the HTL process because it has higher growth rates and more resistance to unfavorable conditions in comparison with high-lipid species. *Spirulina* also present higher BCO production yields because the HTL technique process the whole algae, thus the oil is produced also from the protein and carbohydrate fraction, not only from the lipid fraction.

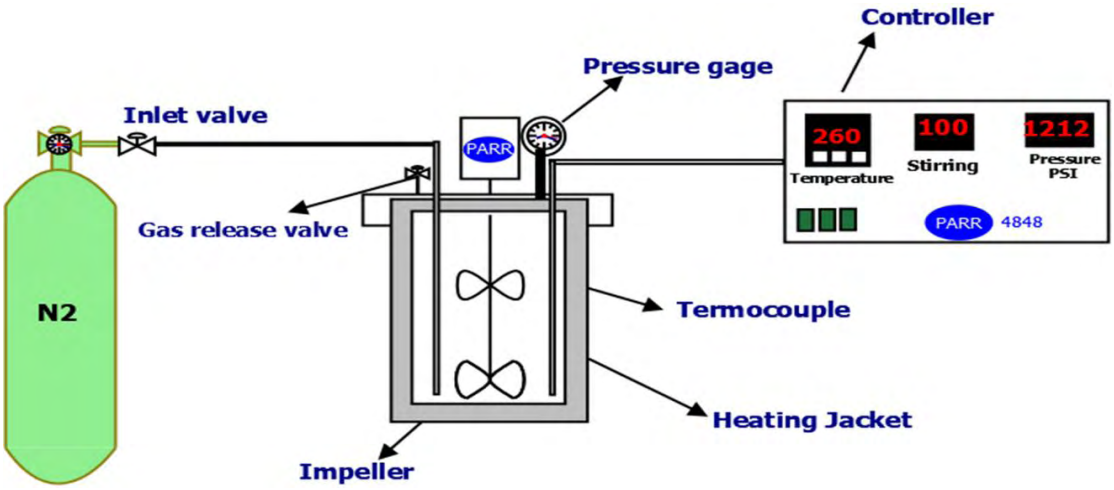
Two PHWW *Spirulina* from were used in this study. The first was performed in the Environmental Enhancing Energy Key Laboratory of the China Agricultural University. The HTL process was conducted based on Vardon et al. (2011) and Tang et al. (2016) in a stainless-steel reactor of 2 L capacity (Parr Instrument Co., Moline, USA), operated in batch mode under constant magnetic stirring. The reaction time was 60 min at 260 °C, with an initial solid content of 20% (w/w). This PHWW was used only in the first anaerobic biodegradability test, meanwhile the HTL process was still being implemented.

Once the HTL process was implemented at the Faculty of Zootechnics and Food Engineering (FZEA/USP - campus Pirassununga), the PHWW used in the other experiments was generated in a stainless-steel reactor of 7 L capacity (Figure 7), also based on Vardon et al., (2011) and Tang et al., (2016). The reactor was equipped with graphite gasket and magnetic stirring (Parr-4575 A) and operated in batch mode under constant magnetic stirring fed with 900 g of *Spirulina* and 4,5L of distillated water. The reaction time was 60 min at 260 °C, with an initial solid content of 20% (w/w) and the reactor was kept under nitrogen atmosphere and 1200 psi of pressure, according to the scheme presented in Figure 8.

Figure 7. HTL Reactor

Source: own authorship

Figure 8. Scheme of HTL process



Source: own authorship

4.2.5. PHWW characterization

The PHWW obtained was vacuum filtered with 0.45 μm pore membrane before being characterized. The characterization obtained are within the ones verified by Gai *et al.* (2015) and it is presented in the Table 7.

Table 7. PHWW characterization

Analysis	PHWW from E2-Energy laboratory	PHWW from FZEA-USP	Method
COD	162.1 ± 2.98 $g.L^{-1}$	120± 5 $g.L^{-1}$	APHA (2017)
Total Alkalinity	41.94 g $CaCO_3.L^{-1}$	25 g CaCO3.L^{-1}	RIPLEY et al.(1986)
HCO_3 Alkalinity	31.2 g $CaCO_3.L^{-1}$	20 g CaCO3.L^{-1}	RIPLEY et al.(1986)
Volatile Acidity	7.5 g $CH_3COOH.L^{-1}$	7 g CH3COOH.L^{-1}	RIPLEY et al.(1986)
pH	8.42	7.9	Potentiometer
Total Nitrogen	16.1 g $N.L^{-1}$	14.8 ± 1.2 g $N.L^{-1}$	APHA (2017)
Ammonia Nitrogen	8.9 ± 0.73 g N - $NH_4^+.L^{-1}$	7.8 ± 0.3 g N- $NH4^+.L^{-1}$	APHA (2017)
Total Phosphorus	0.76 ± 0.055 g P - $PO_4.L^{-1}$	0.33 ± 0.01 g P- PO4.L^{-1}	APHA (2017)
Total Solids	38.3 ± 0.141 $g.L^{-1}$	69 ± 0.45 g.L-1	APHA (2017)
Total Volatile Solids	33.6 ± 0.255 $g.L^{-1}$	52.2 ± 0.5 g.L-1	APHA (2017)
Total Fixed Solids	4.7 ± 0.113 $g.L^{-1}$	16.9 ± 0.25 g.L-1	APHA (2017)
Conductivity	35 $mS.cm^{-1}$	35.7 $mS.cm^{-1}$	Conductivity meter

The PHWW obtained in FZEA-USP were also analyzed in terms VFAs, aniline, phenol and other important target organic compounds by gas cromatograph (GC)

equipped with flame ionization detector (FID). For the preparation of the sample, 750 μL of the sample were transferred to a 1.5 mL vial and 750 μL of pentanol were added for dilution, and acted as an internal standard. 10 μL of formic acid (98%) were added for acidification. The prepared samples were analysed by a GC (Agilenttech 7890A, U.S.) with a capillary HP-FFAP column size of 25 m x 0.32 mm x 0.50 μm (Agilent 19091F-112, U.S.). The GC temperatures were maintained at 225°C for the detector and at 240°C for the injector. The FID was used with helium as carrier gas (pressure = 11 psi, flow rate = 2.45 mL $\cdot$ min^{-1}). The compounds measured (Table 8), corresponded only to 12 $g.L^{-1}$ of organic matter represented in COD, which correspond to only 10% of the total COD in the PHWW.

Table 8. Compounds concentrations found in the PHWW from *Spirulina.*

	$mg.L^{-1}$	$g.L^{-1}$	g $COD.L^{-1}$
Cyclohexanol	350	0,35	0,95
Cyclohexanon	560	0,56	1,46
Acetic acid	4039	4,04	4,44
Propionic acid	610	0,61	0,92
Isobutyric acid	106	0,11	0,19
Butyric acid	231	0,23	0,42
Isovaleric acid	358	0,36	0,75
Valeric acid	150	0,15	0,32
Aniline	127	0,13	0,31
Isocaproic	104	0,10	0,23
Caproic	106	0,11	0,23
Phenol	94	0,09	0,23
p-cresol	28	0,03	0,07
Benzoate	586	0,59	1,17
Total	**7450,6**	**7,5**	**11,7**

4.2.6. Anaerobic biodegradability batch assays

In this study, three anaerobic biodegradability batch assays were performed. The first to assess the sludge biostimulation effect on the methanogenic production and removal of organic matter from AD of PHWW. The second batch assay, aimed to evaluate the use of polyurethane foam as support material for immobilizing biomass, already biostimulated. And the third assay was carried out in order to evaluate the use of methanol as a co-substrate in PHWW AD. All the assays were carried in triplicates, using 120 mL flask reactors, kept in incubator at 37 °C and 150 rpm, under strict anaerobic conditions (Figure 9). After the addition of biomass and substrates, N_2 gas was flowed into the flasks during 2 minutes, and these were closed with rubber caps, which allowed the biogas to be collected and collected, and sealed with aluminum seals.

Figure 9. Anaerobic biodegradability batch assay at the incubator

Source: Own authorship

3.2.6.1. PHWW anaerobic biodegradation with sludge non-biostimulated and biostimulated

The aim of this experiment was to evaluate the effect of biostimulation of the anaerobic microbial consortium on the anaerobic degradability of PHWW. Anaerobic biodegradation batch assays were performed with non-biostimulated and biostimulated sludge. The process was evaluated in terms of methane production and COD removal efficiencies.

PHWW concentrations used were 1, 3.5 7.5, 10, 15 e 30% of the total working volume, corresponding to 2, 4, 7, 11, 16, 20 and 46 g $COD.L^{-1}$, respectively based on Zheng et al., (2017). Basal medium containing macro and micronutrients was prepared according to Angelidaki et al. (2009) and the pH was corrected to 7.5 using HCl solution 10%. Blank flasks were also assembled without any carbon source, to verify the endogenous methane production. Table 9 shows the composition of conditions applied to the assays with both sludge.

Table 9. Conditions applied to the anaerobic biodegradability assays

	blank	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**
Sludge (mL)	20	20	20	20	20	20	20	20
Basal medium (mL)	20	20	20	20	20	20	20	20
PHWW % (v/v)	0	1	3	5	7.5	10	15	30
PHWW (mL)	0	0.6	1.8	3	4.5	6	9	18
Deionized water (mL)	20	19.4	18.2	17	15.5	14	11	2
COD added (g)	-	0.1	0.3	0,5	0.7	1	1.45	3
COD initial ($g.L^{-1}$)	-	2	4	7	11	16	20	46
COD / VSS ratio	-	0.3	0.9	1.5	2	3	4	8
HCl solution 10% (mL)	0	0.3	0.4	0.6	0.8	1	1.3	1,8
Head Space (mL)	60	59.7	59.2	59.4	59.2	59	58.7	58.2
Total Work Volume (mL)	60	60.3	60.8	60.6	60.8	61	61.3	61.8

3.2.6.2. Anaerobic biodegradability batch assay: immobilized biomass evaluation

The anaerobic biodegradability assay was performed with granular biostimulated and biostimulated immobilized sludge. The objective of this assay was to evaluate the degradation of PHWW using immobilized sludge in polyurethane foams, evaluating the removal of organic matter, the methane production obtained, and intermediate metabolites. For that, fifteen bottles (120mL) were incubated with granular biostimulated sludge and others fifteen bottles with immobilized biostimulated sludge. Three flasks of the set were opened weekly to analyse their liquid phases in terms of COD and VFA quantification. In this sense was possible to calculate COD consumption rate over time and the VFA accumulation.

It was chosen to work with condition C4 from the previously batch assay, with PHWW volumetric inclusion of 7.5%, which had the higher initial PHWW concentration and no inhibitory behaviour. Due to the sludge immobilization and limited space for polyurethane foams, the concentration of solids could not be exactly the same under the conditions tested. Thus, the COD. $(g\ TVS)^{-1}$ ratio and the specific organic load rate were maintained for the two tested conditions and the others parameters from the immobilized sludge were calculated based on that. Three blank flasks (without PHWW) were also incubated for both conditions. Table 10. shows the details of this experiment.

Table 10. Composition of the anaerobic biodegradability batch assay testing immobilized sludge

	Granular Sludge	Immobilized Sludge
PHWW COD ($g.L^{-1}$)	120	120
COD added (g)	0.5	0.4
PHWW (mL)	4.5	3
Sludge (mL)	20	15
Foam (mL)	-	0.01
Basal medium (mL)	20	20
Deionized water (mL)	14.7	15
HCL 10% * (mL)	0.8	0.5
Head Space (mL)	60	66.5
Work Volume (mL)	60	53.5
TVS add (g)	1.33	1
COD/TVS	0.4	0.4
g COD. $gTVS^{-1}.d^{-1}$	0.01	0.01

3.2.6.3. Anaerobic biodegradability batch test: co-substrate addition evaluation

The goal of this experiment was evaluate the benefits of using methanol as co-substrate in the anaerobic degradation PHWW from *Spirulina.* This assay was performed with biostimulated and immobilized sludge in polyurethane foams to test the influence of methanol as a co-substrate in the methanogenic production. Three conditions were tested: without methanol (C1), the same organic load used in the C1 coming from just PHWW but with the methanol addition in the proportion of 1: 6 (C2), which correspond to 15% of COD inclusion from methanol and half the COD added coming from the PHWW and the other half coming from methanol (C3), which correspond to 50% of COD inclusion from methanol. Table 11 shows the parameters of each condition.

Table 11. Composition of the anaerobic biodegradability batch assay testing methanol as co-substrate.

	C1	C2	C3
PHWW-COD inclusion	100%	85%	50%
COD.L^{-1}	120	120	120
Volume added (mL)	4.5	4.5	2.25
COD added (g)	0.54	0.54	0.27
Methanol-COD inclusion	0%	15%	50%
Density g.L^{-1}	792	792	792
g COD. (g methanol)$^{-1}$	1.5	1.5	1.5
COD added (g)	0	0.081	0.27
Mass (g)	0	0.054	0.24
Volume (mL)	0	0.1	0.3
Total COD added	**0.54**	**0.62**	**0.54**
TVS add(g)	0.6	0.6	0.6
Foam quantity	80	80	80
g TVS. (foam)$^{-1}$	0.008	0.008	0.008
g COD. (g TVS)$^{-1}$	0.9	1.0	0.9
Sludge (mL)	20	20	20
Basal medium (mL)	20	20	20
Deionized water (mL)	14.7	15.7	167
g COD.L^{-1}	9.1	10.3	9.1
Head Space (mL)	60.8	59.7	60.8
Work Volume (mL)	59.2	60.3	59.3

4.2.7. Kinetics parameters estimation

The Gompertz model (equation 1) was adjusted to the values of methanogenic productions resulting from all the anaerobic biodegradability batch assays performed. From this adjustment it was possible to estimate the maximum methanogenic production rate (k) and the duration of the latency phase (λ) of the processes.

$$P_{CH_4}(t) = P_{CH_4} exp\left\{-exp\left[\frac{k.e}{P_{CH_4}}(\lambda - t) + 1\right]\right\}, \quad (1)$$

In this equation, $P_{CH_4}(t)$ corresponds to the methanogenic production over time t; and is exp (1), i.e. 2.71828. Once k values were obtained, it was divided by the volatile suspended solids (VSS) value added in each assay, to obtain the maximum specific methanogenic production rate ($NmLCH_4.g^{-1}VSS.h^{-1}$). The model was adjusted using

the Levemberg Marquardt method (Microsoft Origin 9.0). It is important to note that all the parameters estimated in this study are apparent, since no questions regarding the resistance to mass transfer were verified.

4.2.7. Anaerobic Continuous Treatment

For studying the anaerobic continuous treatment of PHWW from *Spirulina*, two Horizontal Anaerobic with Immobilized Biomass (HAIB) reactors were operated, one fed with only PHWW (HAIB-R1) as carbon and energy source and the other fed also with methanol as co-substrate (HAIB-R2). The reactors were built on glass bench scale, with a length of 100 cm and an internal diameter of 5 cm, resulting in a ratio of length to diameter (L / D) of 20. Figure 10 shows the reactor before the sludge immobilzation.

Figure 10. HAIB reactor before the sludge immobilization

Source: Own authorship

The total reactors volume was, approximately, 2 litters each. Along the length there were samplers for the collection of biogas and liquids. Polyurethane foam cubes with 0,5 cm of side were used as support material for the sludge immobilization, as described by Zaiat et al. (1994). The reactors were kept in a thermostatic chamber with a temperature of 37ºC. Figure 11 show the reactors in during the operation at the chamber. The substrates were kept in a refrigerator at 19ºC and heated to 35ºC by a water bath before entering the reactor.

Figure 11. HAIB Reactors configuration during the operation.

Source: Own authorship

The monitoring was based on analyses of COD, alkalinity to bicarbonate and methane production. The hydraulic retention time (HRT) chosen was 24 hours, respecting the limitations of the peristaltic pumps available, however, based on work done by Tommaso et al. (2003) and Tommaso et al. (2013). Table 12 summarizes the operational parameters applied in the HAIB reactors.

Table 12. Parameters applied in both HAIB reactors

Parameter	Value
Sludge TVS ($g.L^{-1}$)	66
Foam (g)	17
g TVS.$(g\ foam)^{-1}$	2.7
g TVS add	47
Q pump ($L.d^{-1}$)	0.8
HTD (h)	24
Reactor work volume (L)	0.8
PHWW ($g\ COD.L^{-1}$)	120

For the beginning of the reactors operation, it was decided to use specific organic load rate similar to that used in the batch test using immobilized sludge (0.01 g $COD.g^{-1}$ $TVS.d^{-1}$), in order to do not cause stress in the microbial consortium. When the sludge is immobilized in the foams and transferred to the reactor, the microorganisms may pass through a period of adaptation to the new condition. PHWW was the only source of COD. The dilution, volumetric and specific organic load rates applied in each operational condition, in both reactors, are presented in Table 13. Each organic load was applied to the reactor until the dynamic equilibrium state verified from the constancy in the organic matter removal efficiency and alkalinity production. Before the load increase, samples were taken along the reactor from collect points for substrate and metabolites concentration profiles determination.

Table 13. dilutions and specific organic load rates applied in each operational condition

Operational Condition	Dilution	VOLR ($gCOD.L^{-1}.d^{-1}$)	SOLR (g COD. g $TVS^{-1}.d^{-1}$)
OC1	146	0.8	0.014
OC2	73	1.6	0.028
OC3	36	3.2	0.041
OC4	146	0.8	0.014

The condition which the best performance was obtained in the batch assay testing methanol as co-substrate, C3, was chosen to be applied in the continuous treatment. Therefore, half of the COD supplied was from PHWW and the other half from methanol. Table 14 shows methanol and PHWW proportion used in the feed at each condition applied in HAIB-R2

Table 14 Methanol and PHWW proportion used in the feed at each condition applied.

	CO1	CO2
Feed concentration (g COD.L^{-1})	0.82	1.64
Total COD added (g)	0.66	1.28
Methanol-COD added (g)	0.33	0.64
Methanol density g.L^{-1}	792	792
gCOD.(g methanol)$^{-1}$	1.5	1.5
Methanol added in the feed (g)	0.22	0.44
Methanol volume (ml)	0.3	0.55
PHWW volume (ml)	2.75	5.5
Fresh water (mL)	1000	1000

4.2.8. Analytical methods

COD and solid analyses were performed based on the methods 5220 and 2540, respectively, from Standard Methods for Examination of Water and Wastewater (APHA -, 2017). COD removal efficiencies ($\mathcal{E}_{COD}$) were calculated relative to the initial concentration of soluble COD (C_i) and the final concentration of soluble COD (C_f), according to equation 2:

$$\mathcal{E}_{COD} = \frac{C_i + C_f}{C_i} \; x \; 100 \qquad (2)$$

The COD consumption rate (R_c) obtained in a certain period of time of the batch tests (t), were calculated according equation 3:

$$Rc = \frac{C_i - C_f}{t} \qquad (3)$$

Alkalinity to bicarbonate analyses were performed according to Ripley et al. (1986), and pH values were measured with a calibrated potentiometer.

The volatile fat acids produced during the anaerobic process were determined according to the methodology described by Adorno et al. (2014) on GC-2014 Shimadzu GC with FID, with HP INNOWAX 30m x 0.25mm (inner diameter) x 0.25μm (film thickness) column.

Biogas production was monitored by a pressure transducer model Datalogger GN200. CH_4 quantification was measured using a gas chromatograph equipped with a thermal conductivity detector (2014, Shimadzu, Japan). Gas chromatography used a 30 m Carboxen 1010 PLOT column with 0.53 mm inner diameter. The carrier gas was helium at a flow rate of 10 mL/min. The injector temperature was 100 °C. The column was initially held at 40 °C for 3 min, increased at a rate of 60 °C/min to a final temperature of 150 °C, and held for 1 min. Injection of standard containing CH4 and CO2 (50.032: 49.968) was performed periodically to obtain the conversion factor between area of the peaks and mass of methane present in the samples.

The methane production data expressed in NmL were obtained by converting the obtained area by chromatographic analysis to CH_4 mass (mol) by correlating these values with the areas of the standard. volume of The methane produced volume (V_{CH_4}) was calculated at normal conditions of pressure (P) and temperature (T) by Clapeyron equation 4,in which R is the perfect gases constant:

$$V_{CH_4} = \frac{n.R.T}{p} \qquad (4)$$

Methane yields (Y_{CH_4}) were calculated by equations 5, taking into account the produced volume of methane (V_{CH_4}), the initial COD concentration in each assay (*Ci*), and the work volume of the reactor flask (V_u):

$$Y_{CH_4} = \frac{V_{CH_4}}{C_i \cdot V_u} \quad (5)$$

4.2.9. Statistic

Data obtained in the biodegradability assay evaluating non-biostimulated and biostimulated sludge had the statistical significance determined by analysis of variance using Minitab (Version 18, Minitab, Inc., PA, USA). Comparison of means was performed using the Fisher's LSD test with a confidence level of 95%.

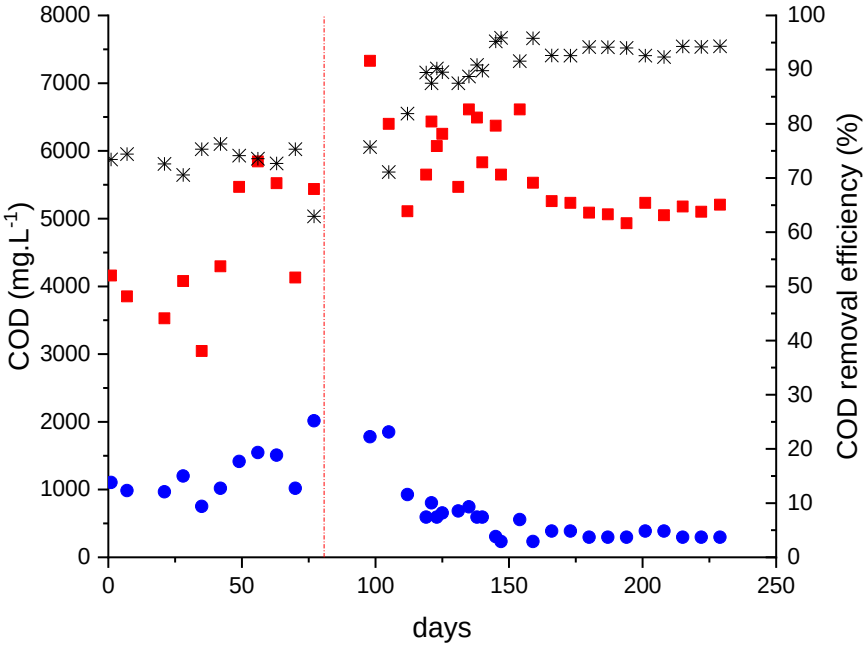
Data obtained in the biodegradability assay evaluating the methanol addition the statistical analysis of variance (ANOVA) was determined using Minitab (Version 18, Minitab, Inc., PA, USA). Comparison of means was performed using the Fisher's LSD test with a confidence level of 95%. And in order to verify if the differences found between the assays using granular and immobilized sludge were significant, and for the data obtained in the continuous reactors, Student's t-test was applied at 95% confidence level using Minitab (Version 18, Minitab, Inc., PA, USA).

Chapter 5. Results and discussion

5.1. Biostimulation Process

The biostimulation process was conducted for 230 days. COD and bicarbonate alkalinity analyses were performed to verify the organic matter removal efficiency of the reactor. The reactor was fed with a mixture of VFA and methanol (corresponding to 5g COD. L^{-1}). After 80 days, it was obtained 70% of COD removal, which represented, 3g COD.L^{-1} removed, as it can be seen in Figure 12. From the 80th day on, further 2 g COD. L^{-1} of COD derived from whey powder, were added according to Table 6 (section 4.2.3). After 150 days, the COD efficiency increased, reaching values higher than 90%, with an average removal of 5 g COD.L^{-1}.

Figure 12. COD removal efficiency of biostimulation reactor: ■ input; ● output, * removal efficiency, (---) whey powder addition.



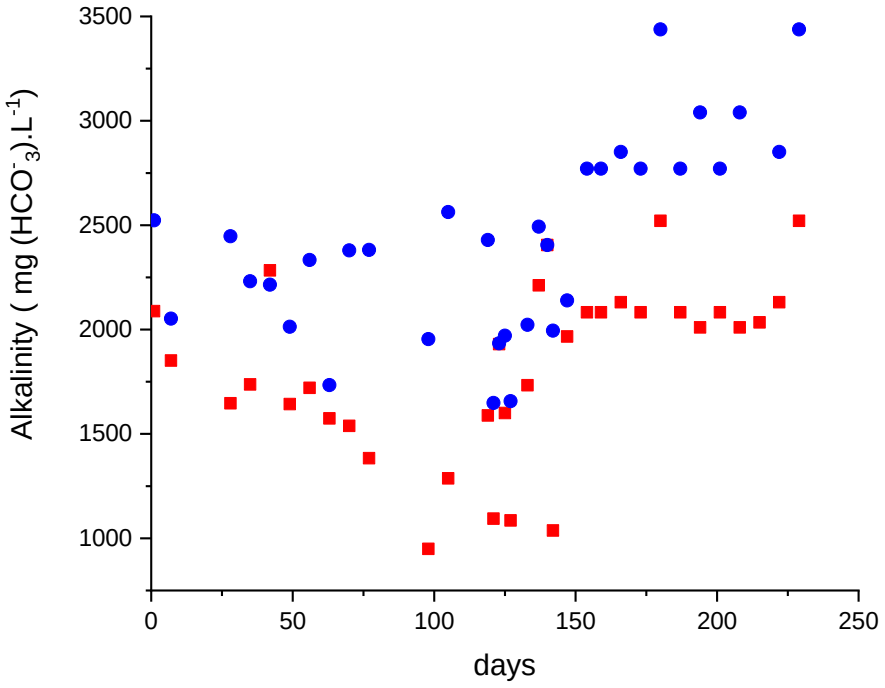
Source: Own authorship

The increase in the COD removal efficiencies after the addition of whey powder may be related to a better balance of the C/ N ratio since previously no source of

nitrogen was being supplied to the microbial consortium. It is believed that the initial stages of anaerobic digestion, such as hydrolysis, were favoured.

The reactor presented increased alkalinity in its effluent with respect to the initial condition, presenting behaviour within the normal one since the alkalinity increases in response to the acids that are produced and consumed throughout the anaerobic process. Figure 13 shows the results obtained in the bicarbonate alkalinity analyses over the time of biostimulation.

Figure 13. Bicarbonate alkalinity response along the biostimulation process: ■ inlet and ● outlet



Source: Own authorship

During the biostimulation reactor monitoring, the bicarbonate alkalinity increased in the output compared to the input, presenting behaviour within the normal since the alkalinity increases in response to the protein from the whey degradation,

generating NH_4^+ and to the acids consumption throughout the anaerobic process. The bicarbonate alkalinity values found were varying from 1600 to 3500 g $CaCO_3.L^{-1}$.

SMA tests were performed before and after the biostimulation process. The specific methanogenic activity increased from 1.6 to 2.2 mL CH4. (g TVS. h) $^{-1}$ after the biostimulation, which means that the biostimulation process has increased in 40% the sludge methane production potential.

5.2. Anaerobic biodegradability assays

5.2.1. PHWW anaerobic biodegradability

The PHWW anaerobic degradation assay was performed with the non-biostimulated sludge to verify the occurrence of inhibition, and with biostimulated sludge to verify if the biostimulation interfered in the methane production. Figure 14. shows total and net methane production (NmL) obtained throughout the assays.

Using non-biostimulated sludge (Fig.14 a), concentrations higher than 11 g $COD.L^{-1}$ (C4) presented total methane production lower than the production of the blank flasks, thus suggesting the inhibition of methanogenesis, even endogenous, under these conditions. The highest total methane production (222 ± 33 NmL) was achieved in the condition with initial organic matter concertation 7 g $COD.L^{-1}$ (C3). When the initial concentration was 4 g $COD.L^{-1}$ (C2) the total methane production was 164 ± 18 NmL, statistically equal to the obtained with 11 g $COD.L^{-1}$ (C4), 163 ± 51NmL.

Using biostimulated sludge (Fig. 14 b), C4 had the highest total methane production, 221 ± 33 NmL, 35% higher than the production obtained with non-biostimulated sludge. However, the production was statically equal to the production obtained at C3 (201 ± 30 NmL), in both assays, and at C5 using biostimulated sludge (219 ± 23 NmL). C5 presented total methane production five times higher when

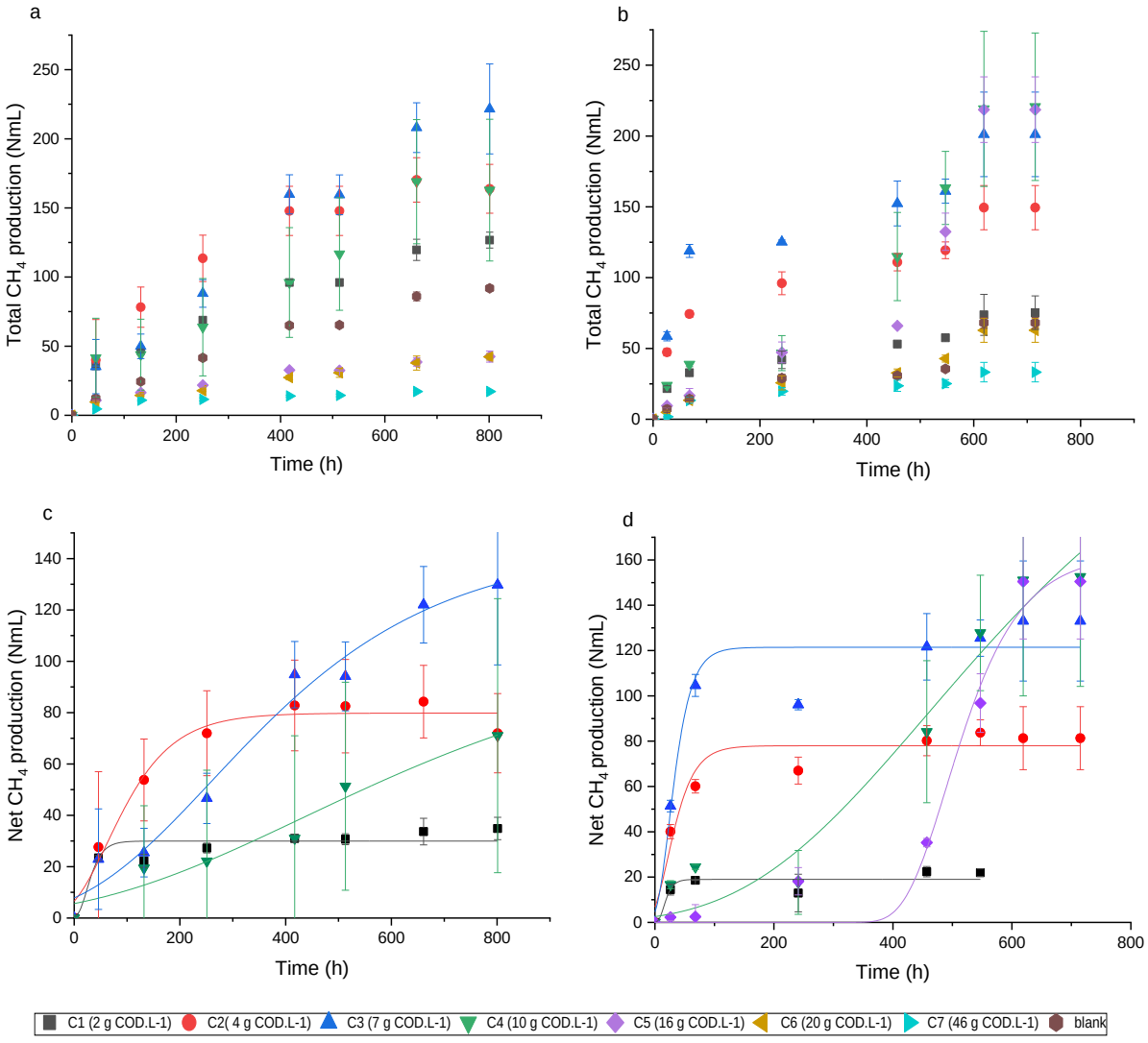
biostimulated sludge was used. Although the assays using the two higher PHWW concentrations, C6 and C7, still presented methane production similar to the blank, there was increase in their methanogenic production in relation to the obtained using non-biostimulated sludge, suggesting that the biostimulation process could potentially increase the methane production. The net methane production was obtained discounted the blank methane production.

Figure 14c and 14d present net methane production obtained using non-biostimulated from C1 to C4, and using biostimulated sludge from C1 to C5. In such figures is possible to observe the advantages of using biostimulated sludge.

Although for C1, C2 and C3 the use of biostimulated sludge did not show significant improvement, the opposite was observed in C4 and C5. C4 presented the double of net methane production obtained with non-biostimulated sludge. C5, which did not present net methane production using non-biostimulated sludge, with biostimulation presented one of the higher values of net methane production (150.5 ± 25.4 NmL) and statistically equal to the production obtained in C4 (152.6 ± 48.4 NmL).

Therefore, it is possible to state that biostimulated sludge was able to cope with higher PHWW concentrations, however, it did not bring benefits to the processes with initial concentrations higher than 16g $COD.L^{-1}$.

Figure 14. Total methane production over time of the anaerobic biodegradability test performed with non-biostimulated (a) and biostimulated (b) sludge and net methane production over time of the anaerobic biodegradability test performed with non-biostimulated (c) and biostimulated (d) sludge.



Source: Own authorship

Methane yields (Y_{CH4}) were calculated in relation to the initial COD applied and are presented in Table 15. When non-biostimulated sludge was used, the highest were observed in the conditions with lower PHWW concentrations, C1, C2 and C3 (289 ± 36, 298 ± 64 and 306 ± 74 $NmL.g^{-1}COD^{-1}$, respectively), since the methane production

decreased or stopped when initial concentrations where higher than 11 g COD.L^{-1} (C4, C5, C6 and C7). On the other hand, except for C1, all the values of Y_{CH4} obtained when biostimulated sludge was used similar or higher compared to the values obtained when non biostimulated sludge.

Table 15. Methane yield (Y_{CH4}) obtained in the anaerobic biodegradability tests, specific maximum methane production rate (k_{sp}) and lag phase (λ)obtained in the Gompertz adjust, and the COD removal efficiency obtained using non-biostimulated (NB) and biostimulated (B) sludge.

	Y_{CH_4} (mLCH_4. g^{-1} COD added)		k_{sp} (mL CH_4.(g VSS.h)$^{-1}$)		λ (h)		COD Removal Efficiency (%)	
	NB	B	NB	B	NB	B	NB	B
C1	289 ± 36^{a,b}	182 ± 14^{c,d}	1.6 ± 1.0^{c,d,e,f}	1.95 ± 0.4^{c,d}	26 ± 7^{c}	15 ± 2^{c}	42 ± 14^{a,b,c}	45 ± 5^{a,b}
C2	298 ± 64^{a,b}	337 ± 58^{a,b}	1.7 ± 0.03^{c,d,e}	3.25 ± 0.9^{b}	61 ± 29^{c}	22 ± 4^{c}	42 ± 8^{a,b,c}	56 ± 12^{a}
C3	306 ± 74^{a,b}	366 ± 73^{a}	0.7 ± 0.06^{e,f}	6.4 ±0.7^{a}	274 ± 63^{b}	23 ± 1^{c}	36 ± 2^{b,c}	32 ± 5^{b,c}
C4	106 ± 80^{d}	251 ± 80^{b,c}	0.6 ± 0.3^{f}	0.9 ± 0.5^{d,e,f}	415 ± 91^{a}	458 ± 55^{a}	26 ± 16^{c,d}	32 ± 3^{b,c}
C5	IP*	154 ± 26^{c,d}	IP*	2.3 ± 0.5^{b,c}	IP*	489 ± 12^{a}	28 ± 14^{c}	32 ± 6^{b,c}
C6	IP*	IP*	ND*	ND*	ND*	ND*	27 ± 4^{c,d}	56 ± 2^{a}
C7	IP*	IP*	ND*	ND*	ND*	ND*	8 ± 2^{e}	10 ± 6^{d,e}

Values are mean ± SD (n = 3).
Means followed by the same letter in columns or rows do not differ statistically (p>0.05)
*IP: insufficient methane production for the calculation.
*ND: not determined

Although there was no statistically significant improvement in conditions with initial COD lower than 11 g COD.L-1 (C1, C2 and C3), for conditions C4 and C5 significant higher yields were observed when biostimulated sludge were used. It is important to state, nonetheless that C4, which presented approximately the double of the using biostimaltion, did not present a significant difference between the C1, C2 and C3 from the assay using non-biostimulated sludge and from C2 from the assay using biostimulated sludge.

According to Watson et al. (2020), methane yields ranging from 14 to 314 mL.g^{-1} COD were observed in previous studies, and most studies reported a yield of around 200 mL.g^{-1} COD which corresponded to an energy recovery of 57%.

Zheng et al. (2017) treated PHWW derivate from Spirulina with approximately 90 g COD.L^{-1} obtained at 300ºC for 30 minutes using inoculum from an anaerobic reactor treating secondary sludge. At volumetric inclusion of 6.5%, which resulted in initial concertation of 6940 ± 399 mg. L^{-1} (similar to C3 from the present study), a methane yield of 216 ± 18 NmL.g^{-1} COD was observed.

Posmanik et al. (2017) studied the anaerobic degradability of PHWW from conversions of binary mixtures (1:1) and ternary mixture (1:1:1, w/w) of carbohydrates, proteins and lipids, using temperature ranging from 200 to 350 °C. Using inoculum from an UASB reactor treating brewery waste and initial COD of 2.5 g. L^{-1} (among C1 and C2) for the binary mixture composed by protein and lipids, with HTL conditions similar to the used in the present study, the authors found a yield of 250 mL.g^{-1}COD similar to the production obtained in C4 from the present study.

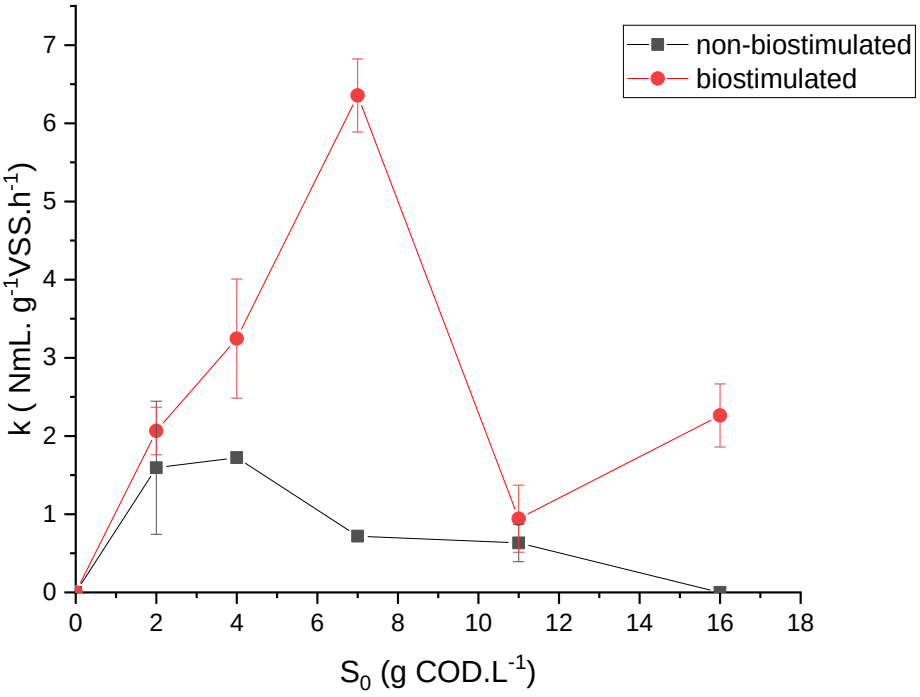
Chen et al. (2017) investigated the methane potentials of PHWW obtained from HTL of rice straw at different temperatures (170–320 °C), the initial COD value of all the anaerobic assays were controlled at 0.75 g. L^{-1}. The inoculum used in the study was obtained from a lab-scale biogas reactor treating sewage sludge. The highest methane yield (314 NmL CH4.g^{-1} COD) was obtained for the PHWW generated at 200°C however, using the PHWW obtained at 260°C, the same used in the present study, the obtained yield was 248 NmLCH4.g^{-1}COD.

Gompertz model was adjusted to the values of net methane production from the assays performed with both sludge and the parameters obtained from the adjustment are presented in Table 15.

Regarding the lag phase (λ), the effect of biostimulation can only be visible at C3, since the λ of this condition decreased significantly for the biostimulated sludge. C1, C2 and C4 did not presented significant difference from their peers using biostimulated sludge. In other hand, high specific maximum methane production rates were obtained with biostimulated sludge at all conditions. However, only at C2, C3 and C4, the increase was statistically significant.

Figure 15 shows how the specific maximum methane production rate (k_{sp}) vary with the initial substrate concentration. Using non-biostimulated sludge, the rate increases with increasing initial concentration until 4 g COD.L^{-1} (C2) and then decreased, reaching zero at 16 g COD.L^{-1} (C5), suggesting again the inhibition of the anaerobic process under the conditions tested with higher PHWW concentrations.

Figure 15. Maximum methane production rate (k_{sp}) varying with initial organic concentration (S_0) tested in the anaerobic biodegradability assay.



Source: Own authorship

COD determination were measured in the conditions before and after the anaerobic biodegradability test (Table 16). Analyzing COD removal, it was observed that the greatest COD removal efficiency occurred at the conditions C1, C2 and C3 (42%,42% and 36%, respectively) using non-bio-stimulated sludge, and conditions with C4, C5 and C6 obtained similar removal efficiency, around 27%, although they presented different behaviour in relation to the methanogenic production, since C4 was the condition that presented higher yields and C5 and C6 obtained production below the blank flasks production. Using biostimulated sludge, the greatest COD removal efficiency occurred at the conditions C2 and C6 which achieved COD removal efficiencies superior to 50%.

However, the positive effect of biostimulation on COD removal was only observed in condition C6, in which a significant difference was found in removal efficiencies. In the other conditions, no significant differences were observed. The condition with the highest concentration of PHWW (C7) obtained the lowest removal efficiency, below 10%, in both tests, which is consistent with its methanogenic production behaviour, where the yields were lower than the blank. The low efficiencies of COD removal found are in line with what was found by Zheng et al (2017).

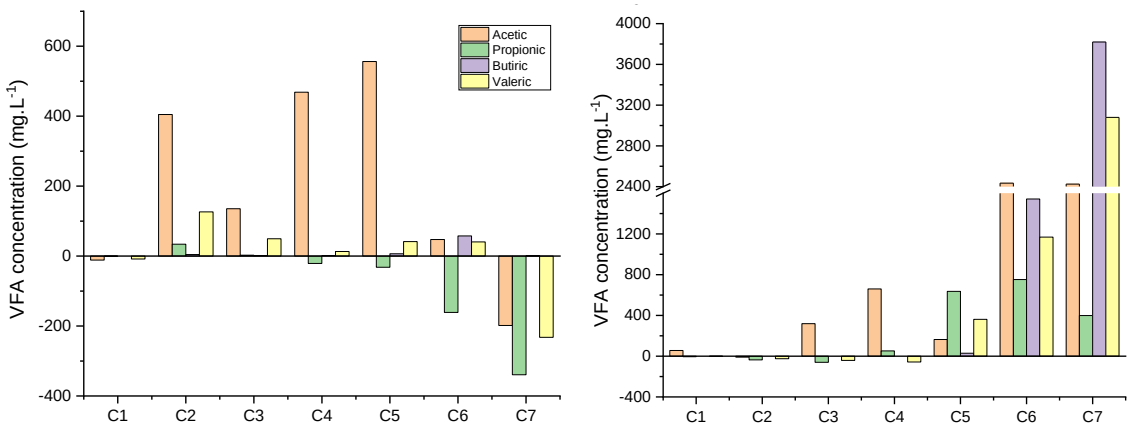
The COD consumption rates calculated were higher using biostimulated sludge at all conditions except to C3 (Table 16), which also agree with the COD removal efficiency (15).

Table 16. COD consumption rate (R_c) obtained in the biodegradability assay with non-biostimulated (NB) and biostimulated (B) sludge.

	R_c (g COD.L^{-1}.h^{-1})	
	NB	**B**
C1	0.0011	0.0012
C2	0.0021	0.0033
C3	0.0031	0.0028
C4	0.0036	0.0047
C5	0.0056	0.0072
C6	0.0070	0.0195
C7	0.0044	0.0068

Figure 16 presents de variation of the volatile fatty acids (VFA) after the anaerobic digestion for both conducted assays. For the assays performed using non-biostimulated sludge, it is possible to see that acetic acid had positive variation in conditions C2 to C6, with emphasis on the conditions C2, C4 and C5. C3 registered the lower variation, which is coincident to the higher value for Y_{CH4}. The final concentrations of acetic acid are in the range observed by Yang et al. (2018). Valeric acid had positive variation from C2 to C6, while propionic acid positive variation was observed only in C4. The valeric acid positive variation is in accordance to the observed by Zheng et al. (2017), being indicative of the process inhibition according to Pind et al. (2003). When biostimulated sludge was used, positive acetic acid variation was observed in from C3 to C7, but with higher values in C6 and C7, together with higher productions of propionic, butyric and valeric. It is possible to see that acidogenic production was favoured in higher concentrations when biostimulated sludge was used, probably due to the modifications in the biomass composition. In general, when biostimulated sludge was used, valeric acid presented lower positive variation inferring that the biostimulation favoured the acetogenic route of valeric acid degradation, seen by Zheng et al. (2017) as a possible limiting step of the anaerobic degradation of PHWW.

Figure 16.Volatile fatty acids variation during the anaerobic biodegradability assay using non-biostimulated (a) and biostimulated sludge (b).



a **b**

Source: Own authorship

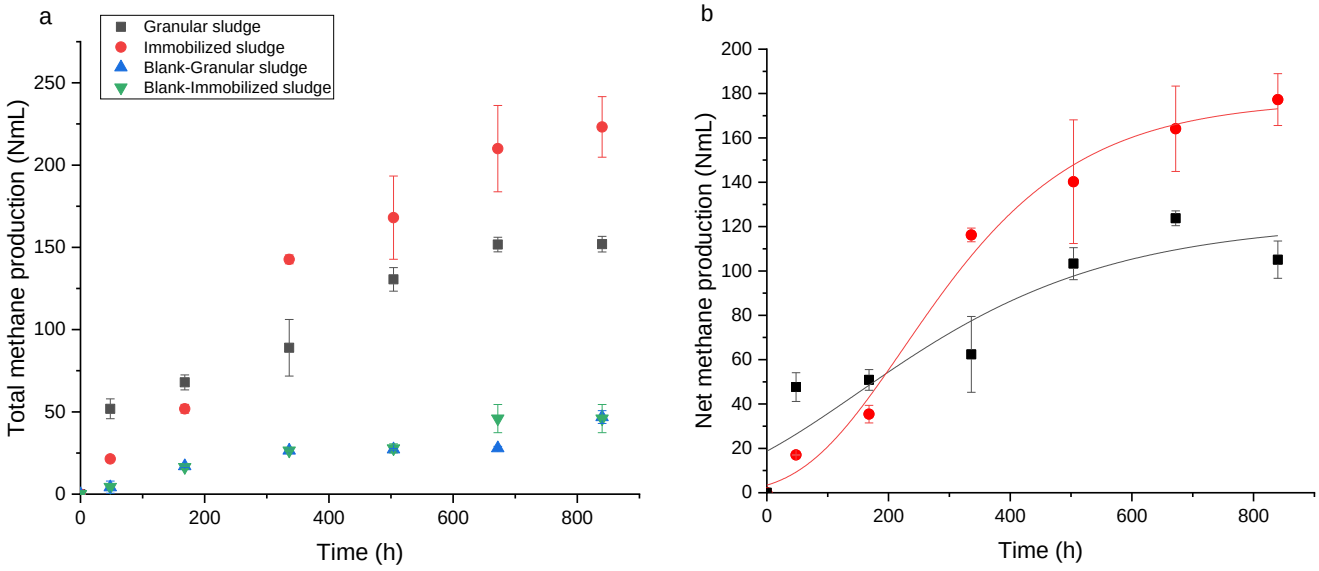
In the real field, biostimulation is widely used for bioremediation of several contaminants, including those from petroleum licks. According to da Silva and Maranho (2019), although mechanical and physical-chemical methods are widely used in remediation of petroleum contamination, biological remediation strategies, including biostimulation and bioaugmentation, are a promising alternative. The approach presents low cost, good social acceptance and minor environmental impacts when it is necessary to decontaminate large areas with diffuse contamination. In order to save resources, for PHWW degradation purposes, the biostimulation process could be made using the effluents from the first reactor of a two-phase anaerobic treatment system composed by a acidogenic reactor followed by a methanogenic reactor. Such reactors may present high biomass yield than methanogenic reactors and methanol could be obtained from such biomass gasification, as preconized by Kumabe et al. (2008).

5.2.2. Immobilized biomass evaluation

In the Figure 17 it is possible to observe the total methane production (a) and the net methane production (b) obtained from the PHWW anaerobic degradation over the 35 days (840 hours) at the two conditions, using immobilized and granular sludge. The total methane production reached using immobilized sludge was 223 NmL, and using granular sludge, 152 NmL.

The net CH_4 production showed the same behavior, the condition using immobilized sludge reached 177 NmL and, the condition using granular sludge reached 105NmL. Student's t-test confirmed that the difference between the CH_4 production of these two assays (suspended and immobilized biomass) was statistically significant ($p < 0.05$). According to Zheng et al. (2017), the polyurethane foam can temporally store nutrients and slowly release them to the microbes, which could contribute to a higher methane production.

Figure 17. Total (a) and net (b) methane production obtained in the biodegradability assay comparing granular and immobilized sludge in polyurethane foam.



Source: own authorship

The net CH4 production values were fitted by a modified Gompertz equation, using Levenberg–Marquardt method (Origin 9.0). The lag phase *(λ)* and maximum methane production rate (*ksp*) stimated are presented in Table 17. The kinects parameters seems to be greater for immobilizated sludge, which means that, besides the lag phase is higher, the specific maximum methane production is greater than using granular sludge. However, according to Student's t-test, the difference between both assays for kinetic parameters was not statistically significant ($p > 0.05$).

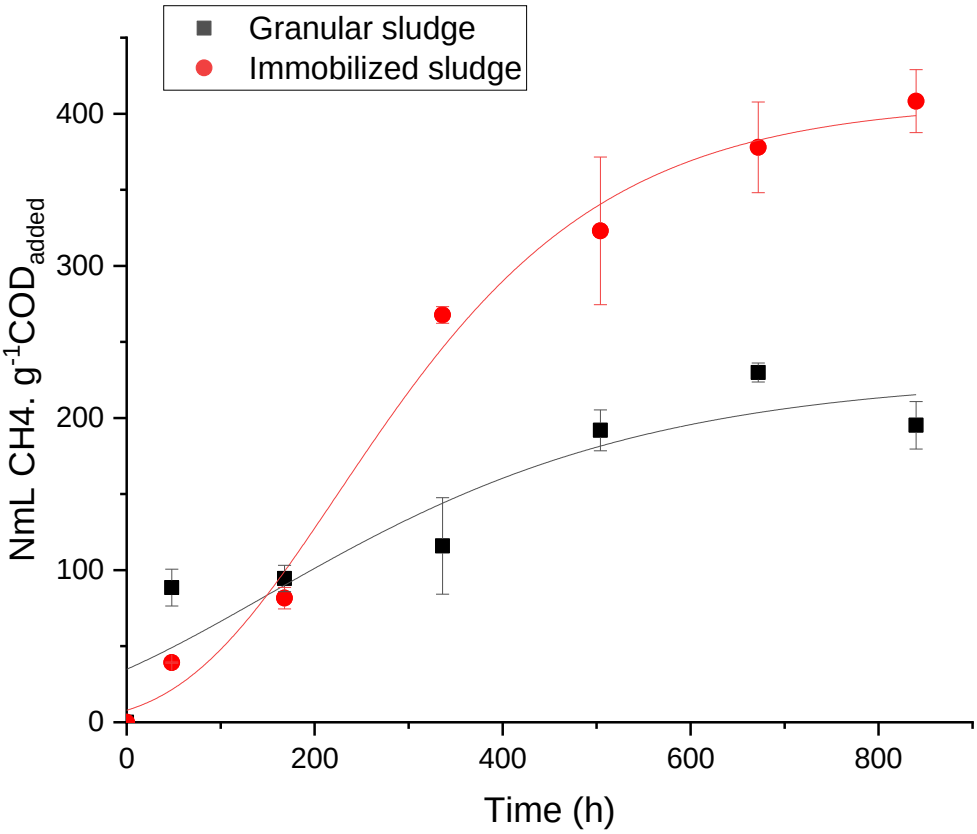
Table 17 also presented the methane yield in terms of COD added. Using immobilized sludge, the theoretical methane yield, 350mL.g^{-1}COD added, was achieved in, approximately, 500 hours, while using granular sludge, this yield value was never achieved (Figure 18).

Table 17. Methane yield and the kinetic parameters, lag phase (λ) and specific maximum methane production rate (k_{sp}) predicted by the modified Gompertz model for both assays.

Y_{CH_4} **(NmLCH$_4$. g^{-1} COD added)**		k_{sp} **(NmL CH$_4$.(g VSS.h)$^{-1}$)**		λ **(h)**	
Granular sludge	Immobilized sludge	Granular sludge	Immobilized sludge	Granular sludge	Immobilized sludge
195±15	408±21	0.48 ± 0.81	1.02 ± 0.2	154 ± 38	228 ± 9

Values are mean ± SD (n = 3).

Figure 18. methane yield obtained in batch tests using granular sludge and immobilized sludge

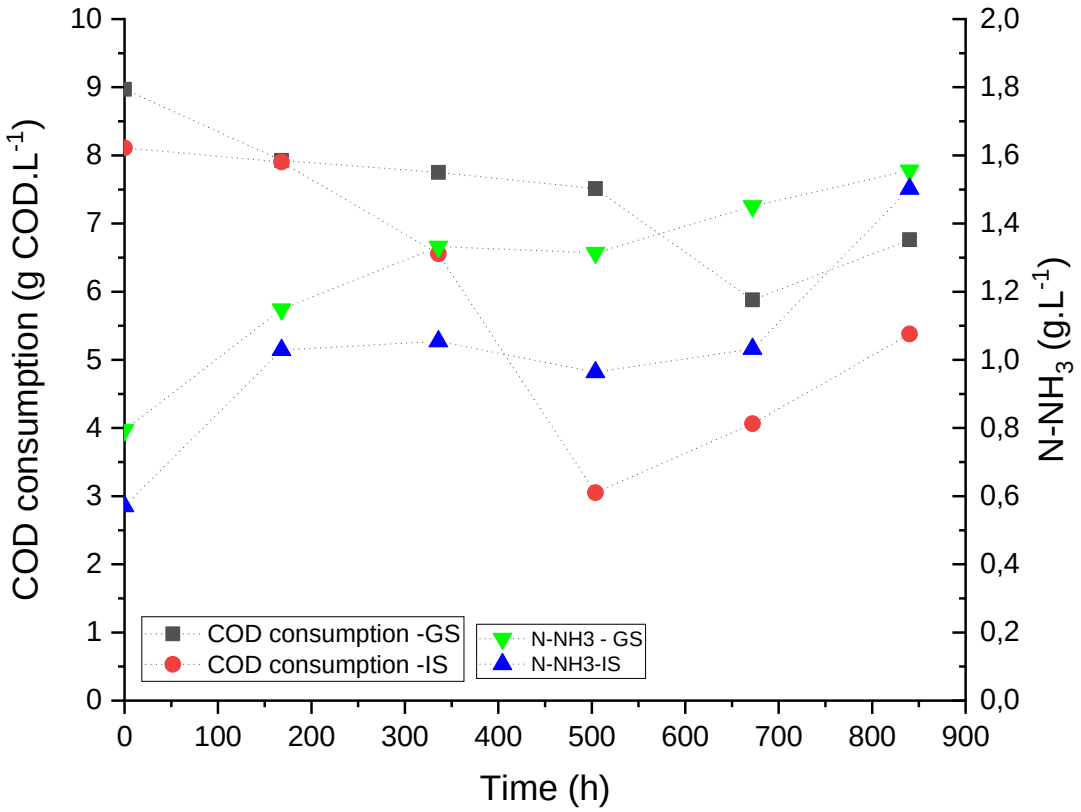


Source: own authorship

The COD removal and the ammoniacal nitrogen production along the time are shown in Figure 19. Using immobilized sludge, the concentration of ammoniacal nitrogen increased along the time specially up to 336 hours and after 504 hours. A similar trend, however delayed, was observed for granular sludge.

Using immobilized sludge, it was possible to verify the reduction of COD up to 504 hours (54.16±3.41% of COD removal) and then a great increase in the concentration of organic matter in the liquid medium. Similar behaviour happened using granular sludge, the reduction of COD happened up to 672 hours (39.64±1.53 % of COD removal) and then a great increase in the concentration of organic matter in the liquid medium.

Figure 19. COD removal and N-NH_3^+ production obtained with granular sludge (GS) and immobilized sludge (IS).

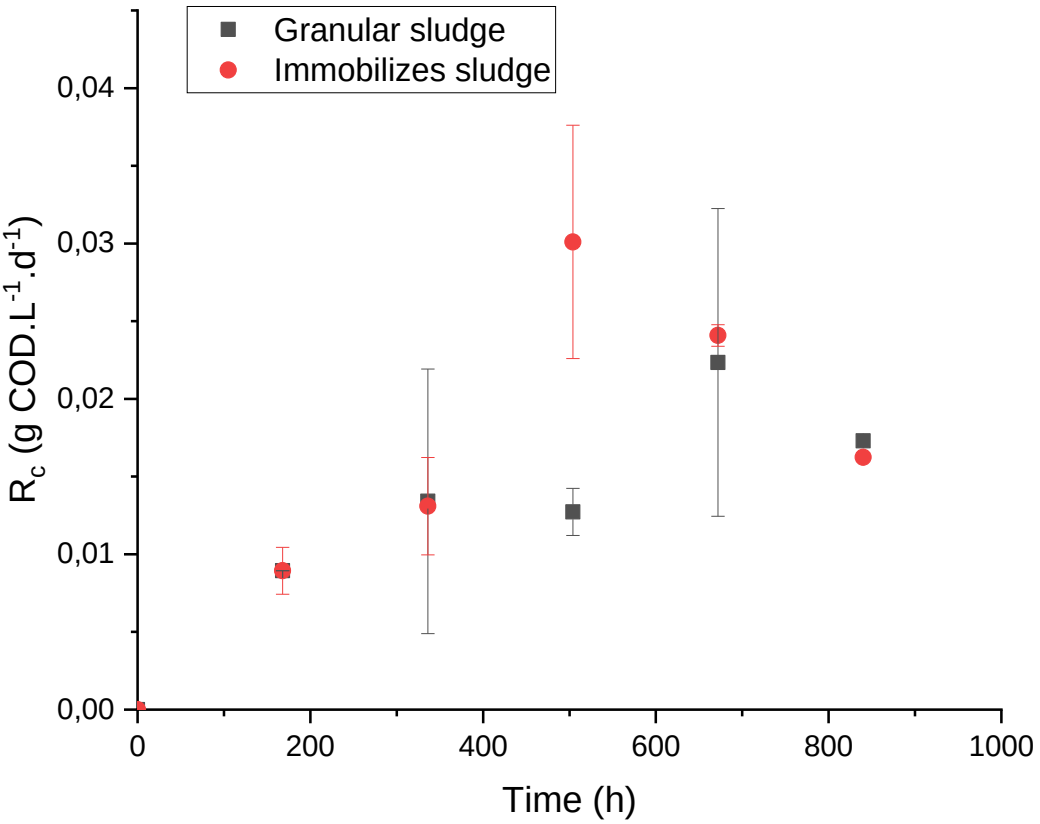


Source: own authorship

In this way, it is stimated that the removal of organic matter is only occurring up to half the time of the tests, and what has occurred after this period may be only the dissolution of the biomass because of cellular lise. The final COD removal efficiency achieved in the end of the assays was 30.72±2.43% and 33.66±0.78% using granular and immobilized sludge, respectively. According to Student's t-test the difference of final COD removal efficiency was not statistically significant ($p > 0.05$) for both assay.

The COD consumption rates for both assays were calculated (Figure 20). It possible to clearly see that the highest COD consumption happened at 504 h, using immobilized sludge (0.03±0.0075 g $COD.L^{-1}$. d^{-1}), and at 672 h using granular sludge (0.022±0,01 g $COD.L^{-1}$. d^{-1}).

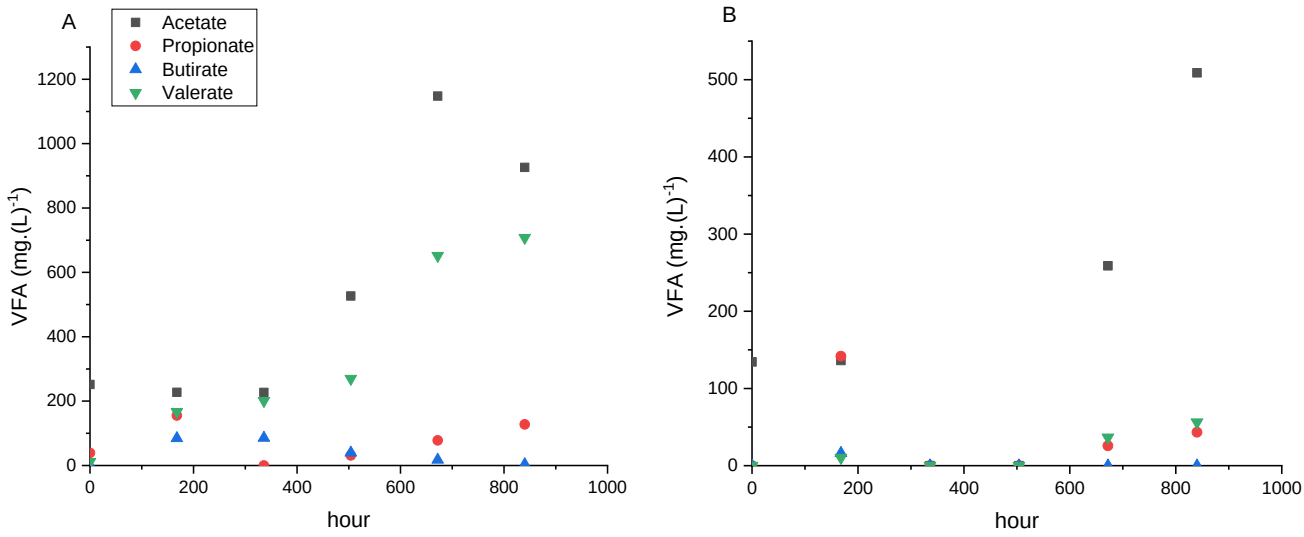
Figure 20. COD consumption rates along the assay comparing granular and immobilized sludge in polyurethane foams.



Source: own authorship

This assumption is confirmed in Figure 21, which shows the volatile acid profiles. In the tests with granular biomass (Figure 21A), diverse behaviour was verified. Acetate and valerate increasing along the time while butyrate and propionate have unstable behaviour. In the tests using immobilized sludge (Figure 21B), all compounds appear in lower concentrations, almost half of the concentration observed in the test with granular sludge. Reduction of each compounds is observed up to 504 hours, using immobilized sludge, and up to 672 using granular sludge, and then a very high production of acetic acid is observed.

Figure 21. VFA variation over time: granular sludge (A) and immobilized sludge (B).



Source: own authorship

However, even the highest acetate concentration achieved in the end of the test using immobilized sludge was half of the highest concentration obtained in the end of the test using granular sludge. The decrease in VFA concentrations, even in accumulation situations (as at the end of the test) is an indicative that the use of immobilized sludge was more efficient than granular sludge for anaerobic digestion of PHWW. The biomass immobilization in polyurethane foam facilitated the substrate diffusion, allowing microorganisms to have greater accessibility to the VFA, increased the consumption of these compounds.

Regarding the methane production and the COD consumption rate, the sludge immobilized in polyurethane foams has potential to the anaerobic degradation pf PHWW which is in line to the results obtained by Zheng et al. (2017).

5.2.3. Anaerobic biodegradability batch assay testing methanol as co-substrate using immobilized sludge

The PHWW anaerobic degradation was evaluated using immobilized sludge in polyurethane foams and 0, 15 and 50% of methanol COD inclusion. The condition with

50% of methanol COD inclusion (C3) achieved higher methane productions (Table 18). However, according to ANOVA, statistical difference was not observed ($p > 0.05$) for CH_4 production nor COD removal and COD consumption rates. It seems that the amount of methanol added was not enough to improve the methane production and the organic matter removal in the PHWW anaerobic degradation in those conditions.

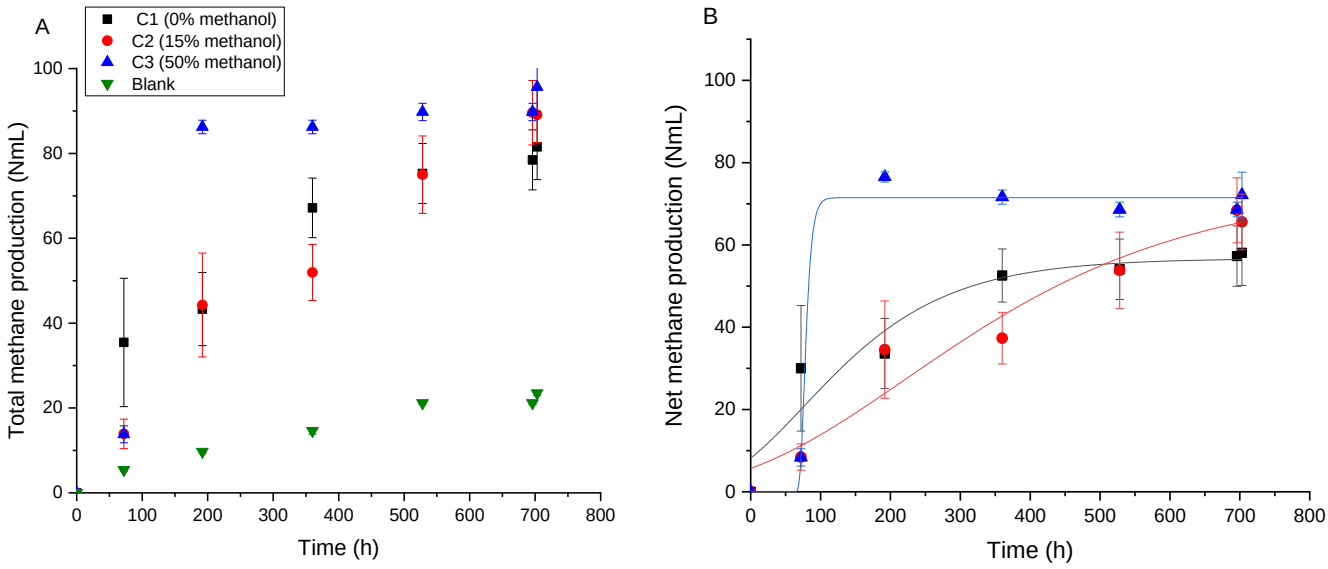
Table 18. Total e net methanogenic production obtained in the batch test evaluating the methanol addition as co-substrate and the COD removal obtained in each condition tested.

Condition	Methanol COD inclusion	Total CH_4 (NmL)	Net CH_4 (NmL)	Y_{CH4} ($NmL.g^{-1}COD_{add}$)	COD removal (%)	R_c
C1	**0%**	82±8	58 ± 8	146 ± 76	64 ± 3	0.0086±5E-04
C2	**15%**	89±7	66 ± 7	119 ± 12	59 ± 10	0.0075±4E-04
C3	**50%**	96±4	72 ± 6	124 ± 10	59 ± 3	0.0084±1E-03

Values are mean ± SD (n = 3).

In the Figure 22, the total (A) and net (B) methane production obtained by the conditions tested during the 30 days (700 hours) of the assay are presented. Although the methane production reached same amount, it seems that condition C3 presented lower lag phase and higher methane production rate.

Figure 22. Total (A) and net (B) methanogenic production obtained from the anaerobic biodegradability of PHWW with immobilized sludge in polyurethane foams and methanol addition.



Source: own authorship

The net CH_4 (NmL) values were fitted by a modified Gompertz equation, using Levenberg–Marquardt method (Origin 9.0), and, the lag phase (λ) and maximum methane production rate (ksp) were estimated (Table 19).

It was confirmed that condition C3 (50% of methanol COD) presented the lower lag phase than C2 (15% of methanol COD), however statistically equal to C1 (no methanol). Thus, the methanol inclusion did not affect the lag phase. However, regarding the specific maximum methane production rates, condition C3 presented the highest methane production rate and statistically differ from the other condition. It can be implying that 50% methanol COD inclusion improve the methane production rate.

Table 19 Kinetic parameters predicted by the modified Gompertz model to duration of the lag phase (λ), maximum methane production rate (*k*).

Condition	Methanol COD inclusion	λ (h)	k_{sp} ((NmL CH4.(g VSS.h)$^{-1}$)
C1	0%	83 ± 44^{b}	0.72 ± 0.79^{b}
C2	15%	254 ± 120^{a}	0.23 ± 0.11^{b}
C3	50%	83 ± 12^{b}	6.36 ± 4^{a}

Values are mean ± SD (n = 3).
Means followed by the same letter in columns do not differ statistically (p>0.05)

5.4. Anaerobic continuous treatment

The HAIB-R1 was operated for approximately 200 days. Table 20 presents the details about the volumetric and specific organic load rates applied in each operation condition (OC), the COD concentration in the influent and effluent and COD removal efficiencies.

Table 20 Operational conditions applied to the PHWW continuous treatment and COD removal efficiencies.

Operational Condition (OC)	**VOLR (gCOD.L^{-1}.d^{-1})**	**COD influent* (g.L^{-1})**	**COD effluent * (g.L^{-1})**	**COD removal* (%)**
1	0.8	0.84± 0.2	0.25± 0.01	69 ± 1.5
2	1.6	1.38 ± 0.13	0.58± 0.09	58 ± 6.7
3	3.2	2.5 ± 0.10	1.5± 0.26	40 ± 9.9
4	0.8	1.02 ± 0.15	0.49 ± 0.14	41 ± 12

* average obtained at system stability period or in the last 15 days of operation in that condition.

Figure 23 shows the influent and effluent COD concentrations and the COD removal efficiencies obtained at each condition tested in the HAIB-R1. In general, COD

removal efficiencies decreased when the applied organic load rate was increased. The COD removal values obtained from OC1 to OC3 were analyzed by Student's t test.

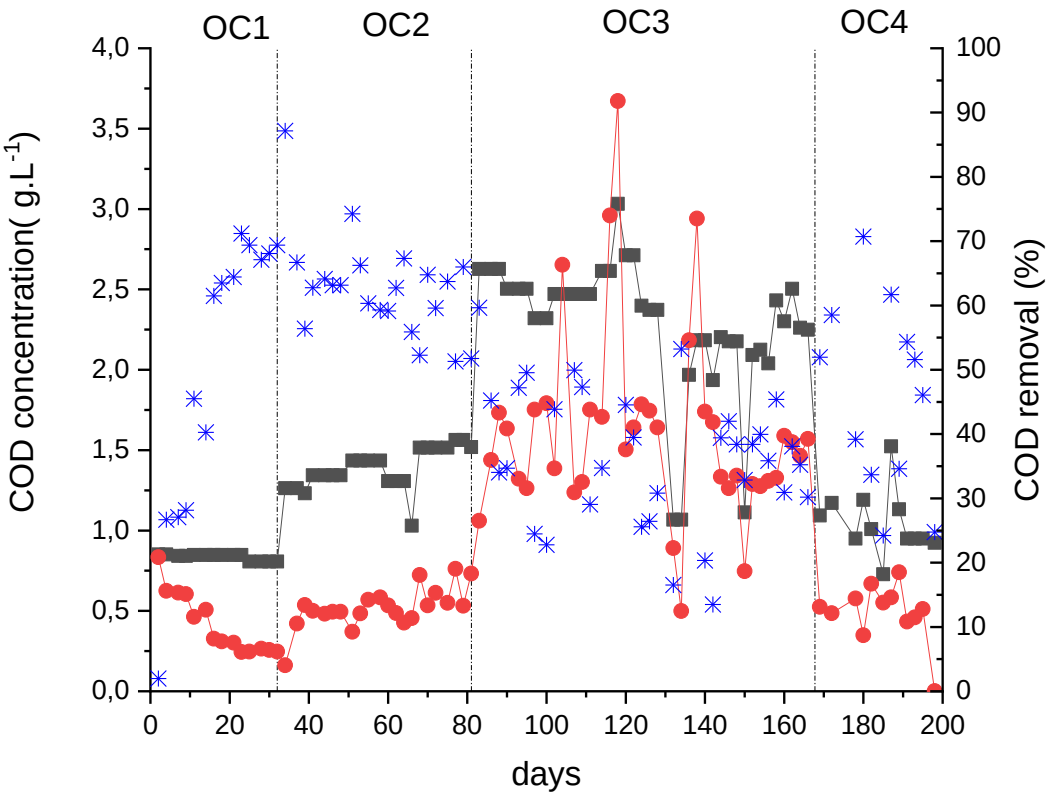
At OC1 the COD removal stability was established after 16 days of the reactor operation. The average of COD removal efficiency (confidence interval from Student's t test) was 66.8% (varying from 64 to 69.6%). Also, the Student's test showed (with 95% reliability) that COD removal was above 60%. At OC2, the COD removal became more stable after 66th day. The average value (confidence interval from Student's test) was 58.16% (varying from 53 to 63.3%). The Student's test also showed (with 95% reliability) that COD removal was above 50%.

At OC3, which higher organic load rate were applied, the reactor did not showed stability for COD removal. The average of COD removal efficiency (confidence interval from Student's test) was 36.8% (varying from 32.6 to 40.9%). Also, the Student's test showed (with 95% reliability) that COD removal was above 30%. Although, no system stability was obtained at OC3, the average COD obtained in the last 15 days of operation is presented in Table 20. Biomass samples were collected from the foams collected in the first collecting point of the reactor at the end of the operation under condition OC3 for molecular microbiology analysis.

The VOLR was then reduced in OC4 in an attempt to recover the previously efficiencies obtained and the reactor was operated during a further 40 days. OC4 had the same VORL applied in OC1, however, the process stability was not achieved, and it was no possible to achieve the same COD removal efficiency as previously, probably because the microorganisms present in the biomass were not still recovered from the organic load rate applied in OC3 and, therefore, weak or had their cells damaged, thus the reactor operation was interrupted.

Analyzes of total volatile solids were performed at the beginning and at the end of the reactor operation. The TVS concentration dropped from 2.7 to 1.5 g TVS. g foam^{-1}. The microorganism decay may be indicative of intoxication by the PHWW. Biomass samples were collected from the foams for molecular microbiology analyzes, however the DNA quality of the cells was not sufficient for the analysis, therefore it was not performed, thus confirming that the cells were damaged.

Figure 23. Organic concentration in the influent (■) and in the effluent (●) and a COD removal efficiencies (✳)

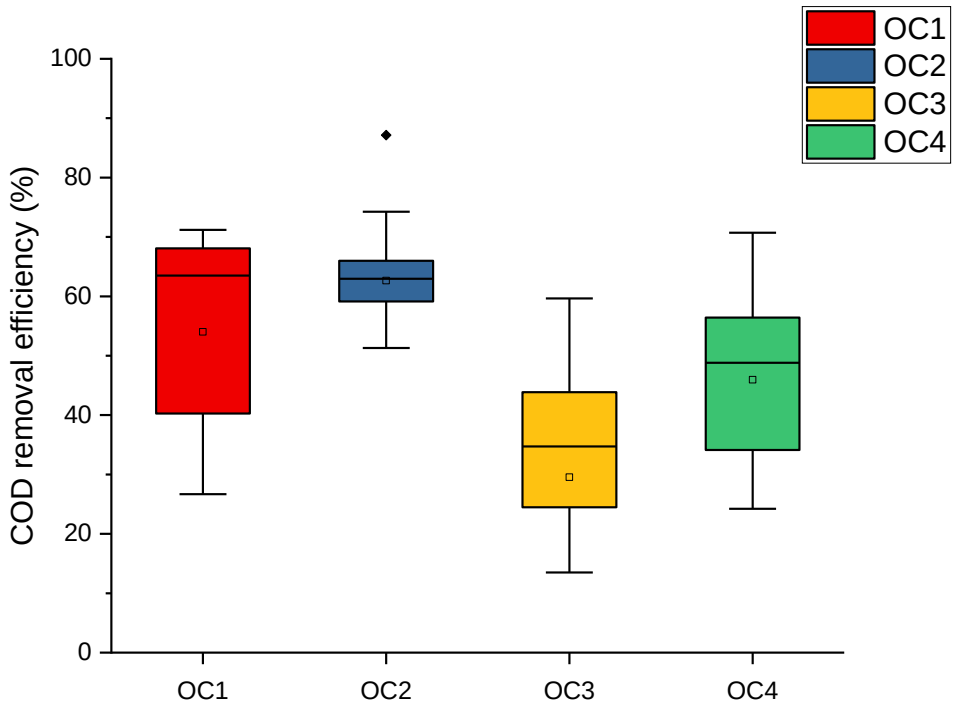


Source: own authorship

Figure 24 shows the boxplot graphic for the COD removal obtained along the HAIB-R1 operation in each operational condition. The boxplot shows the variation of COD removal efficiencies obtained. The whisker extends horizontally from the box,

indicating variability outside the upper and lower quartiles. The outliers are plotted as individual points, as can be seen in OC2. The spaces between the different parts of the box indicate the degree of dispersion, the obliquity in the data and the outliers. It is possible to notice clearly the variation of COD removal in OC1 and OC3. OC2 is clearly the condition in which the reactor had better stability and higher COD removal efficiencies. In OC4, although the COD removal efficiencies increase comparing to OC3, greater variations and lower efficiencies were obtained comparing to OC1, which had the same organic loading rate.

Figure 24. Boxplot graphic for COD removal efficiency in HAIB-R1.



Source: own authorship

The low COD removal efficiencies obtained with the low organic loads tested may be related to the fact that the PHWW used was derived from *Spirulina*, which has a relatively high organic nitrogen content, and part of the nitrogen content is in the form

of cyclic nitrogen compounds, which are often not susceptible to degradation only by anaerobic route (Pham *et al.*, 2013; Quispe-Arpasi *et al.*, 2018).

PHWW derived from other lower nitrogen feedstock produces lower total nitrogen PHWW and probably has different toxicity effects on anaerobic digestion. For instance, Si et al. (2018) performed the continuous anaerobic digestion of PHWW from hydrothermal liquefaction process of cornstalk which has lower protein content and lower heterocyclic nitrogen compounds concentration, as well the inhibitory potential, compared to PHWW from algal biomass. The continuous reactor was fed with the PHWW during 8 days under organic loading rate of 8 g $COD.L^{-1}$. d^{-1} after acclimatization period of 25 days using synthetic substrate with glucose as the carbon source. The COD removal efficiency obtained was around 67%.

Furthermore, according to Tommaso et al. (2015), the characteristics of PHWW are directly related to the variations of the conditions applied in HTL process, the authors found that lower temperatures (260 and 280ºC) led to lower methane production potential, different from that observed by Posmanik et al. (2017) who found that the anaerobic biodegradability of the PHWW was lower when the temperature of HTL process increased (320-350ºC). However, the chemical composition of the hydrothermal aqueous phase affected the anaerobic biodegradability, and, they used food waste as feedstock in the HTL process. Tommaso et al. (2015) used algal biomass, as it was used in the study presented. Gai et al. (2015) observed that HTL using *Chlorella pyrenoidosa* as raw material at 280 and 300 ° C had higher concentrations of organic acids. However, at 260 °C, the same temperature used in the HTL to produce the PHWW for this study, higher concentrations of N & O-heterocyclic cyclic compounds were found, which means that the PHWW used probably had high concentrations of these compounds, which are toxic.

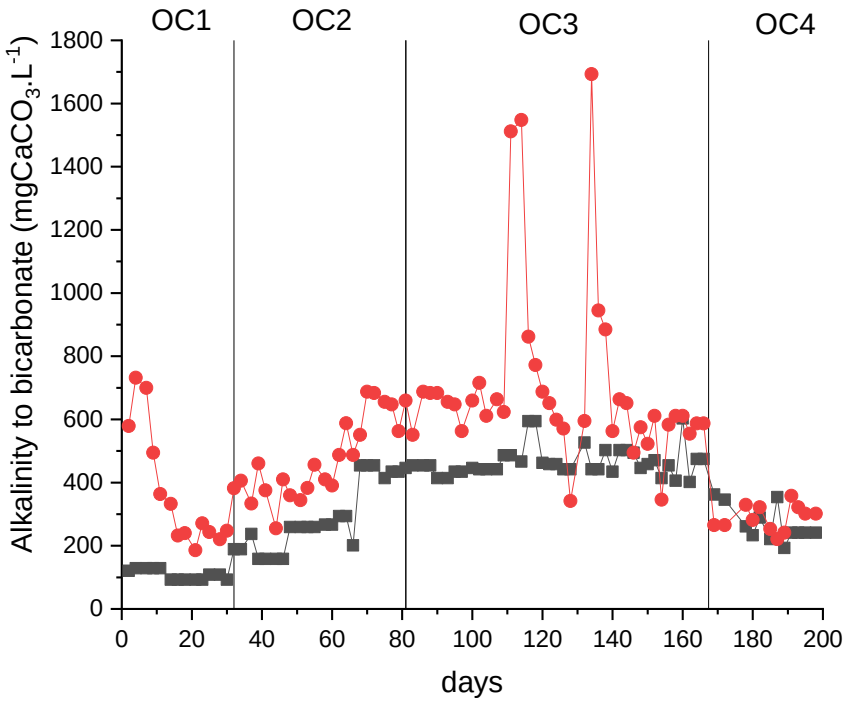
Although the reactor has been in operation for approximately 200 days, the time between each organic rate tested may not be sufficient to recover the reactor's stability. Also, the reactor configuration may not have been adequate as the HAIB reactor is a piston flow reactor type which was receiving all the toxic load at once. The decay of the microorganism shows that the reactor was suffering from this toxic load shock.

Regarding the alkalinity to bicarbonate (Figure 25), its monitoring was important to attest to the stability of the reactor. In all OC tested the alkalinity to bicarbonate was higher in the effluent than in the affluent, as can be seen in the Table 21. The pH of the effluent was more alkaline in relation to the affluent in all conditions, except for OC2 in which the values are similar. According to Ripley et al. (1986), the relationship between intermediate alkalinity (IA), which is related to the volatile acids, and partial alkalinity (PA), which is the alkalinity to bicarbonate, greater than 0.3 is an indicative of instability of the process. However, due to the particularities of each substrate, some reactors may not show instability even presenting IA/PA greater than 0.3 (CHERNICHARO, 2007). In all conditions tested the IA/PA calculated was equal or higher than 0.3, taking the standard deviation in account. All alkalinity indicators showed obvious signs of response delay under PHWW load.

The alkalinity analysis shows the ability of the process to neutralize acids, preventing pH variations due an increased concentration of acids. The alkalization capacity of the reactor is an indication that anaerobic digestion is properly happening. The balance of anaerobic digestion is associated with the ability of the system's alkalinity to neutralize the acids formed and buffer the pH when volatile acids accumulate. At OC4, the alkalinity of the affluent was practically the same as the effluent. Once the ORL was reduced, the alkalinity production also reduced. However, in general the values obtained concern the alkalinity production along the whole

operation were not stable, which is an indicative that the process stability was not robust since alkalinity production is a response to the accumulation of acids produced during the process.

Figure 25. Alkalinity to bicarbonate in the reactor during the operation: influent (■) and effluent (●)



Source: own authorship

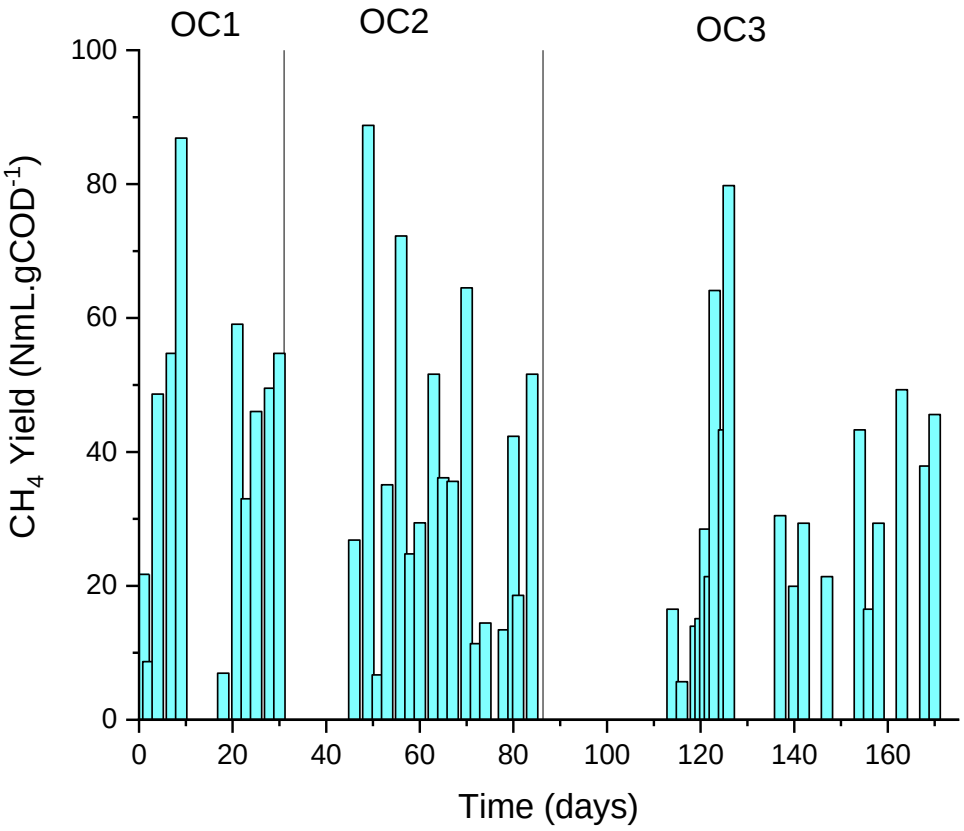
Table 21 Alkalinity and pH obtained in each operational condition

Operation Condition (OC)	Initial pH	Final pH	Alkalinity to bicarbonate (mg $CaCO_3.L^{-1}$) influent	Alkalinity to bicarbonate (mg $CaCO_3.L^{-1}$) effluent	AI/AP
1	7.65	7.8	97± 6	242± 17	0.5±0.1
2	7.78	7.74	259 ± 45	406± 55	0.39±0.11
3	7.86	8.1	460 ± 31.8	708±186	0.36±0.07
4	7.82	7.92	278 ± 53.22	287 ± 36	0.3±0.05

* average obtained at system stability period or in the last 15 days of operation in that condition.

Methane production was measured over the reactor operating time by liquid displacement. Figure 26 presents the methane production obtained from OC1 to the end of OC3. During OC4 no methane production was measured. The daily production was as stable as the COD removal, being the OC" the condition in which higher methane production were achieved. In each change of operation condition, which meant increasing the organic load rate, a reduction in methane production was observed. The decrease or complete cease in the production of methane is probable due the increase of PHWW compounds concentration. It could have caused a delay in the methane production rates by methanogenic microorganisms.

Figure 26. Methanogenic production during the continuous operation of the HAIB-R1.



Source: own authorship

The COD balance was calculated for each condition which methane production was obtained (Table 22). For each OC, the methane production obtained was lower than the theoretical methane production calculated considering the initial COD added and taking into account that 1g COD generates 350 mL. The COD balance results were inferior to 1, which means that part of the COD converted was not converted in methane gas. The COD missing could had been converted in others components of biogas, as H_2 and CO_2, and the process could be indicated for hydrogen production.

Table 22. COD balance regarding the CH_4 production and COD removal achieved in HAIB-R1 operation.

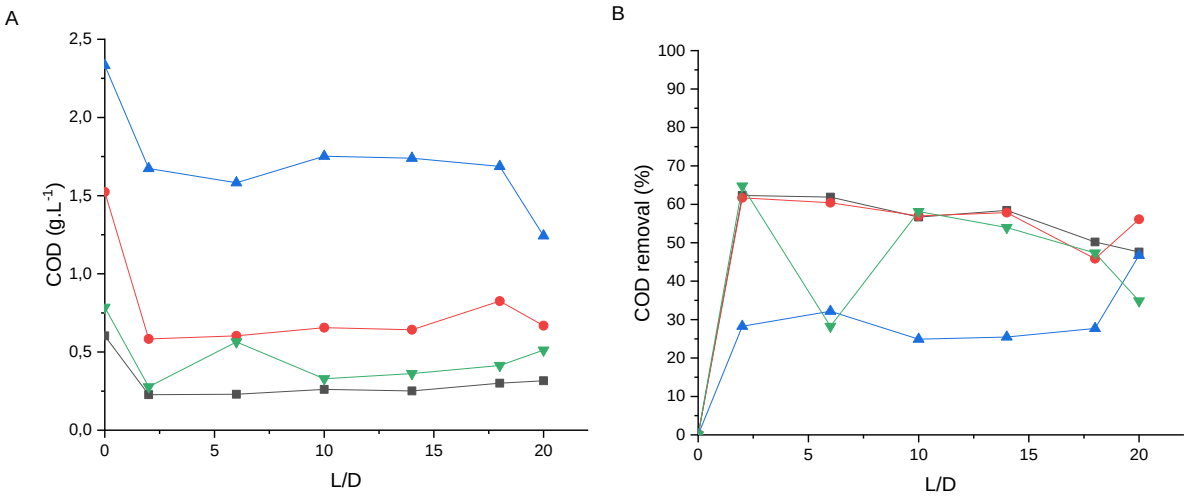
OC	initial COD ($g.L^{-1}.d^{-1}$)	final COD ($g.L^{-1}.d^{-1}$)	initial COD (g)	Final COD (g)	theoretical CH_4 (mL)	experimental CH_4 ($mL.d^{-1}$)	Experimental CH4-gCOD	COD balance
1	0.84	0.25	0.672	0.20	235	11.5	0.03	0.35
2	1.38	0.58	1.104	0.46	386	15.9	0.05	0.46
3	2.5	1.5	2	1.20	700	33.2	0.09	0.65

Samples along the HAIB reactor length were collected in the end of the operational condition, for COD, volatile fatty acids (VFA), alkalinity and nitrogen spatial analyses. The spatial variation of the filtered COD is presented in figure 27A, while the removal efficiencies based on the filtered COD along the reactor length are presented in figure 27.B. In the first two OC applied, the effluent COD concentrations at the last collecting point before the exit (L/D = 18) were higher than those obtained in some intermediate ports along the reactor length during operation. This behaviour was also observed by Zaiat et al. (1997), indicating the presence of preferred cannel in the reactor, allowing to conclude that instantaneous samples of intermediate ports were not representative of the total section due to axial flow and concentration gradients. Under

these conditions, OC1 and OC2, the maximum COD removal efficiency achieved was 62% in L/D=18 (Figure 27.B).

The third OC applied, presented decreasing COD concentration along the length, but with small increases in the middle of the reactor and the maximum COD removal, 46.7%, occurred at the last point, L/D =20. In OC4 similar behaviour was observed to OC1, however showing slight increase in the COD concentration at L/D equal to 6, and, consequently, lower COD removal in the same point. In view of the results obtained, it can be seen that the reactor length did not have an effective contribution in the removal of COD.

Figure 27. Spatial variation of filtrated COD (A) and COD removal efficiency (B) in HAIB reactor when it was operated with 0.8 g COD. $(L.\ d)^{-1}$ (■), 1.6 g COD. $(L.\ d)^{-1}$ (●) 3.3 g COD. $(L.\ d)^{-1}$, (▲) and the second operation with 0.8 g COD. $(L.\ d)^{-1}$ (▼).

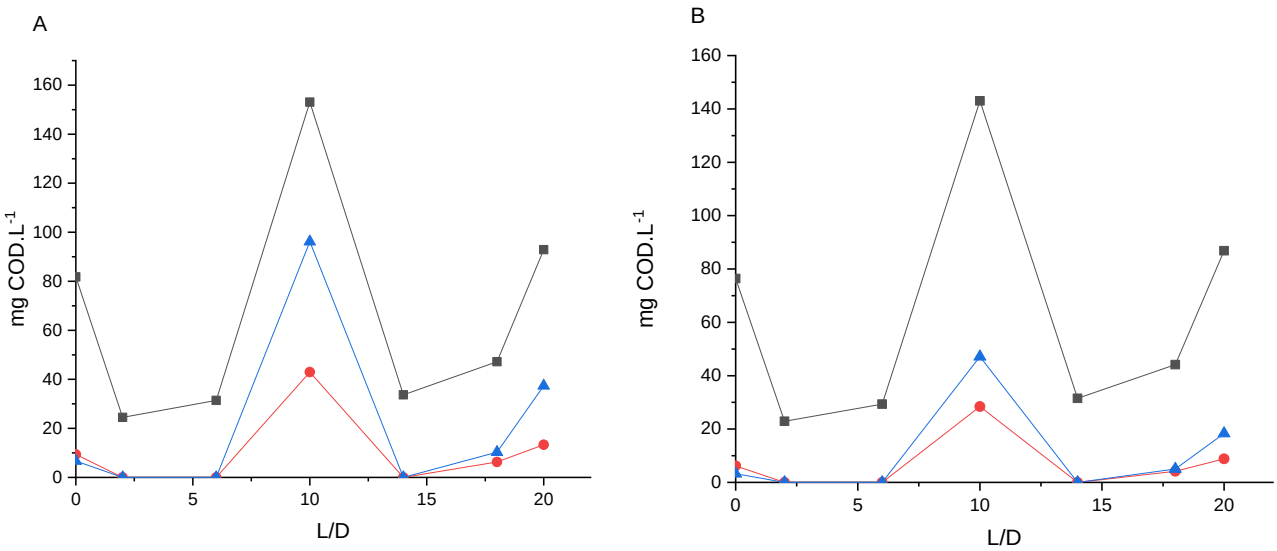


Source: own authorship

The spatial variation of accumulated VFA concentration throughout the reactor during OC1 (0.8 g COD. $(L.\ d)^{-1}$) and OC2 (1.6 g COD. $(L.\ d)^{-1}$) operation are presented in Figure 28A and 28B, respectively. In OC3 (0.8 g COD. $(L.\ d)^{-1}$) and OC4 0.8 g COD.

(L. d)$^{-1}$), the acid peaks obtained were below the calibration curve and therefore it was not possible to present these data. The VFA profiles presented similar behaviors in all the conditions. The maximum concentration of propionic and valeric acids accumulated decreased approximately half when the OLR were increased. However, the acetate concentration was practically the same at both conditions. The spatial (horizontal) profiles along the length of the reactor demonstrated that VFA concentrations increased in the flow direction in a manner similar to that of COD. This result was attributed to the presence of preferential channels.

Figure 28. Spatial variation of VFA in HAIB reactor when it was operated with 0.8 g COD. L^{-1}. d^{-1}(A) e 1.6 g COD. L^{-1}. d^{-1} (B): acetic acid (■), propionic acid (●) and valeric acid (▲).



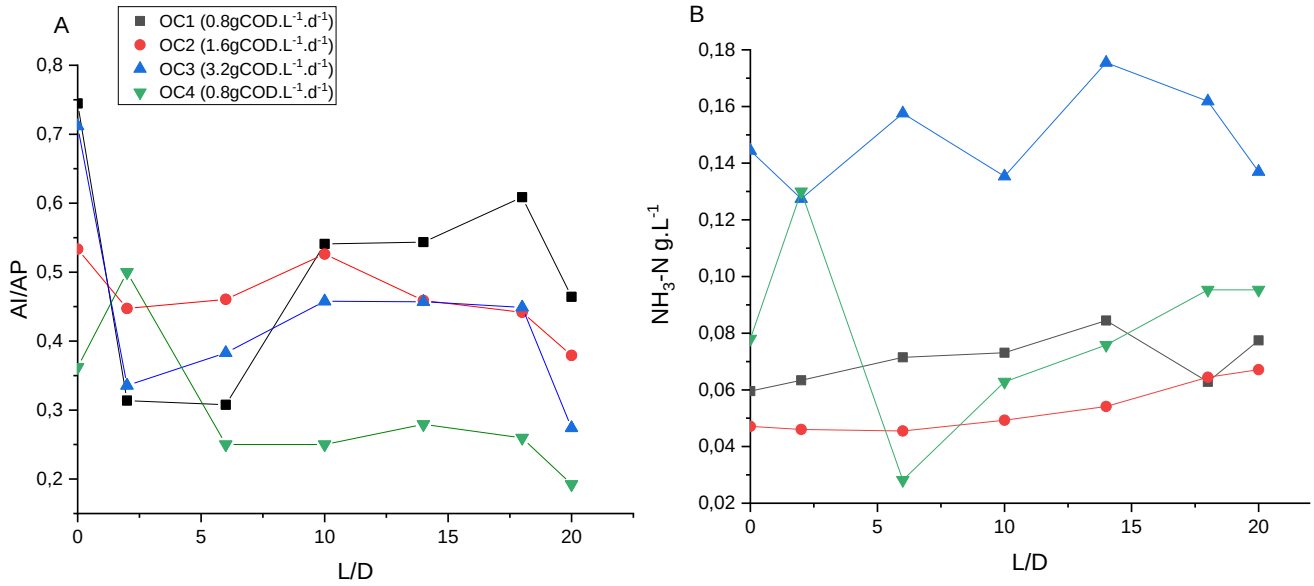
Source: Own authorship

Figure 29 presents the IA/IP (A) and NH_4-N production (B) obtained in the realized profiles analyses for each OC tested. In OC1, there was increase of alkalinity to bicarbonate in relation to the input, showing that the acids were produced, and then, decrease until the end of the reactor´s length, maybe due to difficulties in consuming

those acids previously produced, with a slight increase in the output sample. The values in OC2 maintained constant behavior with only slight alkalinity to bicarbonate increase in the middle of the reactor (L/D= 10), indicating that in that point acids could have been accumulating and the system answered well producing alkalinity. In OC3, there was increase in the alkalinity to bicarbonate, and then, stability along the reactor with no significant variations along the reactor lengths. In all the first 3 OC applied, the relation IA/PA were above 0.3 along the reactor, which was already discussed, could be an indicative of instability of the process. However, it is interesting to notice that, although the COD was not totally removed, there was alkalinity production and the reactor was not acidified at those conditions.

In the last condition applied, OC4, it was observed decrease of the alkalinity to bicarbonate compared to the input in L/D=2, and then, continuous increase throughout the entire reactor, decreasing again at the reactor output. The IA/PA resulted in value inferiors to 0.3 This behavior reflects, the instability of the process in that condition and probable accumulation of acids, indicating that the consumption of acids by the microbial consortium would not be occurring. About the ammoniacal nitrogen production, in OC1 and OC2 were verified the most stable behavior with no significant variations along the reactor lengths. In condition OC3, the behavior is more varied, with increases and decreases in the ammoniacal nitrogen production throughout the reactor but without large variations in values. Unlikely, in OC4 was observed high variations in the ammoniacal nitrogen production since the beginning of the reactor length. However, at all conditions, the ammoniacal nitrogen concentration at the reactor inlet was similar to that at the outlet. If the nitrogen compounds coming from PHWW were being degraded by the anaerobic consortium, a reduction in the ammonical nitrogen was expected.

Figure 29. Spatial variation of IA/IP relation (A) and NH_4-N (B) in the HAIB-R1



Source: own authorship

The HAIB-R2 was operated for evaluating the anaerobic continuous degradation of PHWW with methanol as co-substrate. The reactor was fed with half of the supplied COD coming from PHWW and the other half from methanol following the results obtained in the batch test in section 5.2.3.

The HAIB-R2 was operated for approximately 160 days. COD and alkalinity to bicarbonate analyses were performed every 48 hours. Table 23 presents the organic loading rates applied, the COD removal efficiencies and the alkalinity in the influent and effluent obtained during the reactor operation. Contrary to what was found in the literature, the addition of methanol as co-substrate did not bring benefits for the PHWW anaerobic degradation. The reactor presented instability, high variation in COD removal values and lower COD removal efficiencies, and stable alkalinity production was not achieved. This behavior is an indicative that the anaerobic process was not occurring properly. The solids concentration decreased from 2.7 g TVS. g foam^{-1}, at the

beginning of the operation, to 0.92 g TVS. g foam^{-1} at the end of the process, which is an indicative of microorganism decay.

Table 23. Operational conditions applied to the continuous reactor, COD removal efficiencies and alkalinity production obtained in each condition.

Operation Condition (OC)	VOLR (g COD. $L^{-1}.d^{-1}$)	SOLR (g COD. g VTS.d^{-1})	COD removal* %	Alkalinity to bicarbonate (mg $CaCO_3.L^{-1}$) influent	Alkalinity to bicarbonate (mg $CaCO_3.L^{-1}$) effluent	AI/AP
1	0.8	0.014	36 ± 0.9	82±8.9	678.5±394.5	0.54±0.2
2	1.6	0.028	55 ± 10	133.6±18.8	176±38.4	0.4±0.18

* average obtained at system stability period or in the last 15 days of operation in that condition.

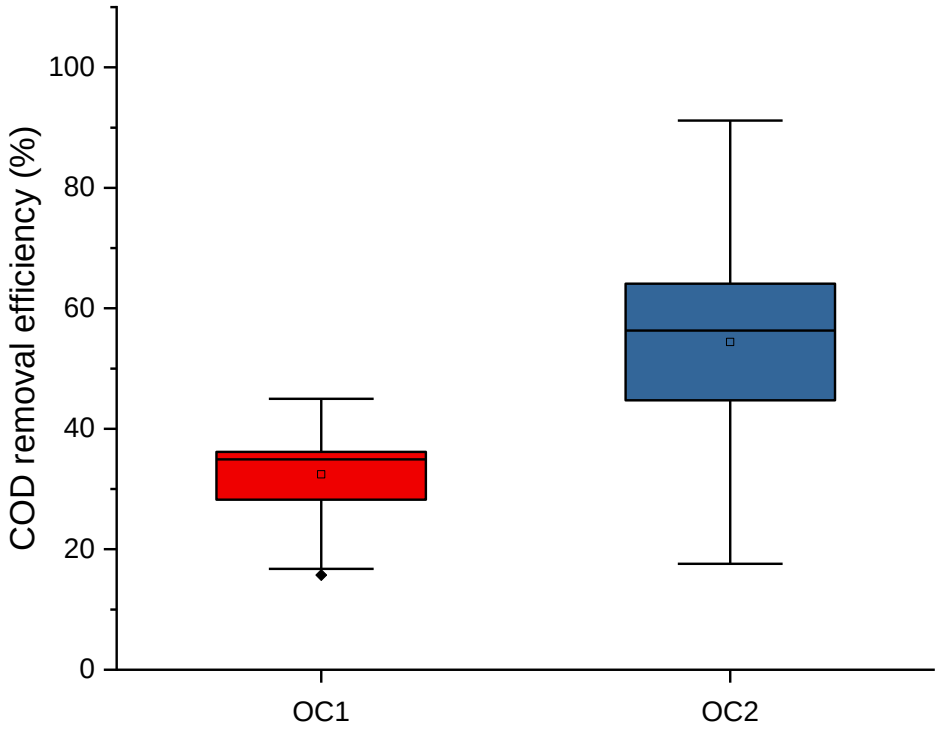
At OC1, the COD removal was stabilized since day 21 and the average value (confidence interval from Student's test) was 36.16% (varying from 35 to 37.1%). The Student's test also showed (with 95% reliability) that COD removal was above 30%. At the OC2, no COD removal stability was achieved and the average COD removal efficiency (confidence interval from Student's test) was 63.5% (varying from 52.6 to 74.4%). Also, the Student's test showed (with 95% reliability) that COD removal was above 50%. Differently from R1, the removal efficiencies did not decrease with the organic load increasing. However, the stability of the process was not achieved.

Figure 30 shows the boxplot graphic for the COD removal obtained along the HAIB-R2 operation in each operational condition. It is possible to notice that in OC2 the variation of COD removal was greater than in OC1. However, in OC2 it was possible to achieved higher COD removal efficiencies.

Regarding the alkalinity to bicarbonate, the reactor presented higher alkalinity production in the OC1, although with high variation. In the OC2, the alkalinity

production decreased, and was similar in the input and output of the reactor, if the deviation standard is take into account. The relation AI/AP obtained in each OC was superior to 0.3 in OC1 and varying from 0.22 to 0.58 in OC2.

Figure 30. Boxplot graphic for COD removal efficiency in HAIB-R2.



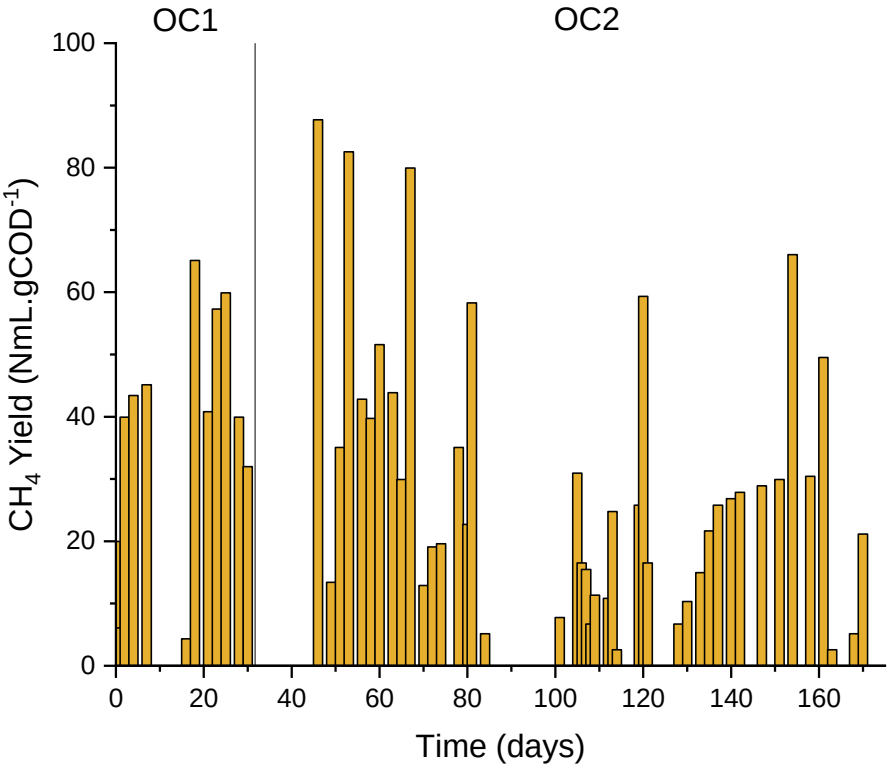
Source: own authorship

Methane production was measured over the reactor operating time by liquid displacement (Figure 31). OC1 presented more stability in the methane production, however lower amounts. When the organic load rate was increased to OC2 the methane production stopped, approximately 20 days. During OC2, the methane production was stable and had some periods of complete cease.

The COD balance was calculated for each condition which methane production was obtained (Table 24). For each OC, the methane production obtained was lower than

the theoretical methane production calculated considering the initial COD added. The COD balance calculated was inferior to 1 in both OC tested. Thus, HAIB-R2 also presented a potential tendency to hydrogen production. However, it cannot be affirmed once H_2 was not measured in the present study.

Figure 31. Methanogenic production during the continuous operation of the HAIB-R2.



Source: own authorship

Table 24. COD balance regarding the CH_4 production and COD removal achieved in HAIB-R1 operation.

OC	initial COD ($g.L^{-1}.d^{-1}$)	final COD ($g.L^{-1}.d^{-1}$)	initial COD ($g.d^{-1}$)	Final COD ($g.d^{-1}$)	theoretical CH_4 ($mL.d^{-1}$)	experimental CH_4 ($mL.d^{-1}$)	Experimental CH4-gCOD	COD balance
1	0.84	0.298	0.672	0.238	235.2	10.6	0.03	0.40
2	1.38	0.552	1.104	0.441	386.4	17.5	0.05	0.44

The possible explanation for the non-positive performance of methanol as co-substrate in the continuous treatment of PHWW could be the low concentration of methanol and of organic load as a whole. In the batch tests, the condition which had the best performance was that one containing 50% of the COD coming from the methanol. The adding COD from methanol was 0.33g, which was similar to the COD from methanol added in the continuous reactor in the first condition, 0.33g. However, the methanol concentration was different since the work volume in the batch tests was 60mL and in the continuous reactor was 800mL. This results in a methanol concentration of 6 g $COD.L^{-1}$ (or 4g methanol. L^{-1}) in the batch tests and 0.4 g $COD.L^{-1}$ (or 0,6 g methanol. L^{-1}) in the HAIB reactor. In terms of organic load rate, 0.4 g $COD.L^{-1}$. d^{-1} was applied in the continuous reactor, and 2.8 g $COD.L^{-1}$. d^{-1}, in the batch test the continuous reactor. Higher methanol concentration and organic load rates were used in the batch test than in the continuous process. Thus, the addition of methanol at these concentrations did not had the same positive effect on the continuous reactor than it had in the batch tests.

The application of the same concentrations used in the batch test and in the biostimulation process was not possible in the HAIB reactor since the methanol adding corresponded 50% of the total COD added, which would imply in the adding also higher PHWW concentration.

Due to the process instability and lower COD removal efficiencies obtained, only one profile analysis was performed, at the end of the experiment. The results obtained for all the analysis performed with the profile samples are presented in Figure 32. No VFAs were detected in this profile. The spatial variation of the filtered COD is presented in Figure 32A, and the removal efficiencies based on the filtered COD along the reactor length are presented in Figure 32B. The effluent COD concentration at the

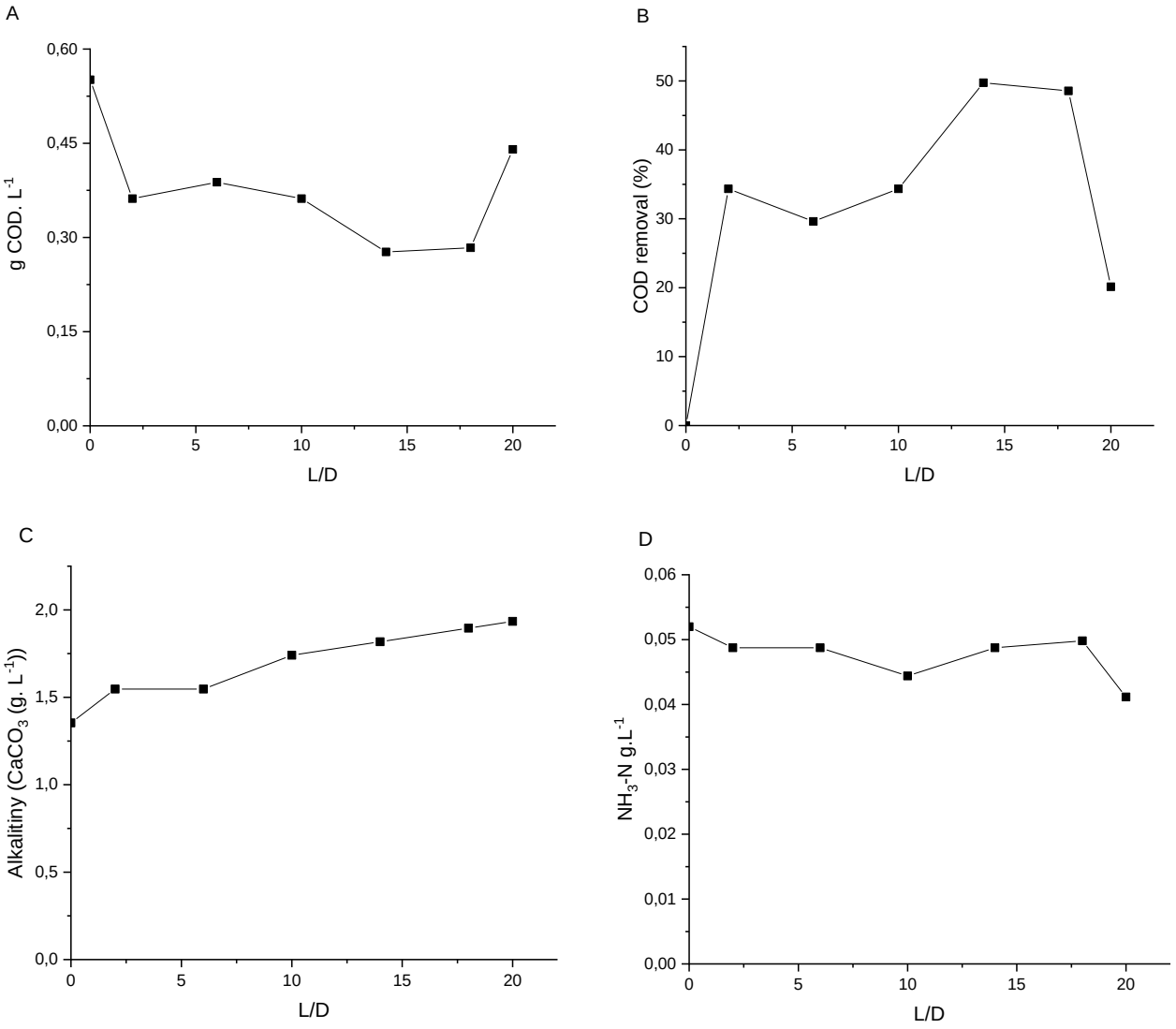
last collect point (L/D = 20) was higher than those obtained in some intermediate ports along the reactor length during operation.

This behavior was also observed by Zaiat et al. (1997), indicating the presence of preferred cannel in the reactor, allowing to conclude that instantaneous samples of intermediate ports were not representative of the total section due to axial flow and concentration gradients. The maximum COD removal efficiency achieved was 50% in L/D =14 (Figure 25B). However, in the output, the COD removal percentage dropped to 20%.

Regarding the alkalinity production (Figure 25C), there was a slight increase along the reactor. The alkalinity production in the input increased from 1.35 g CaCO3. L^{-1} to1.93 g CaCO3. L^{-1} in the output of the reactor. Stable behavior with no significant variations along the reactor lengths was verified from the ammoniacal nitrogen production (Figure 25D).

Biomass samples were collected after the samples profile collecting to analyze molecular biology; however, the DNA quality of the cells was not sufficient for the analysis, so it was not performed.

Figure 32. Profile analysis performed for R2 in the end of the operation with CO2: g $COD.L^{-1}$ (A), COD removal efficiencies (B), alkalinity production (C) and ammoniac nitrogen (D).



Source: own authorship

5.5. Microbial profile

Samples from the three sludge used to compose the inoculum, as well the inoculum, were observed under a phase and fluorescence contrast optical microscope for the visualization of methanogenic archaea. The images obtained during the analysis can be observed in Figure 33.

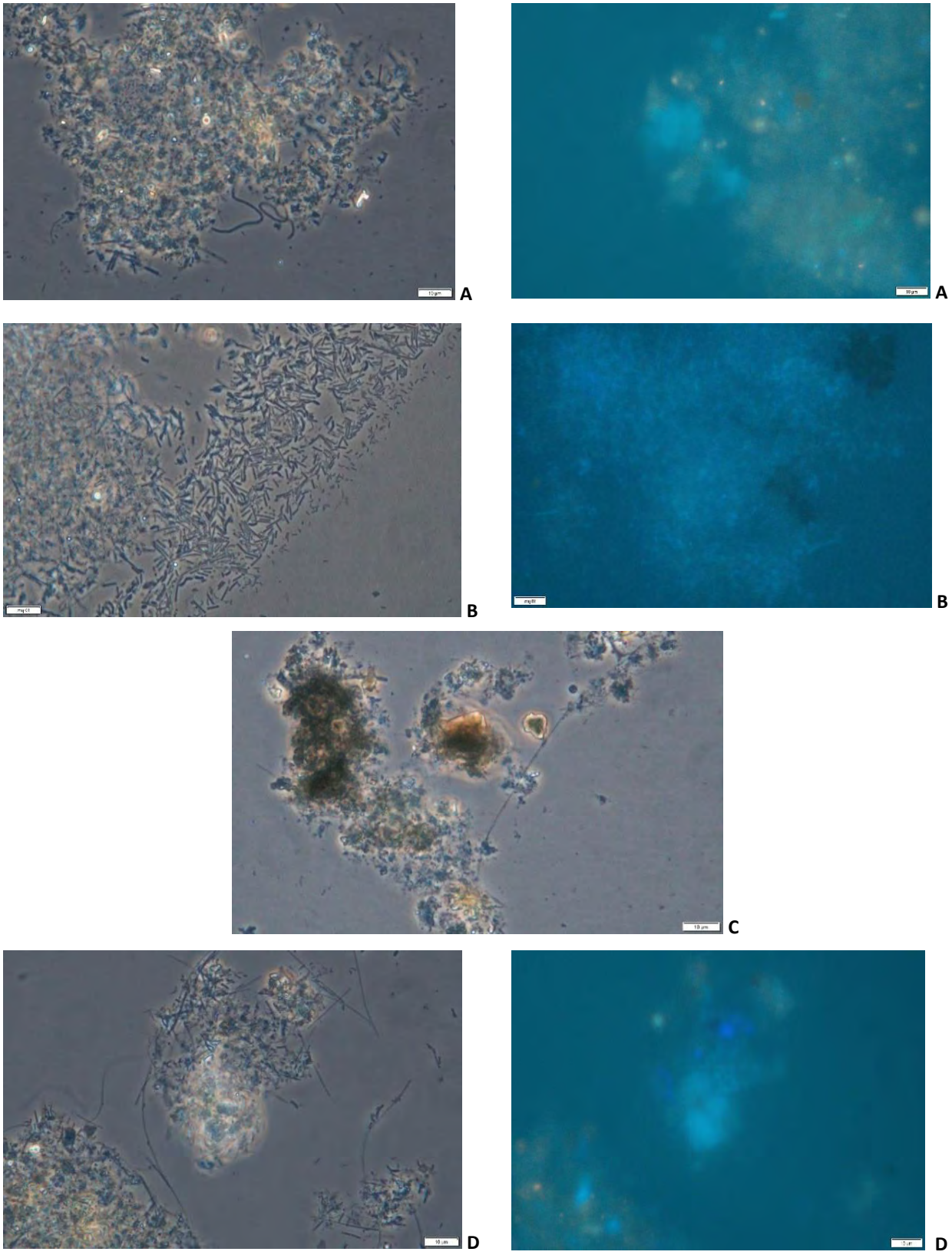
The sludge from the reactor treating effluent from poultry slaughterhouse (A) showed many morphologies similar to *Metanosaeta sp*., being the main morphology observed, besides coccus, spirochetes, thin filaments. In the fluorescence analyses the presence of morphologies similar to *Methanosarcina sp.* was verified.

Sludge from the effluent treatment of starch production (B) showed bacilli, thin filaments and many fluorescent bacilli. Consequently, in the composite sludge many filaments, few cocci, many structures similar to *Methanosaeta sp*., fluorescent bacilli and the presence of *Methanosarcin sp.*

Sludge from the sewage treatment plant (C) did not show fluorescent structures, but it was possible to observe structures of bacilli, thin filaments and sulphate reducing bacteria.

The intention of mixing the 3 different sludge was to obtain a very diverse sludge in terms of microbial morphologies. Through the images observed by the optical microscope analysis, it is possible to notice that the sludge obtained after the mixture (D) presented very different morphologies, being a promising sludge to the biostimulation experiment.

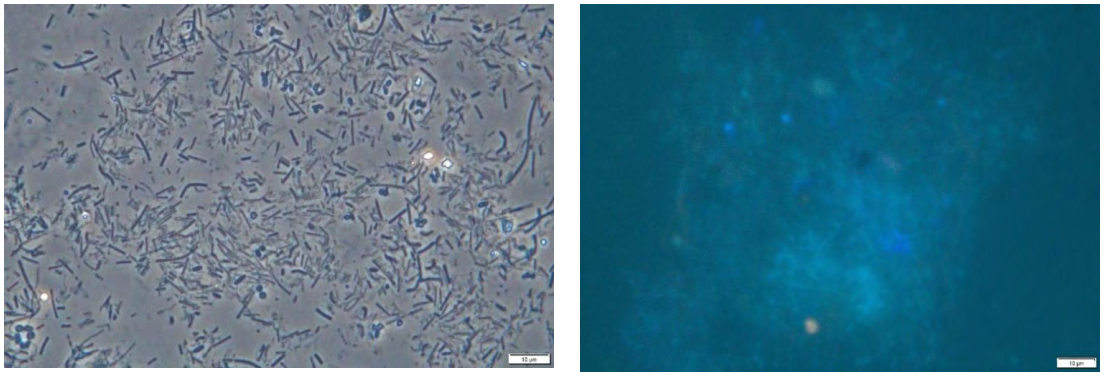
Figure 33. Images obtained in the optical microscopy analysis of the sludge used for the composite sludge composition: A: DACAR sludge; B: Ingredion sludge, C: ETE sludge and D: mixed sludge



Source: Own authorship

After the biostimulation process, biostimulated sludge samples were also observed under a phase contrast optical microscope and fluorescence. The images obtained during the analysis can be observed in Figure 34. A very diverse population, rich in coccus arrangements, sulfurous bacteria (comma format), fluorescent bacilli and morphologies similar to *Methanosaetas* sp, was observed.

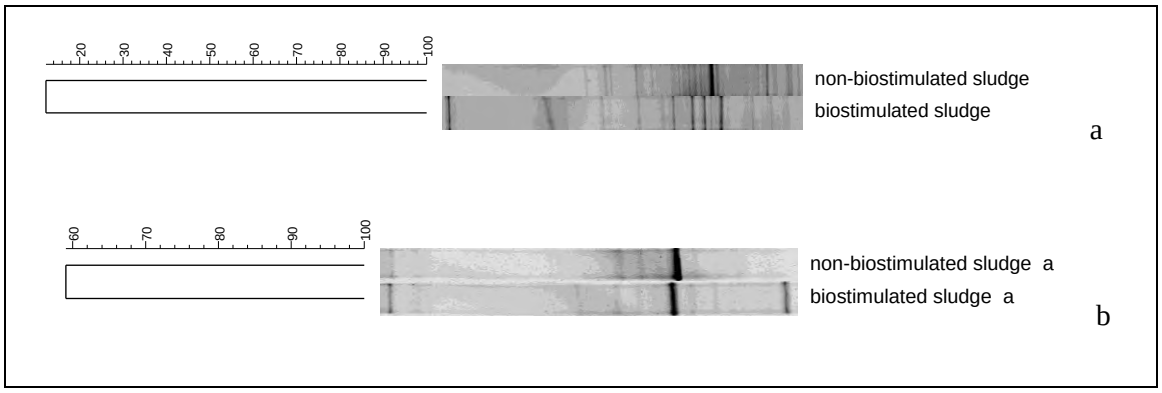
Figure 34. Biostimulated sludge images obtained in the optical microscopy analysis.



Source: Own authorship

The polymerase chain reaction technique and denaturing gradient gel electrophoresis (PCR / DGGE) were used to analyse the diversity of the microbial community and the comparison between the inoculum and biostimulated sludge. For the bacterial populations, the dendrogram shown in Figure 35a was obtained. Using the similarity index Pearson's correlation, a similarity index of 20% was obtained between the two analysed populations. For archaeal populations, the dendogram presented in Figure 35b was obtained and the similarity index by Pearson's correlation was 59% between the two analysed populations. Diversity among populations was analysed by the ecological indexes of Shanon (H) and Dominance (D) presented in Table 25.

Figure 35. Dendrograms obtained in the test: a (*Bacteria*) and b (*Archaea*).



Source: Own authorship

Table 25. Diversity indexes for non-biostimulated (NB) and biostimulated (B) sludge.

	Bacteria		*Archaea*	
	NB	B	NB	B
Shannon	3.042	2.692	1.43	1.629
Dominance	0.06776	0.08526	0.3266	0.2728

For the bacterial domain, the Shannon diversity index of the biostimulated sludge decreased relative to the non-biostimulated sludge, which means that there is less diversity of bacteria after biostimulation. In other hand, the Dominance index increased, which was a sign that a selection of bacterial populations occurred and the biostimulation favoured the growth of specific populations. However, in the *Archaea* domain the reverse happened. The Shannon index increased for the biostimulated sludge, indicating an increase in the archaeal population diversity, and the Dominance index decreased, indicating that new populations developed after biostimulation.

During the 16S rDNA sequencing of the non-biostimulated and biostimulated sludge, 60748 and 47360 sequences were obtained, which were comprehended into 455 and 423 OTU's, respectively. High coverage was observed (99%) for both samples (Table 26). Around 23% and 27% of the OTUs were unique to non-biostimulated and

biostimulated sludge, respectively. The diversity index (Shannon) and richness estimation (Chao-1) for both sludge are shown in Table 26.

Table 26.Diversity analyses indexes and estimates estimations.

16S rRNA database	Non-stimulated sludge	Biostimulated Sludge
Sequences	60748	47360
OTUs taxonomical classification	455	423
Diversity (Shannon)	4.71	4,80
Richness (Chao-1)	435.23	460.56
Goods_Coverage	99%	99%
Average lengths of effective tags	408	403

In the non-biostimulated sludge, 33.3% of the microbial community was constituted by Archaea and 66.7% was Bacteria. Whereas, for the biostimulated sludge, 54.3% was Archaea and 45.7% was Bacteria, indicating the selection of archaeal communities in the biostimulated sludge probably as a response of acetic acid and methanol addition during the biostimulation process.

Xu and Tay (2002) reported that the addition of methanol to the sludge from UASB reactor accelerated the granules formation, which were around 20% higher than those from the inoculum without methanol. This probably occurred because methanol is a suitable substrate metabolized by many methanogenic archaea and favoured the development of different enzymes and metabolic pathways for methane production. Thus, the increase in the archaea community after biostimulation can be related with methanol addition.

At the phylum level, 39 and 40 distinct phyla were observed for non-biostimulated sludge and biostimulated sludge, respectively. The Euryarchaeota

phylum, which comprises the methanogenic archaea, was the most archaeal representative phyla for both sludge. Cloroflexi, Spirochaetes, Bacteroides, Nitrospira, and Proteobacteria were the most representative bacterial phyla for non-biostimulated sludge. In contrast, Bacteroidetes, Firmicutes and Proteobacteria were the most representative bacterial phyla for biostimulated sludge.

At the genus level (Figure 36), *Methanosaeta* (31.84%), *Treponema* (11.89%), *Caldisericum* (2.83%), *Paludibacter* (2.73%), and *Desulfovibrio* (1.37%) were identified in relative abundance higher than 1% in the non-biostimulated sludge.

For the biostimulated sludge, the genus identified in relative abundance higher than 1% were *Methanosaeta* (43.21%), *Methanomethylovorans* (5.36%), *Mesotoga* (2.49%), *Macillibacteroides* (2.21), *Smithella* (2.11%), *Lentimicrobium* (1.99%), *Methanomassilicoccus* (1.74%), *Syntrophobacter* (1.59%), *and Methanolinea* (1.43%).

The most abundant bacterial genus identified in the non-biostimulated sludge was *Treponema* (11.89%) which is an acetogenic bacteria previously reported in anaerobic digester (GUO *et al.*, 2015). However, the relative abundance of this genus in the biostimulated sludge was only 0.06%, indicating that these bacteria was severely inhibited by the organic acids and methanol addition. The same profile was observed for the *Caldisericum*, *Paludibacter* and *Desulfovibrio* genera, which were in higher relative abundance in the non-biostimulated sludge (2.83, 2.73, and 1.37 %, respectively) than after the biostimulation (0.04, 0.14, and 0.17%, respectively).

Members of the *Caldisericales* were associated with thermophilic biodegradation of complex substrate such as petroleum hydrocarbon, which can be found in the PHWW (CHENG *et al.*, 2014) and black water (ZHA *et al.*, 2019). The *Paludibacter* genus include propionic-producer bacteria able to consume many sugars as substrate and produce acetic acid and propionic acid as major fermentation end-

products with small amount of succinic acid (UEKI *et al.*, 2006). This genus probably was inhibited by the organic acids (acetic, propionic and valeric acids) and methanol added as bio stimulant, once it is acid-producers and could be affected by the acid accumulation.

Desulfovibrio genus is sulphate-reducing bacteria (SRB) which has an essential role in the metabolism of fermentative systems (ZHONG *et al.*, 2017) once these bacteria can stablish syntrophic interaction with *Clostridium* resulting in a continuous and stable hydrogen production (BARCA *et al.*, 2016).

BRS fermentation occurs in the absence or in low sulphate concentrations, competing with acetoclastic microorganisms. In high sulphate concentrations, BRS performed respiration. The BRS, which cannot oxidize acetate, produces acetate and uses additional electrons for sulfate reduction to gain some additional energy through sulfate respiration (PHILIPP and SCHINK, 2012). *Desulfovibrio* can degrade nitro aromatic compounds (IWAMOTO and NASU, 2001) and it is commonly found in anaerobic degradation of hydroxyhydroquinone (CARMONA *et al.*, 2009; PHILIPP and SCHINK, 2012) and coal gasification wastewater (YAJIE LI *et al.*, 2018; ZHU *et al.*, 2018; ZHUANG *et al.*, 2018) which are rich in phenolic and aromatic compounds, as PHWW.

In contrast, many bacterial genera that were in low relative abundance in the non-biostimulated sludge and were favoured after the addition of organic acids and methanol, such as *Mesotoga* (from 0.43 to 2.49%), *Macelliibacteroides* (from 0.04 to 2.21%), *Smithella* (from 0.75 to 2.11%), *Lentimicrobium* (from 0.02 to 1.99%), and *Syntrophobacter* (from 0.74 to 1.59%).

The genus *Mesotoga* include acetoclastic bacteria with potential role on degradation of halogenated aromatic compounds such as polychlorobiphenol, a toxic

pollutants generated by industries (BEN HANIA *et al.*, 2011). This genus was related to organic acid, mainly lactic acid, in reactors fed with hydrothermal liquefaction wastewater (CHEN *et al.*, 2016) the same wastewater used as substrate in the present study.

The genus *Lentimicrobion* comprehends fermentative bacteria, which produces mainly acetate, hydrogen, and butyrate (ZHANG *et al.*, 2019). This genus is widely reported in methanogenic reactors (YANG *et al.*, 2018; ZHANG *et al.*, 2019; ZHENG *et al.*, 2019). Other fermentative bacteria identified in the biostimulated sludge belongs to *Macellibacteroides* genus. The main end products of glucose fermentation developed by this genus are lactic, acetic, and butyric acid (JABARI *et al.*, 2012).

Bioconversion of complex compounds such as aromatic compounds into methane, requires interaction between syntrophic substrate-oxidizing bacteria and methanogenic archaea. Bacteria from the genera *Smithella* and *Syntrophobacter* are related to syntrophic acid bioconversion, such as propionic acid, into methane by association with methanogenic archaea (equations 2, 3, 4 and 5) (AQUINO and CHERNICHARO, 2005; NARIHIRO *et al.*, 2018).

		ΔG°(kJ/reaction)
$CH_3CH_2COO^- + 3H_2O \rightarrow CH_3COO^- + 3H_2 + H^+ + HCO_3^-$	Eq 2	76.1
$C_3H_7COO^- + 2H_2O \rightarrow 2CH_3COO^- + 2H_2 + H^+$	Eq 3	48.1
$CH_3(CH_2)_3COO^- + 4H_2O \rightarrow 2CH_3COO^- + CO_2 + H^+ + 5H_2$	Eq 4	159.5
$4H_2 + HCO_3^- + H^{\mp} \rightarrow CH_4 + 3H_2O$	Eq 5	-101.7

The microbial communities from the biostimulated sludge had potential to develop these metabolic pathways once as *Smithella* and *Syntrophobacter* genera were favoured after the addition of propionic acid and the methanogenic archaea were

favoured by the addition of acetic acid. Propionate is degraded by the combined action of acetogenic bacteria and methanogenic archaea, due to the unfavourable energetics under standard thermodynamics. The methanogenic microorganisms make propionate oxidation energetically feasible by keeping the H_2 concentration low (AQUINO and CHERNICHARO, 2005; CHEN, LIU and DONG, 2005). Methane-producing archaea are one of the key populations in methanogenic bioreactors, developing the final step of the anaerobic digestion of fermentation end products such as H_2/CO_2, acetate and methylated compounds (EVANS *et al.*, 2019).

In the present study, the potential pathway for methane production was acetoclastic once *Methanosaeta*, an acetate-consuming archaea, was the predominant genera in both sludge (31.84 and 43.25% in the non-biostimulated and biostimulated sludge, respectively). In the non-biostimulated sludge, *Methanosaeta* was the only methanogenic archaea identified in relative abundance higher than 1%. After the biostimulation, *Methanomethylovorans* (5.36%), *Methanomassiliicoccus* (1.74%), and *Methanolinea* genera (1.43%) were favoured.

Methylotrophic methanogenic archaea are able to transfer the methyl groups from methylated compounds to the thiol group pf the coenzyme M, in the penultimate step of the methanogenesis, resulting in its bioconversion into methane. In the biostimulated sludge this was the second most important pathway for methane production developed by *Methanomethylovorans* archaea. This genus was in low relative abundance (0.01%) in the non-biostimulated sludge and it was favoured by the methanol addition increasing to 5.36% in the biostimulated sludge, indicating the efficiency of the biostimulation, which has used methanol as one of the bio stimulants, and the potential of this sludge in the methanol biodegradation.

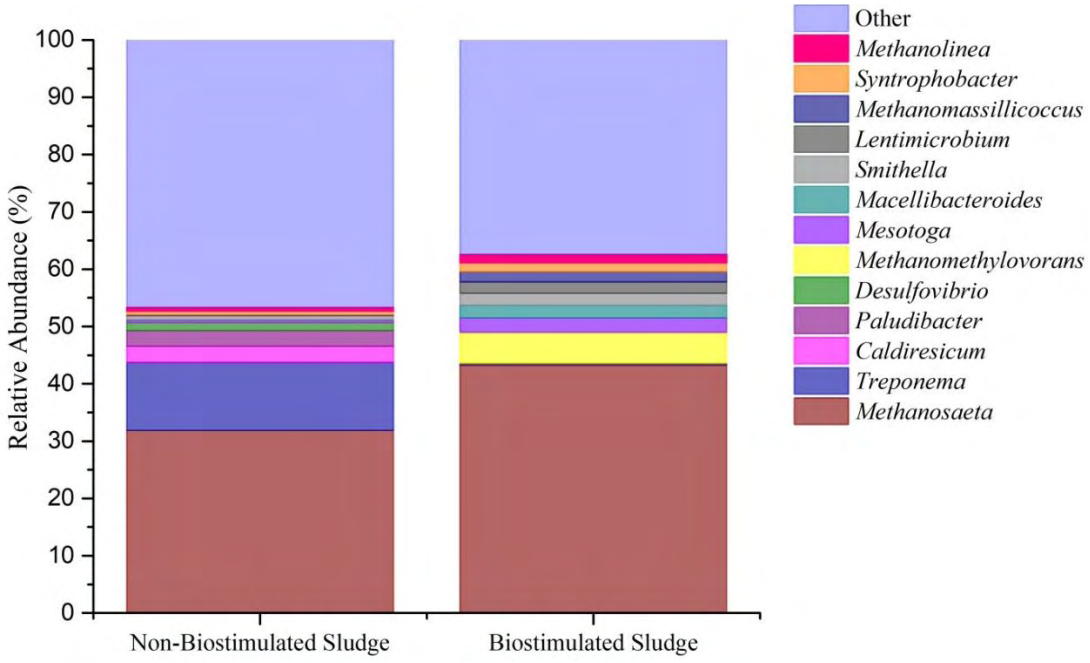
In the same way, *Methanomassiliicoccus* uses methanol or methylamines as substrate with H_2 as electron donor to grow but does not reduce CO_2 to methane (Dridi *et al.*, 2012). In the methanogenesis developed by this genus, the methyl group from methylated compounds is transferred by substrate-specific methyltransferases to 2-mercaptoethanol (HS-CoM) forming methyl-CoM which is, by mean of methyl coenzyme M reductase, reduced to methane (equation 6) using 7-mercaptoheptanoyl-threonine phosphate (HS-CoB) as electron donor Kröninger et al. (2017).

$$\text{Methyl} - \text{CoM} + \text{CoB} \rightarrow \text{CoM} - \text{S} - \text{S} - \text{CoB} + \text{methane} \qquad \text{(Eq. 6)}$$

In contrast, the genus *Methanolinea* belongs to the Methanomicrobiales ordem and comprehend mesophilic hydrogenotrophic archaea (METHANOBACTER, 2006) able to degrade propionic acid (SAKAI *et al.*, 2012). This genus was identified in many systems treating complex substrate such as alkylbenzene linear sulfonate (MOTTERAN *et al.*, 2016), terephthalate (NOBU *et al.*, 2015), and artificial food waste (Ying Li *et al.*, 2018) and had a potential role in H_2/CO_2 consume in the present study.

Based on the microbial profile, important shifts were observed in the bacterial and archaeal communities after the biostimulation process, which selected different microorganisms able to develop potential metabolic pathways to bioconversion of complex substrates. The biostimulated sludge was able to deal with higher PHWW concentrations that has been inhibitory before the biostimulation, probably because of the favouring the PHWW-degrading bacteria growth, such as Mesotoga and methylotrophic methane-producing archaea, such as Methanomethylovorans, which probably were a key tool to tackle inhibition of anaerobic digestion.

Figure 36. Taxonomic affiliation of the reads in the non-biostimulated and biostimulated sludge



Source: Own authorship

The biostimulated sludge was, thus, immobilized in polyurethane foams and used and the anaerobic continuous treatment using HAIB reactor. Biomass samples were collected at the L / D = 2 point of the HAIB-R1 at end of the OC3. During the 16S rDNA sequencing 42491 sequences were obtained. The results obtained from the reactor sludge were compared with the results obtained from the biostimulated sludge, once this was the sludge used for the immobilization in foams for the HAIB reactor.

In the biostimulated sludge, 54.3% was Archaea and 45.7% was Bacteria. After the continuous process until OC3, the sludge presented 90.8% of Bacteria and only 9.2% of Archaea. It shows that a major change in the microbial community occurred during the PHWW continuous treatment in the HAIB reactor.

At the phylum level, 40 and 35 distinct phyla were observed for biostimulated sludge and the reactor sludge, respectively. The Euryarchaeota phylum, which

comprehend the methanogenic archaea, was the most archaeal representative phyla for biostimulated sludge. The most representative bacterial phyla were Bacteroidetes, Firmicutes and Proteobacteria. The phyla Firmicutes and Proteobacteriaare linked to syntrophic phenol degradation (NA *et al.*, 2016; WANG *et al.*, 2017; MUÑOZ SIERRA *et al.*, 2018). In contrast, in the sludge after the reactor operation, the Synergistetes and Firmicutes phylum were the bacteria phylum most representative, followed by the Euryarchaeota phylum, the only archaeal representative phyla for that sludge.

At the genus level (Figure 37), *Methanosaeta* (43.21%), *Methanomethylovorans* (5.36%), *Mesotoga* (2.49%), *Macillibacteroides* (2.21), *Smithella* (2.11%), *Lentimicrobium* (1.99%), *Methanomassilicoccus* (1.74%), *Syntrophobacter* (1.59%), *and Methanolinea* (1.43%) were identified in relative abundance higher than 1% in the biostimulated sludge. After the reactor operation, the genus identified in high abundance in the sludge were *Anaerobaculum* (39.5%), *Coprothermobacter* (31.2%), *Methanosaeta* (7.34%), *Fervidobacterium* (3.9%) and *Methanobacterium* (1.64%).

Regarding the Bacteria domain, the most abundant bacterial genus identified in biostimulated sludge comprehend acetogenic bacteria with potential role on degradation of halogenated aromatic compounds, *Mesotoga* (BEN HANIA *et al.*, 2011), fermentative bacteria which produces mainly acetate, hydrogen, and butyrate, *Macillibacteroides* and *Lentimicrobium* (JABARI *et al.*, 2016; ZHANG *et al.*, 2019), and syntrophic substrate-oxidizing bacteria which are related to syntrophic acid bioconversion, such as propionic acid, into methane by association with methanogenic archaea, *Smithella* and *Syntrophobacter* (NARIHIRO et al., 2018).

However, after the reactor operation, the fermentative bacteria were favored. *Coprothermobacter* and *Anaerobaculum* are part of the phylogenetic group *Firmicutes* and *Synergistetes,* respectively, and they are described in the literature as proteolytic

bacteria or peptide fermenter (RAINEY and STACKEBRANDT, 1993; MENES and MUXÍ, 2002).

Both genera were found in microbial cultures enriched with LCFA (HATAMOTO et al., 2007; PALATSI et al., 2011). Nevertheless, some authors justify their presence by the ability to use protein substrates, resulting from deteriorated cells (SOUSA *et al.*, 2009). It can be another indication that the reactor was already facing microbial cells deterioration at the end of operation with OC3.

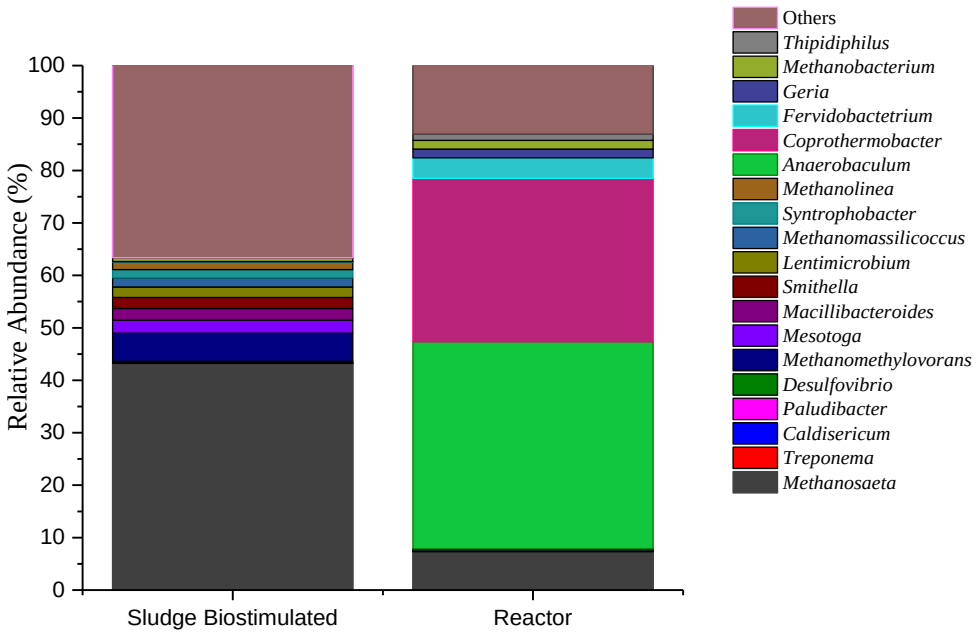
Anaerobaculum is reported in the literature as a moderately thermophilic microorganism, organic acids fermeters and hydrogen producers (MENES and MUXÍ, 2002; MAUNE and TANNER, 2012; BEN HANIA *et al.*, 2016). The reactor operation was kept at mesophic temperatures (37ºC), however this genus was also also found in an anaerobic lagoon treating wool-scouring wastewater (MENES and MUXÍ, 2002) which normally is kept at enviremetal temperatures. Anaerobaculum was also found in methanogenic packed-bed reactor treating protein solid waste (SASAKI *et al.*, 2007) and petroleum reservoir (GIEG *et al.*, 2010).

Coprothermobacter is describe in the literature as an anaerobic, proteolytic, thermophilic bacterium and associated with hydrogen production (ETCHEBEHERE and MUXÍ, 2000; KAWAGOSHI *et al.*, 2005; TANDISHABO *et al.*, 2012; GAGLIANO, BRAGUGLIA and ROSSETTI, 2014; GAGLIANO *et al.*, 2015). *Coprothermobacter* was identified in non-conventional anaerobic systems as in petroleum reservoir (NAZINA *et al.*, 2006), which has similar compounds also found in PHWW. Tatara et al. (2008) found that biofilm is an important niche for *Coprothermobacter*, thus besides the temperature, HAIB reactor treating PHWW from Spirulina seems to have conditions to favoured the *Coprothermobacter* growth. The

potential hydrogen production by *Anaerobaculum* and *Coprothermobacter* could also explain the COD balance values found inferior to 1.

The potential pathway for methane production in the biostimulated sludge was acetoclastic once *Methanosaeta*, an acetate-consuming archaea, was the predominant genera, and methylotrophic methanogenic was the second most important pathway for methane production developed by *Methanomethylovorans* and *Methanomassiliicoccus* archaea. After the continuous operation, in the biomass from the HAIB reactor, *Methanosaeta* was the only methanogenic archaea identified in relative abundance higher than 1%, but still in lower abundance (7.34%) compared to the biostimulated sludge (43.25%). It indicates that the conditions in the PHWW continuous treatment process using HAIB reactor did not favored the growth of methanogenic archaea. Furthermore, sludge samples for sequencing were collected after the final operation, OC4, nevertheless they did not have sufficient DNA minimum quality to be sequenced.

Figure 37. Comparison between the top genera with relative abundance higher than 1% in the biostimulated sludge and the sludge collected from the HAIB reactor.



Source: own authorship

Final Conclusions

Based on the objectives initially proposed and the results obtained, the main conclusions from this work answer the sub-hypotheses initially formulated and are presented:

Sub-hypotheses 1: Solution consisted by volatile fatty acids (acetic, propionic and valeric) and methanol, would stimulate the development of microorganisms that would facilitate the anaerobic degradation of PHWW from *Spirulina*.

Conclusion 1: many bacterial genera that were in low relative abundance in the non-biostimulated sludge were favoured after the addition of organic acids and methanol, such as *Mesotoga* (from 0.43 to 2.49%), *Macelliibacteroides* (from 0.04 to 2.21%), *Smithella* (from 0.75 to 2.11%), *Lentimicrobium* (from 0.02 to 1.99%), and *Syntrophobacter* (from 0.74 to 1.59%). Those bacteria include aromatic-degraders, fermentative and syntrophic acid bioconverters. Besides that, acetoclastic (*Methanosaeta*) and methylotrophic (*Methanomethylovorans*) archaea were favored which favored the degradability of PHWW, reduced the degree of inhibition, and enhanced the methane production. Higher methane production was obtained using the biostimulated sludge under 11, 16 and 49 g COD.L-, and higher maximum methane production rates were observed at 11 and 16 g COD.L-1 as a function of the biostimulation

Sub-hypotheses 2: Biomass immobilized on support material is beneficial for anaerobic degradation of PHWW from *Spirulina.*

Conclusion 2: Batch tests have shown that the use of immobilized biomass in polyurethane foams can be more efficient than granular biomass for the degradation of PHWW from Spirulina. Higher methane production potential and less volatile fatty acids accumulation were obtained using immobilized biomass in polyurethane foam.

Sub-hypotheses 3: Horizontal anaerobic with immobilized biomass reactor would be suitable for anaerobic degradation of PHWW of *Spirulina*.

Conclusion 3: The continuous treatment of PHWW of Spirulina in HAIB reactor, achieved COD removal efficiencies of 69% and 58% in specific organic load rates of 0.014 and 0.028 g COD. g VTS.d^{-1}, respectively. However, HAIB reactor did not favor the methanogenic archaea development and did not show stability in specific organic load rates higher than 0.028.

Sub-hypotheses 4: The use of methanol as a co-substrate could be beneficial to the anaerobic degradation PHWW from *Spirulina*.

Conclusion 4: The use of methanol as co-substrate in the anaerobic degradation of PHWW from *Spirulina* was beneficial in batch tests, with 50% of COD inclusion. However, in the continuous treatment using HAIB, methanol did not present positives contribution due to the characteristics of the substrate (PHWW), operational conditions and the organic loads rates applied.

Final Considerations

Studies regarding the anaerobic treatment of compounds found in industrial wastewater are important for better understanding the degradation process and the performance of the anaerobic reactors. Since experiments on continuous treatment of effluent from the hydrothermal liquefaction process are scarce in the literature, this study contributes to the anaerobic treatment of algae HTL effluent supporting future studies aiming the implementation of this process for obtaining renewable and alternative fuels.

However, future studies aiming different operational conditions and /or reactor configurations and different compounds as co-substrate, as well, must be carried out.

In view of the difficulties faced along this study, it is suggested two different approaches for future work:

1. Better approach, based on Donlon et al. (1996), would be to start the continuous reactor still feeding with the biostimulation solution and, after a certain adaptation period, the reactor could be fed slowly with PHWW.
2. Another option could be using other easily biodegradable compound, during the continuous reactor start-up. Razo-Flores et al. (2003), for example, used only acetate during the UASB reactor start-up for the treatment of waste water with phenolic mixture. After the addition of the phenolic compounds, the acetate dosage was gradually reduced and then completely eliminated from the feed.

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