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INSIGHTS INTO IMPACTS OF TEMPERATURE AND ORGANIC LOAD IN ANAEROBIC REACTORS TREATING EFFLUENTS FROM FISH PROCESSING INDUSTRY

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Belo Horizonte/MG
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Area of concentration: Preventive Veterinary Medicine

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To João Paulo, Luiz Mário, Helen, Zé, Junior, Kauanna,
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“I don’t know what I may seem to the world, but as to myself, I seem to have been only like a boy playing on the sea-shore and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me”

Isaac Newton

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LIST OF ABBREVIATIONS

BOD	Biochemical Oxygen Demand
CH_4	Methane
CO_2	Carbon Dioxide
COD	Chemical Oxygen Demand
DNA	Deoxyribonucleic Acid
DGGE	Denaturing Gradient Gel Electrophoresis
EGSB	Expanded Granular Sludge Blanket
FAO	Food and Agriculture Organization of the United Nations
FISH	Fluorescence in situ Hybridization
H_2	Hydrogen
H_2S	Hydrogen Sulfide
H_2SO_4	Sulfuric Acid
IA	Intermediate Alkalinity
IA/PA	Alkalinity Ratio
IPEA	Brazilian Institute of Applied Economic Research
kg	Kilogram
MG-RAST	Metagenomics Rapid Annotation using Subsystem Technology
NaOH	Sodium Hydroxide
NGS	Next Generation Sequencing
NH_3	Ammonia
NO_3^-	Nitrate
OLR	Organic Load Rate
OTU	Operational Taxonomic Units
PA	Partial Alkalinity
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PGM	Personal Genome Machine
pH	Potential of Hydrogen
QIIME	Quantitative Insights into Microbial Ecology
rRNA	Ribosomal Ribonucleic Acid
SO_4^{-2}	Sulfate
HDT	Hydraulic Detention Time
UASB	Upflow Anaerobic Sludge Blanket
UFMG	Federal University of Minas Gerais
VFA	Volatile Fatty Acids

RESUMO: O aumento da produção mundial de pescado resulta em um consequente aumento de efluentes gerados pela indústria de processamento. Devido à sua composição, esse tipo de efluente requer soluções particulares de tratamento. Diversas alternativas têm sido sugeridas, como o uso de processos biológicos para a degradação da matéria orgânica através de microrganismos. Entretanto, estudos sobre o microbioma de lodos de reatores anaeróbios que tratam efluentes de processamento de peixes ainda são escassos. Com o objetivo de avaliar os efeitos da temperatura no tratamento da digestão anaeróbia de efluentes de processamento de pescado, reatores anaeróbios replicados em escala piloto foram operados a 20°C e 37°C por 60 dias, sendo avaliados por análises físico-químicas e metagenômica. Os resultados demonstraram que os reatores a 37°C foram estatisticamente superiores no dia 50 na remoção da demanda química de oxigênio (DQO) e o enriquecimento das famílias hidrolíticas e acidogênicas *Porphyromonadaceae, Rikenellaceae* e *Spirochaetaceae* pode ter contribuído para que os reatores a 37°C tenham tido melhor desempenho. No entanto, para uma melhor compreensão do processo, um segundo ensaio experimental foi desenhado para avaliar não apenas a temperatura, mas a influência da taxa de carga orgânica aplicada (COV), realizado utilizando-se a mesma metodologia. De maneira diferente, os reatores a 37°C apresentaram acúmulo de ácidos graxos voláteis (AGV), o que levou a uma remoção superior da matéria orgânica a 20°C. A família *Anaerobaculaceae*, presente apenas a 37°C, foi sugerida como intimamente ligada aos altos níveis de AGV nesses reatores. Diferentemente do primeiro experimento, as principais famílias envolvidas em ambas as temperaturas foram *Ectothiorhodospiraceae, Syntrophorhabdaceae, Dethiosulfovibrionaceae* e *Synergistaceae,* apresentando diferentes abundâncias. Em conjunto, esses resultados fornecem dados sobre a regulação de comunidades biológicas complexas pela temperatura e COV em reatores anaeróbios usados para tratar águas residuárias do processamento de peixes.

Palavras-chave: digestão anaeróbia, lodo, sequenciamento metagenômico, comunidade microbiana, águas residuárias de processamento de pescado.

ABSTRACT: The increase of fish production worldwide results in a consequent increase of generated effluents by processing industries. Due to its composition, this type of wastewater requires particular treatment solutions. Several alternatives have been suggested, such as the use of biological processes for the degradation of organic matter through microorganisms. However, studies on microbiome from anaerobic reactors sludge treating fish processing wastewater are still scarce. In order to evaluate temperature effects on anaerobic digestion treatment of fish processing effluents, replicated pilot-scale anaerobic reactors were operated at 20°C and 37°C for 60 days, being evaluated through physicochemical analysis and a metagenomic approach. The results demonstrated that 37°C reactors were statistically superior from day 50 in chemical oxygen demand (COD) removal and the enrichment of hydrolytic and acidogenic *Porphyromonadaceae*, *Rikenellaceae*, and *Spirochaetaceae* families may have contributed to 37°C reactors improved performance. However, to a better understanding of the process, a second experiment trial was designed to evaluate not only the temperature, but the influence of the applied organic load rate (OLR), which was done using the same methodology. In a different way, the 37°C reactors showed volatile fatty acids (VFA) accumulation, which lead to superior organic matter removal at 20°C. The *Anaerobaculaceae* family, present only at 37°C, was suggested as closely linked to high VFA levels in these reactors. Differently from the first trial, the main involved families in both temperatures were *Ectothiorhodospiraceae*, *Syntrophorhabdaceae*, *Dethiosulfovibrionaceae* and *Synergistaceae*, appearing with different abundances. Taking together, these results provide insights about the regulation of complex biological communities by temperature and OLR in anaerobic reactors used to treat fish processing wastewater.

Keywords: anaerobic digestion, sludge, metagenomic sequencing, microbial community, fish processing wastewater.

1. GENERAL INTRODUCTION

Agribusiness related activities are often pointed out by their polluting potential to the environment. Due to their scarcity, water resources are increasingly drawing attention of society and the scientific community to its preservation, which translates into the research for the effective control of the factors and processes that lead to water contamination. The use of water in animal production systems has changed a lot over the last decades, due to factors such as the development of different management techniques and expansion of the food industry.

Regardless of the animal species and the type of production, agricultural activities always generate residues, which may compromise the environment on a lesser or greater degree. Physicochemical characteristics of this kind of wastewater are highly variable and should be evaluated in a particular way, however, in general they have diverse contaminants in soluble and particulate form, presenting high organic load. Thus, recognizing the importance of water for the maintenance of life, agrarian systems that generate excessive waste or water consumption will become unviable on our planet.

In this context, evaluating fisheries and aquaculture activities nowadays, it is observed that they are one of the most-traded food commodities worldwide. Fish production and exportation are essential to the economy of many developing countries, being the growth of this market remarkable during the past years. However, despite trade expansion and technological advances in preservation, transportation and processing, the sector still has a lot of challenges. One of them refers to the sustainable development, which includes the management of effluents generated by the fish processing industry.

About fish processing wastewater, proteins and lipids are the main components of these effluents, with their variations related to some factors such as the unit process, water quality and processed fish species. For the maintenance of the environment and public health, waste from these activities must be treated and final disposed in order to have the lowest impact as possible. Therefore, biological processes that aim at the degradation of organic matter have been suggested as an alternative for the treatment of these kind of wastewater, being some anaerobic alternatives suggested.

Anaerobic digestion is a process that reduces biomass and can mitigate an ample aspect of environment problems. The process is performed in reactors, which are closed environments designed according to system-related variables. The digestion can be severely affected by some factors, which may be related to system operating conditions or environment variations. These interferences occur due to the intensification or inhibition of certain parameters, such as the growth rate and microbial decay and the consumption of the substrate with the consequent formation of products from biochemical reactions. Since this is a biological process, it is essential that the active microorganisms have suitable environmental conditions for their development, so that

they can perform an efficient conversion of the organic matter. Therefore, it is extremely important that these critical factors are properly monitored.
From the factors that have great impact of anaerobic digestion, temperature and organic load rate (OLR) are commonly cited as important to the dynamics of the process. Temperature in one crucial factor, once it influences the thermodynamics and the microbial population, being a key determinant of its composition. Brazil is a country of continental dimensions, subject to a wide temperature range throughout the year. As the reactors remain in external areas and are subjected to ambient temperature, the climate in the different seasons directly affect the system. Similarly, OLR is a parameter of great influence, being related to organic matter concentration and influence the structure of microorganisms. As microbial species respond differently to changes in temperature and OLR, their study can bring substantial data about how involved communities are altered while are treating different effluents categories.

Currently, studies have offered important contributions to optimize this type of treatment and present it as a technically feasible and economically advantageous alternative. Although being commonly used in the treatment of urban sewage treatment, the application of anaerobic processes in the treatment of effluents from fish slaughterhouses is incipient. In this context, the evaluation of impacts of different temperatures and OLR in the microbial communities from anaerobic reactors treating fish processing wastewater can provide more accurate data and thus lead to a better understanding of the process. Therefore, comprehend the mechanisms that rule these microbial interactions is fundamental to the development of process management strategies and may allow suggestions for future research.

2. OBJECTIVES

This study aims to investigate and evaluate pilot scale reactor performances and the structure of sludge microbial communities involved in the anaerobic digestion process treating effluents from the fish processing industry, subjected to different temperatures and organic load rates, through physicochemical analysis and metagenomic sequencing.

3. CHAPTER 1. LITERATURE REVIEW

3.1. AQUACULTURE PROSPECTS

With the continuously increase in world population, in the next decades developed and developing countries will face some challenges to meet the enhanced food demand. According to projections of the Food and Agriculture Organization of the United Nations (FAO), it is estimated that the world population will be around 9.7 billion people by 2050. To feed this growing number of individuals, annual meat output is expected to raise considerably (FAO, 2018). In this scenario, Brazil, one of the main players in the international meat market, along with China, the European Union and the United States, is expected to be repositioned, not only in the production of beef, pork and poultry, but also in the fish aquaculture (IPEA, 2017).

In the last 50 years, the annual global growth in fish consumption exceeded the population increase in the same period, with the fish per capita consumption raising from 9.9 kg per year in the 1960s to 20.3 kg per year in 2016. This was favored by several factors, such as the expansion of urban areas with more efficient storage, distribution and marketing, in addition to the significant development of aquaculture itself. Besides that, tastes of modern consumers are also characterized by an emphasis on healthy living, and fish is a source of high biological value proteins and unsaturated fatty acids. Currently, it is estimated that fish represents 17% of all animal protein consumed by humans on the planet and 7% of all protein, considering animal and vegetable origins (FAO, 2018; Brabo et al., 2016).

In response to the demand, global fish production reached about 171 million tonnes in 2016, being 47% of this value obtained through aquaculture (FAO, 2018). Brazil is a country with great aquaculture potential, since it has 8500 km of coastline, 13.7% of all freshwater available on the planet, tropical climate in most territory and a significant grain production (Brabo et al., 2016; Suplicy, 2007; Garreta, 2003). Confirming this, data published in 2017 by the Brazilian Institute of Applied Economic Research (IPEA) showed that although Brazil is a major producer of chicken, cattle and pigs, aquaculture was the meat sector that presented the highest percentage increase in production between 2004 and 2014, with an average annual growth of almost 8%, against 5.1% for cattle, 4.1% for chicken and 2.9% for pigs (IPEA, 2017; Kubitza, 2015).

Considering production values, Brazilian pisciculture reached 485.2 thousand tons in 2017. Among states, Paraná headed the list with 20.2% of the total production in the country (98 thousand tons). São Paulo, in turn, produced 47.5 thousand tons, becoming the second in the ranking. In Rondônia, a state that had significant values in 2016, production fell by 56% in 2017, being at the third position. Thus, it can be observed that the North region declined, but Northeast, South and Central-West regions increased their participation (Carvalho Filho, 2018; IBGE, 2017). For a better understanding of each federation unit role, their individual productions are described in Table 1.

Table 1. Fish production in the Brazilian states in 2017 and its percentage variation in relation to the previous year.

State	Production		Variation to 2016 (%)
	Total (tonnes)	%	
Paraná	98.004	20.2	+28.84
São Paulo	47.539	9.8	-1.67
Rodônia	39.884	8.2	-56.0
Mato Grosso	36.609	7.5	-9..41
Santa Catarina	31.796	6.6	-8.38
Minas Gerais	31.327	6.5	-4.50
Maranhão	27.775	5.7	+13.71
Pernambuco	20.594	4.2	+213.03
Mato Grosso do Sul	18.041	3.7	+161.81
Goiás	16.502	3.4	+6.66
Bahia	16.038	3.3	+49.04
Rio Grande do Sul	13.741	2.8	-6.45
Pará	12.164	2.5	-5.77
Tocantins	11.542	2.4	+20.93
Alagoas	10.970	2.3	+150.97
Ceará	10.229	2.1	-41.11
Piauí	9.379	1.9	-10.45
Roraima	9.379	1.9	-10.45
Amazonas	7.574	1.6	-64.07
Acre	3.899	0.8	-11.73
Espírito Santo	3.737	0.8	-30.23
Sergipe	2.690	0.6	-13.73
Paraíba	2.394	0.5	+12.39
Rio Grande do Norte	2.172	0.4	-9.12
Rio de Janeiro	1.402	0.3	-12.92
Distrito Federal	820	0.2	-23.00
Amapá	754	0.2	-23.00

Source: IBGE, 2017.

Currently, Nile Tilapia (*Oreochromis niloticus*) is the most cultivated species (58.4% of the total) and the main producing states are Paraná, São Paulo and Minas Gerais. Some features such its adaptation to different environments, the superior results achieved in intensive systems and the demand, have led Tilapia to be the most important species in the national production. Tambaqui (*Colossoma macropomum*) remained the second most cultivated fish species in the country, with a participation representing 18.2% of the total. Even with the mentioned drop in overall production, the North Region continued to be the largest producer of Tambaqui, with prominence for Rondônia, Maranhão and Roraima (IBGE, 2017; IPEA, 2017; Brabo et al., 2016; Kubitza, 2015).

The number of industries destined to fish processing has increased significantly over the last years. These establishments include slaughterhouses, which in its turn demand inspection services at municipal (SIM), state (SIE) or federal (SIF) level, defined by the Brazilian sanitary legislation according to the geographical area where products will be marketed (Brasil, 2017; Kubitza e Campos, 2006). Both large industrial conglomerates and smaller enterprises currently exist in the aquaculture sector. However, there is a trend towards the regionalization of the activities of food production and inputs, leading to the use of local and regional market niches, with better logistics and lower distribution costs (Kubitza, 2010). Therefore, fish industrialization process valorizes an extremely perishable raw material, increasing the shelf life of the product and thus bringing new consumption options (IPEA, 2017).

Although positive aspects of Brazilian fish production, there are challenges in developing the chain as a whole in order to sustain its growth. A fundamental point concerns to waste management, since the increase of fish production consequently leads to an augment of the residues from activities inherent to aquaculture, such as slaughter and processing. With the scarcity of natural resources observed nowadays, this production must be based in sustainable practices due to its pollution potential (FAO, 2018; IPEA, 2017).

3.2. ANAEROBIC DIGESTION

Anaerobic systems have been applied for the treatment of diverse types of effluents (urban, industrial and agricultural), including fish processing wastewater (Lin et al., 2019; Suárez et al., 2018; Maragkaki et al., 2017; Reynaud and Buckley, 2016; Duda et al, 2015; Chowdhury et al., 2010; Palenzuela-Rollon et al., 2002). Aiming the sustainability at fish production, anaerobic processes allies the development of the sector, however, there is still a demand for studies related to the various possibilities of applying this technology in aquaculture industry. Thus, additional research is needed regarding different processes and fish species, which generate effluents with diverse biodegradability characteristics.

The study of anaerobic microorganisms and the desired conditions for the methane formation obtained remarkable growth from 1930, since then, different aspects regarding anaerobic digestion have been discovered. In the past, the so-called classical systems, such as Imhoff tanks, septic tanks and anaerobic ponds had a low efficiency (Van Haandel et al., 2006). However, in recent decades, scientific publications on the subject have grown worldwide, leading to a development of diverse anaerobic reactors configurations aiming to maximize the efficiency of treatment plants. These new configurations, like the upflow anaerobic sludge blanket (UASB), the expanded granular sludge blanket (EGSB), the anaerobic filter (AF) and the anaerobic baffled reactor (ABR) suggest the containment of a large anaerobic bacterial immobilized mass (attached or not to a carrier material) and the promotion of intensive contact between the influent material and the microbial community (Nguyen and Dao, 2012;).

The anaerobic digestion process is based on the biological degradation of organic matter in the absence of oxygen by microorganisms, where complex molecules are converted into simpler compounds such as methane (CH_4) and carbon dioxide (CO_2). A series of sequential reactions occurs, and the by-products of one reaction become the raw material of the subsequent reactions, which promotes a chemical and biological balance between the different microbial populations (Calusinka et al., 2018; Shah et al., 2014; Manyi-Loh et al., 2013; Chowdhury et al., 2010; Chernicharo, 2007).

The involved microorganisms obtain energy through cellular processes of anaerobic respiration and fermentation. A huge number of microorganisms are involved in the anaerobic process and diverse species have distinct roles in a collective organization, forming an extremely specialized and complex microbiome (Campanaro et al., 2018). The main reactions to energy generation occurring under anaerobic conditions are:

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \text{ (Hydrogenotrophic methanogenesis)} \quad (1)$$

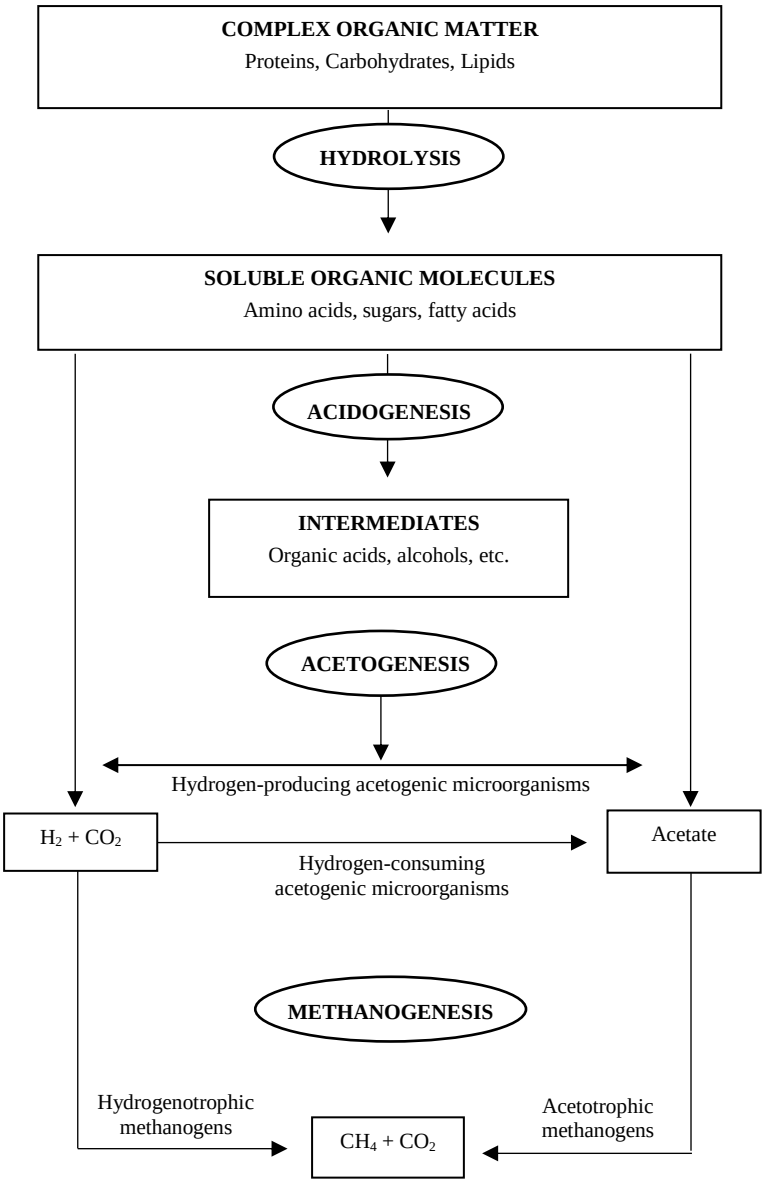
$$CH_3COOH \rightarrow CH_4 + CO_2 \text{ (Acetotrophic methanogenesis)} \quad (2)$$

$$CH_3COOH + SO_4^{-2} + 2H^+ \rightarrow H_2S + 2CO_2 + 2H_2O \text{ (Sulfate reduction)} \quad (3)$$

3.2.1. Stages

The anaerobic digestion is subdivided into four main phases: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Campanaro et al., 2018; Meegoda et al., 2018; Bozan et al., 2017; Shah et al., 2014; Manyi-Loh et al., 2013; Chernicharo, 2007; Van Haandel and Lettinga, 1994; Metcalf and Eddy, 1991). They are illustrated in Figure 1 and its main aspects are described below.

Figure 1. Stages of anaerobic digestion.



Source: Adapted from Van Haandel and Lettinga, 1994.

3.2.1.1. Hydrolysis

In the hydrolysis, phylogenetically diverse microorganisms convert polymerized organic compounds (carbohydrates, proteins and fats) into monomers and dimer (monosaccharides, amino acids, and fatty acids), acting as hydrolytic enzyme producers and end-product utilizers. Hydrolytic bacteria may be strict anaerobes or facultative

(Meegoda et al., 2018; Frankewhittle et al., 2014; Shah et al., 2014; Manyi-Loh et al., 2013).

Under anaerobic conditions, the organic substrate is used at the same time as electron acceptor and donor, being one part of the compound oxidized while the other is reduced. The fermentation of 1 mole of glucose by this process produces 2 moles of pyruvic acid, being its formation involved in the generation of 2 moles of NADH, the reduced form of the NAD^+ electron carrier. As the amount of NAD^+ is limited, anaerobic microorganisms recycle the electron carrier, transferring them to pyruvate, which leads to the formation of several reduced compounds (e.g. propionate, butyrate, ethanol), depending on the type of microorganism and the involved environmental conditions (Aquino and Chernicharo, 2005).

The hydrolysis rate depends on some parameters, such as size of particles, pH, production and adsorption of enzymes (Shah et al., 2014). As hydrolysis is essential to increase the substrate access, this step can be limiting in the process, especially when hardly decomposable polymers are present (Meegoda et al., 2018; Shah et al., 2014). According to Shrestha et al. (2017), anaerobic hydrolytic bacteria can be found within the phyla *Firmicutes*, *Bacteroidetes*, *Fibrobacteres*, *Spirochaetes* and *Thermotogae*. Although these groups are common, each situation should be evaluated separately, since the composition of the involved microorganisms is reflected by other parameters, like the used substrate.

3.2.1.2. Acidogenesis

In this phase, monomers from hydrolysis are absorbed by acidogenic microorganisms, being metabolized and excreted as even simpler compounds. These products include organic acids (formic, acetic, propionic, butyric, and pentanoic), alcohols (methanol, ethanol) and various inorganic compounds, such as CO_2, hydrogen (H_2), H_2S (hydrogen sulfide) and NH_3 (ammonia). Their concentrations may depend on the reactor operations, since volatile fatty acids (VFA) concentrations can fluctuate significantly at different conditions (Meegoda et al., 2018; Shah et al., 2014; Manyi-Loh et al., 2013).

Acidogenesis is commonly referred as the fastest of all anaerobic digestion stages, since acidogenic bacteria have a regeneration time of less than 36 hours. This characteristic should be emphasized, once the VFA production can lead to acidification of the reactors, a commonly reported factor linked to operational failures. Furthermore, it is important to highlight that in protein-rich wastes, such as fish processing wastewater, VFA may be produced from amino acids, which are usually degraded in pairs via the Stickland reaction. The single amino acid degradation is also possible when hydrogenotrophic microorganisms are present (Meegoda et al., 2018; Deublein and Steinhauser, 2008).

3.2.1.3. Acetogenesis

Acetogenesis depicts the efficiency of biogas production, since approximately 70% of methane arises of acetate reduction. In this step, acetogenic microorganisms convert VFA, alcohols and other compounds into acetate, CO_2 and H_2, which serve as substrate for the methane formation. For this purpose, there are two mechanisms, denominated acetogenic dehydrogenation and acetogenic hydrogenation (Frankewhittle et al., 2014; Shah et al., 2014; Deublein and Steinhauser, 2008; Rincón-Mejía and Heras, 2008; Aquino and Chernicharo, 2005).

The acetogenic dehydrogenation process involves acetogenic bacteria that are H_2 producers and needs low hydrogen concentration for their survival. When the hydrogen partial pressure is adequate, they convert acidogenesis end products into acetate, CO_2 and H_2. These microorganisms live in a syntrophic relationship with hydrogenotrophic methanogens, which constantly consumes hydrogen, keeping a suitable hydrogen partial pressure for acetogenic bacteria. The presence of syntrophic microorganisms is essential for efficient anaerobic digestion performance, since they prevent acids accumulation (Meegoda et al., 2018; Frankewhittle et al., 2014; Shah et al., 2014; Deublein and Steinhauser, 2008). On the other hand, in the acetogenic hydrogenation process the acetate is produced from hexoses, CO_2 and H_2, however, the metabolic activity of these bacteria is independent of syntrophic relations (Rincón-Mejía and Heras, 2008)

3.2.1.4. Methanogenesis

Methanogenesis is the final stage of anaerobic digestion, where CH_4 formation takes place under strictly anaerobic conditions. Acetate and H_2 produced in the previous steps are converted into CH_4 and CO_2 by methanogenic microorganisms, which may be acetotrophic or hydrogenotrophic (Meegoda et al., 2018; Shah et al., 2014; Manyi-Loh et al., 2013; Deublein and Steinhauser, 2008; Aquino and Chernicharo, 2005). These processes can be described according to the following equations:

$$CH_3COOH \rightarrow CH_4 + CO_2 \text{ (Acetotrophic methanogenesis)} \quad (4)$$

$$4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \text{ (Hydrogenotrophic methanogenesis)} \quad (5)$$

Acetotrophs are heterotrophs, producing CH_4 and CO_2 from the reduction of acetate. Although they are more important, these microorganisms are slow growing, with a minimum generation time of 2 to 3 days, being also extremely dependent on the maintenance of optimum growth conditions (Meegoda et al., 2018; Shah et al., 2014; Aquino and Chernicharo, 2005). In turn, hydrogenotrophic microorganisms are autotrophs, reducing CO_2 to CH_4 and using O_2 (from CO_2) as acceptor and H_2 as electron donor, forming water molecules. These microorganisms have faster growth, with a minimum generation time of 6 hours. In addition to contribute about 30% of the methane formation, its presence helps to maintain low hydrogen pressures, thus

favoring VFA degradation (Meegoda et al., 2018; Shah et al., 2014; Rincón-Mejía and Heras, 2008; Aquino and Chernicharo, 2005).

It is observed that methanogenic microorganisms depend on the substrate provided by the acetogenic, which in turn are dependent on the acidogenic and the hydrolytic ones, thus establishing a mechanism of interactions between these groups. Thus, in a balanced anaerobic treatment system, the products generated in one step are converted to the next without significant accumulation of intermediate products. The overall conversion efficiency of organic matter depends on the efficiency of each reaction and the balance between the various species of microorganisms. When these populations are in disequilibrium or in unfavorable environmental conditions (which lead to the reduction of methanogenic activity), acid accumulation may occur. This fact can cause acidification of the reactor content, being a common cause of operational failure in anaerobic treatment systems (Chernicharo, 2007; Aquino and Chernicharo, 2005; Van Haandel & Lettinga, 1994).

3.2.1.5. Applications in the treatment of fish processing effluents

Some researchers have evaluated the performance of anaerobic processes for the treatment of wastewater from different types of fish processing industry, finding different results. Puñal and Lema (1999) studied the start-up and optimization of a 380m^3 UASB reactor treating wastewater from a fish-canning industry. the efficiency of the system proved to be highly dependent on the nature of the wastewater canned product (mussel, tuna and sardines). The reactor had better when treating mussel and tuna cooking wastewaters jointly, due to its higher degradable carbohydrate content. Although relatively high values of VFA detected, the IA/PA ratio was always maintained lower than 0.3.

In its turn, Achour et al. (2000) designed a treatment plant including a physical pre-treatment unit, an anaerobic digester and an activated sludge system to treat tuna processing effluents. They founded different efficiencies when evaluating the units in a particular way. The anaerobic system transformed 45% of the dissolved COD into methane gas, the pre-treatment removed 40% and the activated sludge reduced 85% of the COD. Thus, an integrated system combining steps allows the removal of up to 95% of the COD with minimal energy consumption and sludge production.

According to Palenzuela-Rollon et al. (2002) the application of UASB reactor was a promising treatment option for fish processing wastewater. They evaluated this system to treat effluents with different lipids levels, artificially generated simulating the canning of sardines and tuna. In treating a low lipid wastewater (203–261 mg/L, 9% of total COD), the COD removal were 78%, however, wastewater with a higher lipid content (47% of the total COD), the COD were 92%, being a considerable part of the influent total COD removed via adsorption on reactor surfaces and sludge particles. Indeed, as the adsorption of lipids on sludge particles threatens the stability of the UASB operation, the authors recommended a two-step UASB system in these cases.

Nguyen and Dao (2012) carried out a pilot scale anaerobic reactor to treat diluted fish processing wastewater (1000±50 mg L^{-1}). The medium removal efficiency for COD and BOD was 90 and 92%, respectively. The results demonstrated that the system may be used as an effective treatment alternative in tropical regions. They also studied diverse HRT (4 – 24 hours) under an OLR of 4.0 kg $CODm^3d^{-1}$ and concluded that the optimum retention time within this load was 6 hours.

Furthermore, some fish processing effluents has a large amount of salts (mainly NaCl). As several other factors, the presence of high sodium concentrations can interfere in the anaerobic treatment, since methanogenesis is strongly inhibited by a sodium concentration above 10 gL^{-1} (Lefebvre and Moletta, 2006). Omil et al. (1995) using an anaerobic contact system treating fish processing effluent showed that the adaptation of an active methanogenic biomass at high salinity level was possible with a suitable strategy. Anyhow, even though biological treatment may be feasible at high salt concentrations, the performance depends on a proper adaptation of the biomass (Lefebvre and Moletta, 2006).

It is observed that in developing countries, such as Brazil, there is a large diffusion of small scale slaughterers to serve regional markets, and they need low cost solutions for the treatment of effluents. With the expansion of fish processing industries and the concern with its pollutant potential, biological treatment alternatives have been increasingly targeted for research. In addition, due to growing concern about water use, there is a trend that global environmental legislation will become more restrictive in the coming years. There are several classes of effluents attributed to this industry, with greater or lesser degree of contamination. Thus, it is inappropriate to generalize them due to the variables inherent in the process, such as the processing unit and the fish species. Therefore, the specific study of the various types of effluents in this industry through the search for viable low-cost alternatives for its treatment are extreme important for the plants to remain competitively in business.

3.2.2. Interfering factors in anaerobic digestion

Anaerobic digestion can be seriously affected by key determinants that may be related to the operating conditions or to environmental variations. These interferences are due to the intensification or inhibition of some parameters, such as the growth rate and microbial decay and the consumption of the substrate. Therefore, it is extremely important that these critical factors are properly monitored (Beale et al; 2016; Zhang et al, 2014).

3.2.2.1. Temperature

Temperature is one of the most significant parameters that influence anaerobic digestion, since it can directly influence the thermodynamics of the reactions (Lin et al, 2017; Lin et al, 2016; Beale et al; 2016; Zhang et al, 2014). Conventionally, anaerobic bacteria grow at psychrophilic, mesophilic and thermophilic conditions, however, some

authors report that mesophilic and thermophilic operation has higher rates of metabolic activity, higher biogas production and improved destruction of pathogens (Liu et al., 2018; Vanwonterghem et al, 2015; Zhang et al, 2014; Manyi-Loh et al., 2013). In areas with low temperatures or high thermal variation, anaerobic treatments can be impaired, which makes the process more feasible in tropical and subtropical countries, since the artificial increase in temperature generates considerable additional costs. Thus, as important as operating at optimum temperatures is the prevention of abrupt changes in the parameter, once temperature oscillations can also lead to an imbalance between the involved microbial populations (Keating, 2018; Ping et al, 2018; Lin et al, 2016; Manyi-Loh et al., 2013). Furthermore, the effects of temperature variation on the sludge of anaerobic reactors treating effluent from the fish processing industry has not yet been evaluated.

3.2.2.2. Alkalinity, VFA and pH

Alkalinity, VFA and pH also influence directly the anaerobic digestion process. The pH affect the enzymatic activity and toxicity of numerous compounds, such as ammonia and sulfide. In normal conditions, the pH range of anaerobic digestion occurs near neutral values, being low pH associated with high concentrations of VFA, which may lead to methanogenesis failures (Zhang et al, 2014; Manyi-Loh et al., 2013; Chernicharo, 2007). The alkalinity prevents variations in pH and its measurement evaluates the acid neutralization capacity. In anaerobic reactors monitoring, the systematic verification of alkalinity and VFA is as important as the pH evaluation, since pH values vary in logarithmic scale, meaning that small reductions in pH imply the consumption of high amount of alkalinity, decreasing the buffering capacity (Frankewhittle et al., 2014; Chernicharo, 2007; Van Haandel e Lettinga, 1994). When the anaerobic digestion is stable, methanogenic microorganisms use VFA as they are formed, on the contrary, there will be an VFA accumulation in the system. When this occurs, the alkalinity of the medium is rapidly consumed, and the pH of the system is reduced. Thus, the interaction between alkalinity, pH and VFA expresses the dynamic equilibrium of an anaerobic digestion system (Zhang et al, 2014; Chernicharo, 2007).

3.2.2.3. Organic load rate

Another fundamental parameter that interferes in the process is the OLR, which refers to the amount of organic matter to be fed daily in a reactor. Overloading an anaerobic system may cause it to be rapidly hydrolyzed, favoring an accumulation of VFA Reactor acidification is one of the most common reasons for process deterioration (Braz et al., 2019; Meegoda et al., 2018; Frankewhittle et al., 2014; Manyi-Loh et al., 2013). Furthermore, it does not appear to be possible to define an exact VFA value to indicate the state of an anaerobic process. This is due to characteristics of each system, since in certain reactors different levels of VFA may or not cause instability. Although there are great similarities, it is important to evaluate digesters in a particular way (Frankewhittle et al., 2014).

3.2.2.4. Hydraulic retention time

Hydraulic retention time (HRT) is other important operating conditions that may affect the composition of the microbial community and should be periodically monitored in anaerobic reactors (Win et al., 2016; Ziganshin et al., 2016; Manyi-Loh et al., 2013). This parameter is calculated from the working volume of the reactor, divided by the daily feed volume of the substrate, so it is closely related to the organic load rate (OLR) and defines the average residence time of the substrate in the system. In addition, HRT is one of the main parameters for determining the size of the reactor and consequently the costs.

3.2.2.5. Others

Besides that, when present at concentrations exceeding a critical value, some chemical compounds may be biologically toxic to the anaerobic process. The toxicity of a chemical compound is relative, since depending on the concentration, the same substance may or may not be toxic. The sensitivity of an anaerobic processes depends considerably on the HRT, being that the higher this parameter, more a reactor can assimilate toxic loads. Various substances are considered toxic to the anaerobic digestion process, such as ammonia, sulfide, nitrate and heavy metals (Braz et al., 2019; Zhang et al., 2014; Manyi-Loh et al., 2013; Chernicharo, 2007; Omil et al., 1995).

3.3. ANAEROBIC SLUDGE MICROBIOME ANALYSIS

Microorganisms are the most abundant organisms on Earth, have high phylogenetic and metabolic diversity and are responsible for several processes in the existing geochemical cycles (Singh et al., 2009). The knowledge of the predominant communities in the sludge of anaerobic reactors are of great relevance for the design and optimization of wastewater treatment systems, since microorganisms are responsible for promoting the degradation of many compounds (Bozan et al., 2017; Plugge, 2017; Ibarbalz, et al., 2016; Ma et al., 2016; Win et al., 2016). However, despite their importance, the contribution of many microbial species, as well as its interactions, remains unknown, mainly due the fact that vast number of microorganisms is difficult to cultivate by classical microbiological techniques (Campanaro et al., 2018; Jünemann et al., 2017; Madhavan et al., 2017; Stewart, 2012).

This limitation has hampered the knowledge about the subject until the last decades, when molecular tools were developed to prospect data about the potential of microbial communities and their activity in different ecosystems, like soil, ocean water, the human body and wastewater treatment plans (Bozan et al., 2017; Jünemann et al., 2017; Madhavan et al., 2017; Heather and Chain, 2016). These tools include clone library of 16S rRNA genes (Sekiguchi et al, 1998; Schuppler et al., 1995), denaturing gradient gel electrophoresis (DGGE) (Aydin et al., 2015; Hwang et al., 2008; Temmerman et al., 2003) and fluorescence in situ hybridization (FISH) (Benova and Wanner, 2013; Yilmaz et al., 2010; Amann et al., 2001). However, these methodologies only provided

limited information comparing to the high-throughput sequencing based metagenomic approaches that were emerging (Jünemann et al., 2017; Yang et al, 2014).

Metagenomics is a technique that allows the study of deoxyribonucleic acid (DNA) from an environmental sample, which applies a suite of genomic technologies and bioinformatic tools to access an entire or partial community of microorganisms (Madhavan et al., 2017; Sudarikov et al., 2017; Thomas et al., 2012). It allows the recovery of genetic material without any cultivation techniques, making possible to reveal unknown genetic content of complex microbial communities, obtain information about novel enzymes, generate hypotheses of microbial function and infer genomic linkages between function and phylogeny of uncultured organisms (Pyzik et al, 2018; Jünemann et al., 2017; Madhavan et al., 2017; Sidhu et al., 2017; Sudarikov et al., 2017; Ma et al., 2016; Ibarbalz, et al., 2016; Thomas et al., 2012). The method is performed through next generation sequence (NGS), where extracted DNA is fragmented and sequenced, being each sequencing reaction recorded separately. Different from traditional Sanger's capillary electrophoresis (first-generation sequencing), NGS instruments can carry out millions of sequencing reactions in a high-throughput massively parallel manner, being able to detect all the sequencing reads in a single machine run (Besser et al., 2018; Jünemann et al., 2017; Hui, 2014).

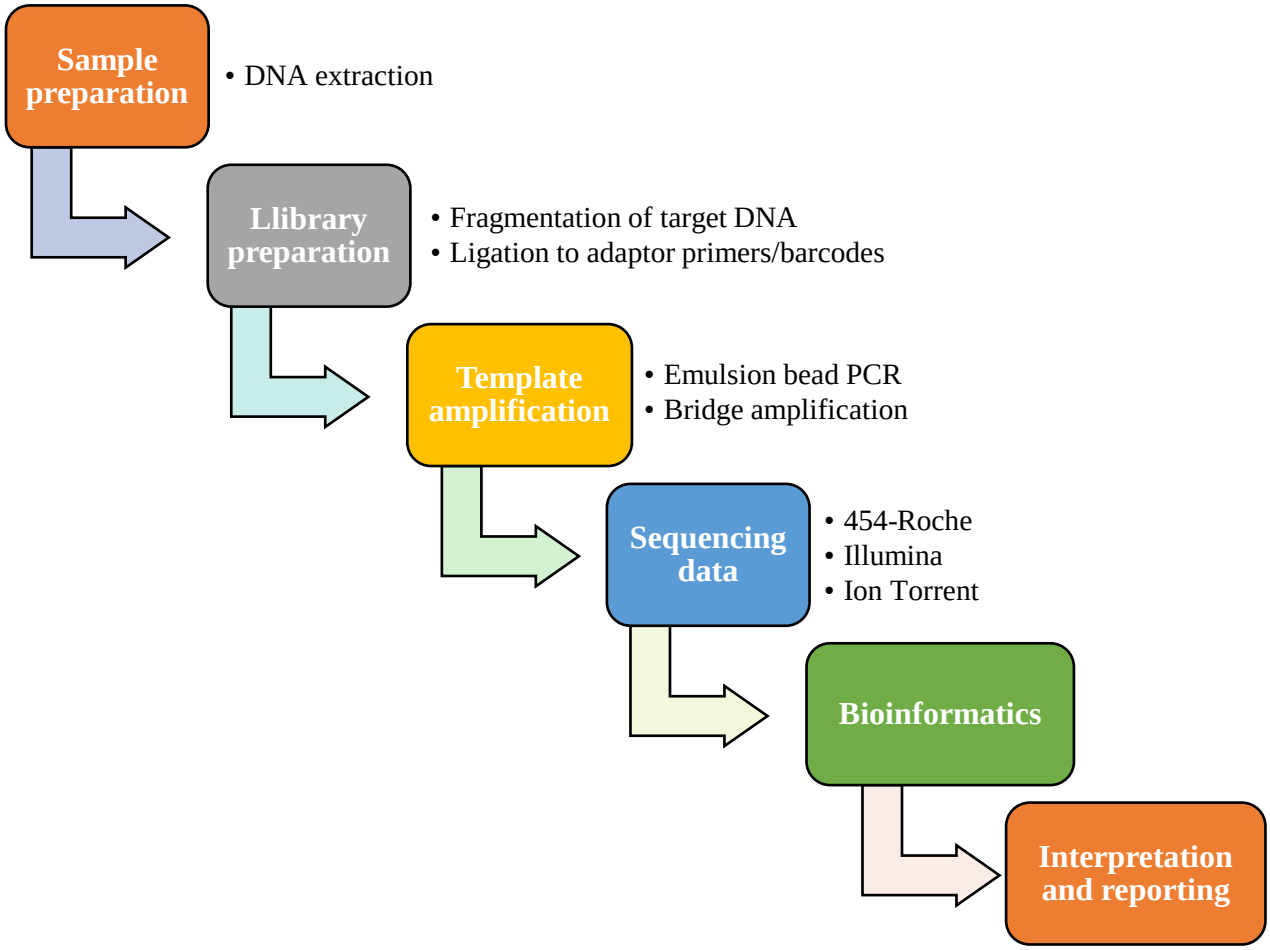
In recent years, several new sequencing technologies providing cheaper, faster, and higher-throughput sequencing have emerged, increasing the feasibility of metagenomic projects (Bragg and Tyson, 2014). There were also a rapid and substantial cost reduction in NGS, which favored the sequence-based metagenomics development, enabling the execution of diverse types of research (Bozan et al, 2017; Jünemann et al., 2017; Madhavan et al., 2017; Ibarbalz, et al., 2016; Techtmann and Hazen, 2016; Thomas et al., 2012).

Even with these advances, it seems plausible to assume that sludge communities are much more diverse from what is currently described and still poorly characterized (Pyzik et al, 2018). Attempting to facilitate the development of more efficient full-scale anaerobic digestion systems, recently several high-throughput sequencing technologies such as 454 pyrosequencing (Koo et al., 2017; Ning et al., 2014), Illumina (Pyzik et al, 2018; Ma et al., 2016; Guo et al., 2015; Yang et al., 2014) and Ion Torrent sequencing (Gwin et al., 2018; Sidhu et al., 2017; Lefevre et al., 2016) have been applied as promising methods to characterize the phylogenetic composition and functional potential of the complex community from sludge samples on pilot and full scale digesters. Each technology has benefits and drawbacks, being the sequencing device very important to success achievement in a metagenomic study (Bragg and Tyson, 2014). It is very important to consider the impact on data quality and relative abundance of taxa when selecting an NGS platform aiming reproducibility and consistency between similar projects (Allali et al., 2017). Therefore, a brief description of used platforms for metagenomics is provided below.

3.3.1. Next generation sequencing Technologies

In 2005, the introduction of the Roche 454 device began the NGS revolution. Since then, many other NGS technologies have emerged, being often referred as second and third generation sequencing according to their years of availability and chemistry (Besser et al., 2018; Jünemann et al., 2017; Bragg and Tyson, 2014; Bronner et al., 2014; Hui, 2014). The platforms differ substantially in terms of their engineering, output (length of reads, number of sequences), accuracy and cost (Besser et al., 2018). Even though their differences, the workflow has similar steps (Figure 2), including DNA extraction; library preparation, which comprise fragmentation of target DNA (either mechanically or enzymatically) and the ligation of the fragments to adaptor primers/barcodes; template preparation by emulsion bead PCR or bridge amplification and the automated sequencing (Besser et al., 2018; Hui, 2014).

Figure 2. Summary of workflow possibilities in next generation sequencing.



Source: Adapted from Besser et al., 2018.

After the sequencing reaction, millions of reads are generated. Bioinformatics analysis generally involves sequence file conversion to readable files, and sequence alignment with reference DNA for final variant identification and annotation. For accuracy and interpretation, is critical to have sufficient coverage of interest regions (Hui, 2014).

3.3.1.1. 454-Roche

The Roche 454 platform (GS20, GS FLX, GS FLX Titanium, GS FLX+) implements the sequencing-by-synthesis approach, where DNA synthesis is monitored in real time (Bragg and Tyson, 2014; Harrington et al., 2013; Liu et al., 2012). DNA templates are affixed to microbeads and amplified using emulsion PCR to achieve clonal amplification of target sequence. Beads and sequencing enzymes are then distributed into individual picoliter-sized pyrosequencing reactors (Heather and Chain, 2016; Bragg and Tyson, 2014; Bronner et al., 2014; Hui, 2014; Harrington et al., 2013). Pyrosequencing uses luciferase to generate light for detection of the individual nucleotide incorporation, being producing light proportional to the amount of pyrophosphate (Heather and Chain, 2016; Hui, 2014; Harrington et al., 201). Reads produced by this platform are significantly longer than that of Illumina, with the latest pyrosequencer producing reads up to 800 bp long. These medium-sized reads are appropriate for several applications, including metagenomics. However, 454-pyrosequencing has a lower magnitude than Illumina, being less applicable when ultra-deep coverage is required (Bragg and Tyson, 2014).

3.3.1.2. Illumina

Illumina, which purchased Solexa in 2007, developed a wide range of improvements and instruments from mid to large scale output in the following years. Among their platforms (MiniSeq, MiSeq, NextSeq, HiSeq, NovaSeq), they achieve diverse read lengths and a throughput range between multiple Gb in a few hours (Besser et al., 2018; Jünemann et al., 2017). Instead of parallelizing by performing bead-based PCR, the Illumina approach molecules of DNA are hybridized to oligonucleotides that are attached to the polymer coated glass surface of a flow cell, and it is based on sequencing by synthesis of the complementary strand and fluorescence-based detection of reversibly-blocked terminator nucleotides (Besser et al., 2018; Jünemann et al., 2017; Heather and Chain, 2016; Bragg and Tyson, 2014; Bronner et al., 2014; Hui, 2014; Liu et al., 2012). In contrast to pyrosequencing, DNA can only be extended one nucleotide at a time. After record of a fluorescent image of the incorporated nucleotide, the fluorophore is chemically removed from the DNA molecule, allowing the next cycle to occur (Hui, 2014). This is very used system due features like the high-quality total sequence obtained and the relatively low average cost per base (Bronner et al., 2014).

3.3.1.3. Ion Torrent

In its turn, the Ion Torrent platform (Personal Genome Machine, S5, Proton) adopts a novel approach to DNA sequencing (Besser et al., 2018; Bragg and Tyson, 2014; Hui,

2014). Similar to 454-Pirosequencing, beads bearing clonal populations of DNA fragments are washed over a plate followed by each nucleotide, however, instead of measuring the nucleotide incorporation by pyrophosphate release, it is done due to the difference in pH caused by the protons release (H^+ ions) during polymerization (Heather and Chain, 2016; Bragg and Tyson, 2014). The pH decrease is proportionate to the number of nucleotides that bound during the flow, and a potential change is recorded as direct measurement of nucleotide incorporation events (Bragg and Tyson, 2014; Hui, 2012).

The Ion Torrent sequencing run itself is shorter when compared to the Illumina instruments. Nevertheless, because of the nature of the semiconductor, the platform it is less able to readily interpret homopolymer sequences due to the loss of signal as multiple matching dNTPs incorporates (Besser et al., 2018; Heather and Chain, 2016; Hui, 2014). Pereira et al. (2016) compared the efficacy of Ion Personal Genome Machine (Ion PGM 400 bp Sequencing Kit and Ion Hi-Q 400 bp Sequencing Kit) and the Illumina MiSEQ Personal Sequencer (Nextera 3 rd version kit). Both Ion Torrent approaches did not show significant modifications on time and cost in manufacture protocols, however, MiSEQ showed the smallest cost per 1 Mb sequenced. Although still requiring a greater depth coverage, the final quality of the genome sequence generated with Hi-Q was closer to the data produced by the MiSEQ technology, showing a similarity in performance. Thus, with so many available competing technologies, platform distributors are constantly searching for longer, less error and greater numbers of reads (Bragg and Tyson, 2014).

3.3.1.4. Data analysis

Given the numerous variables that determine the nature of a metagenome, there is no universally applicable analysis strategy for all datasets (Bragg and Tyson, 2014). Once the sequence read files have been produced, they are analyzed using one (or more) workflows, which are tailored according to how the sample has been processed and the question being addressed. Most workflows involve aspects such as quality filtering, pair-end assembly and clustering via binning the sequencing reads into operational taxonomic units (OTU) based on their sequence similarity to each other (Hoopen et al., 2017; Mysara et al., 2017).

Integration of those single-step tools into pipelines covering the whole processing stage results in different analysis tools, which can affect the prediction of community composition and its functional capacity. Among the bioinformatic available tools, it can be cited web servers, such as Metagenomics Rapid Annotation using Subsystem Technology (MG-RAST) and EBI Metagenomics, and pipelines such as Quantitative Insights into Microbial Ecology (QIIME) and mothur, that allow researchers to perform integrated metagenomic analyses (Mysara et al., 2017; Hiraoka et al., 2016; Lindgreen et al., 2016; Plummer et al., 2015). Moreover, for the performance validation of the NGS process, environmental sequencing studies often include a low-diversity community sample with known composition, denominated ‘mock’. Theses samples may

be used to determine the overall quality of a sequencing run, as well as error rates, such as the insertion, deletion and substitution. With the use of a mock community, experimental protocols can be standardized to ensure consistency between obtained data (May et al., 2015).

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4. CHAPTER 2. EFFECT OF TEMPERATURE CHANGE ON THE EFFICIENCY AND MICROBIAL COMMUNITY IN ANAEROBIC REACTORS TREATING WASTEWATER FROM FISH SLAUGHTERHOUSE

4.1. ABSTRACT

To study temperature effects on the effluents treatment from a fish processing industry by anaerobic digestion, replicated pilot-scale anaerobic reactors were operated for 60 days at 20°C and 37°C. The results demonstrated that BOD removal efficiencies peaked on day 60, reaching 60.15% at 20°C and 61.75% at 37°C. In COD removal, 37°C reactors were statistically superior from day 50. The 20°C reactors had a higher diversity and the communities were taxonomically more similar to each other than at 37°C. Although low archaeal abundance, there was a predominance of hydrogenotrophic methanogens, mainly *Methanobacterium.* The enrichment of hydrolytic and acidogenic *Porphyromonadaceae*, *Rikenellaceae*, and *Spirochaetaceae* families at 37°C may have contributed to their improved performance in COD removal. Even though temperature altered the microbial community, stability and efficiency were similar, demonstrating that under the studied conditions anaerobic digestion could be used to treat wastewater from fish slaughterhouse at both 20 and 37°C.

Keywords: metagenomics, anaerobic digestion, fish processing, sludge.

4.2. INTRODUCTION

Modern society faces the challenge of providing food to a population expected to reach 9 billion people by the middle of the twenty-first century. Reflecting this issue, global fish consumption has increased considerably in the past few decades from 9.9 kg/capita/year in the 1960s to 20.2 kg/capita/year in 2015. In 2016, Nile Tilapia (*Oreochromis niloticus*) represented 8% of total finfish production and was the fourth most produced species in the world, demonstrating the strength of this commodity. As a consequence of aquaculture growth, there is currently an increased demand for industries for processing these products. However, due to the present recognition of the finite nature of natural resources, animal production and slaughter must be based on sustainable practices to minimize the potential pollution inherent in these activities (FAO, 2018).

In fish processing plants, the most common steps are filleting, freezing, drying, canning, smoking, and fermenting (Chowdhury et al., 2010). These activities generate effluents, which are rich in organic content due to the presence of blood and tissues, in addition to the high concentration of nutrients (Palenzuela-Rollon et al, 2002). The volume of generated wastewater and its degree of contamination varies and can be altered depending on fish species, processing unit structure and water quality used for processing. Disposal of fish processing wastewater requires careful management due to the environmental impacts that may be caused by its inadequate disposal (Chowdhury et al., 2010).

In developing countries, the expansion of small-scale slaughterhouses is fundamental to serving regional markets, and thus so is the use of low-cost technologies. Biological processes have often been recommended for the treatment of high organic matter wastewaters, such as effluents from the fish processing industry, as being more economical due to their use of optimized natural pathways (Chowdhury et al., 2010). These processes include the use of reactors inoculated with anaerobic sludge, where microorganisms utilize the available organic matter for their growth and reproduction (Vanwonterghem et al., 2015; Palenzuela-Rollon et al., 2002).

Anaerobic digestion has four steps: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Vanwonterghem et al., 2015; Lyberatos and Skiadas, 1999). These steps can be adversely affected by several factors, which may be related to the operating conditions of the system or to environmental variations. Temperature is one of the most important physical factors affecting anaerobic digestion, since it both directly influences the reactions thermodynamics and is a determinant for the composition of the bacterial populations (Keating et al., 2018; Beale et al., 2016; Vanwonterghem et al., 2015). Thus, the operating temperature regulates fermentation, leading to changes in the composition of the soluble products produced (Vanwonterghem et al., 2015).

Previous studies have compared different operating temperatures to evaluate their effects on the dynamics of the microbial communities in anaerobic reactors (Keating et al., 2018, Liu et al., 2018; Beale et al., 2016; Vanwonterghem et al., 2015). In general, it was observed that an increase in temperature can optimize hydrolysis and digestibility of the substrate, leading to a faster and more stable digestion process (Liu et al., 2018; Ping et al., 2018; Vanwonterghem et al., 2015). However, full-scale anaerobic reactors can seldom reach and maintain these temperatures, even in tropical countries. Therefore, the use of temperature regulating devices would often be necessary, which would result in a more expensive and impractical process (Keating et al., 2018; Ping et al., 2018).

The study of the anaerobic digestion of fish processing wastewater at different temperatures can provide answers regarding the use and application of this technology in industrial scale systems (Keating et al., 2018; Vanwonterghem et al., 2015; Chowdhury et al., 2010). In countries such as Brazil, that have a wide range of temperatures throughout the year, anaerobic digestion performed at ambient temperatures could be a valid alternative. This approach has some advantages: it does not require a specific heating system, can be more simply operated, and has lower costs (Ping et al., 2018).

Determining microbial community structure during bio-monitoring of an anaerobic reactor is crucial for a better understanding of the operational parameters (Keating et al., 2018). Metagenomic analyses therefore have great relevance for the optimization of wastewater treatment reactors, since they can help to establish connections between the microbial structure and the functional characteristics of the system (Guo et al, 2015; Vanwonterghem, 2015).

To better understand the effects of temperature on anaerobic digestion systems, this study aimed to evaluate the efficiency of parallel anaerobic reactors treating fish processing wastewater at two different temperatures (20°C and 37°C), simulating the mean winter and summer temperatures in tropical countries. Physicochemical and metagenomic analyses were used to address the effect of these temperatures and the microorganisms involved in the efficiency of the reactors in removing organic matter.

4.3. MATERIAL AND METHODS

4.3.1. Inocula, reactor set up, and operation

In order to observe changes in microbial community dynamics and thus verify if there are any reproducible effects on reactor performance, eight laboratory scale anaerobic reactors with a working volume of 2.1 liters were operated for 60 days semi-continuously, with a daily schedule of feeding and wasting. Reactors 1, 2, 3, and 4 were biological replicates at 20°C, representing the winter season on tropical countries, whereas reactors 5, 6, 7, and 8 were biological replicates at 37°C, representing the summer season. Temperatures were maintained externally throughout incubators.

Reactor started up consisted of inoculating 30% of the working volume (0.63 L) with sludge from a full-scale UASB (upflow anaerobic sludge blanket) reactor, used for the treatment of poultry slaughterhouse effluents, with the remaining 70% (1.47 L) consisting of raw sifted wastewater from a Nile Tilapia processing plant, located in Minas Gerais, Brazil. The reactors were then acclimatized for 10 days with daily feedings with the same wastewater used at start-up. The HRT was set at 14 days and the applied OLR was 0,08 Kg DQO/m^3.day. To maintain a fixed organic load throughout the experimental period, the fish processing wastewater was collected at a single time, frozen at -20°C, fractionated and brought to room temperature prior to each inoculation.

During the experiment, samples of raw affluent (i.e., the wastewater used for inoculation) and effluent from the reactors were collected and immediately processed for physicochemical analyses. Sludge samples were collected and stored until at -80°C until process for metagenomic analysis. Effluents and sludges were collected every 10 days during the experimental period, denoted as T1 (day 10), T2 (day 20), T3 (day 30), T4 (day 40), T5 (day 50), and T6 (day 60).

4.3.2. Physicochemical analysis

To characterize the physicochemical parameters of the raw affluent and effluents from the reactors, pH, alkalinity ratio (IA/PA), volatile fatty acids (VFA), total chemical oxygen demand (COD) and total biochemical oxygen demand (BOD) were measured. Effluent sampling and sludge collection were carried out simultaneously.

pH values were measured using a pH meter (PHS-3D, Sanxin, Shanghai, China). Alkalinity was measured by the titration method, carried out according to the methodology described by Jenkins et al., (1983) and Ripley et al. (1986). To calculate the intermediate alkalinity/partial alkalinity ratio (IA/PA), a total of 50 mL of sample was titrated with 0.02 N H_2SO_4 (sulfuric acid) to pH 5.75 (partial alkalinity) and a second-stage titration was continued until pH 4.3 (intermediate alkalinity) was reached. Samples for VFA were titrated to pH 3.3 with sulfuric acid (H_2SO_4), boiled to remove CO_2 and bicarbonates, and then back-titrated to between pH 4.0 and 7.0 with 0.05 N NaOH (sodium hydroxide) to determine the volatile acids (DiLallo and Albertson, 1961). Both COD and BOD measurements were carried out according to the Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012). To measure COD method, the sample was added to a standard potassium dichromate solution 0.04167 M and a sulfuric acid reagent (Labsynth, Brazil), followed by digestion at 150°C for 2 h. The solution was then titrated with standard ferrous ammonium sulfate 0.25 M, using ferroin solution as an indicator. The method for measuring BOD consists of filling an airtight bottle with the sample and dilution water and incubating it for 5 days at 20°C. To calculate BOD, dissolved oxygen is measured initially and after incubation, and the value is computed from this difference. Statistical significance determined using an unpaired T test and R software package (R Core Team, 2015). *P* values higher than 0.05 was considered statistically significative.

4.3.3. DNA extraction and 16S rRNA gene amplicon sequencing

For the metagenomic analyses and consequent characterization of the microbial communities present in the reactors, DNA was extracted from the 48 samples using an QIAamp Fast DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions and DNA concentrations were measured using Qubit®2.0 Fluorometer and Qubit® dsDNA BR Assay Kit (both from Life Technologies, USA).

After DNA extraction, the hypervariable V4 region of the bacterial and archaeal 16S rRNA genes were amplified using the fusion primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′) (Bokulich et al., 2013; Bokulich et al., 2012). This method uses 2 pairs of customized primers, 1 reverse primer and 96 fusion primers with barcodes for the V4 region (Ion Amplicon Library Preparation - Fusion Method, Publication Number 4468326, Revision C).

PCR reactions were prepared according to the manufacturer's recommendations (Ion Amplicon Library Preparation - Fusion Method, Publication Number 4468326, Revision C). Each reaction consisted of 1x Platinum® PCR SuperMix High Fidelity; 5 µM of each oligonucleotide primer; approximately 20–50 ng genomic DNA, and sterilized, deionized water. The cycle parameters were: initial denaturation of 3 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 58°C for and extension of 1 min/kb at 68°C. PCR products were confirmed using a QiAxcel Advanced System (Qiagen) and purified with Agencourt® AMPure XP Reagent (Beckman Coulter, USA). The

concentration of each purified amplicon was measured using a Qubit®2.0 Fluorometer and Qubit® dsDNA HS Assay Kit (Life Technologies) and equimolar amounts of the amplicons were pooled to produce a composite sample with a final concentration of 26 pM.

4.3.4. Metagenomic sequencing

The DNA extracted from the 48 samples and from two mocks, was sequenced with an Ion Personal Genome Machine (PGM) ™ Hi-Q™ View OT2 Kit (Life Technologies). PCR sample emulsion, emulsion breaking, and enrichment were performed according to the manufacturer's instructions. Briefly, DNA was added to the emulsion PCR master mix at an input concentration of one template copy/Ion Sphere Particle (ISP), and the emulsion was generated using an OT2 (Life Technologies). Subsequently, the ISPs were recovered, and Dynabeads MyOne Streptavidin C1 beads (Life Technologies) were used to enrich for template-positive ISPs. The sample was prepared for sequencing using the Ion PGM™ Hi-Q™ View Sequencing Kit (Life Technologies). Each composite sample was loaded onto an Ion 318 chip v2 and sequenced on the Ion Torrent PGM system for 850 flows. Two synthetic 16S microbial communities (Mock Communities, HM-782D and HM-783D; BEI Resources, USA) of species with known genomes were used to evaluate the quality of the metagenomic assay.

4.3.5. Bioinformatics' analyses

The reads obtained from the sequencing process, were analyzed as follows: The fastq file with raw data including all barcodes (except the two barcodes with mock communities) were run through an OTUs classification pipeline derived from the 16S profiling data analysis pipeline of the Brazilian Microbiome Project (Pyrlo et al., 2014). First, the raw data was filtered using an in-house script (available at: https://github.com/aquacen/fast_sample) with the following parameters: "-n 100" (test all reads), "-s 160" (include only reads ≥ 160 bp), "-b 310" (to trim reads ≥ 310 bp), "-l 0" (no left clip), and "-q 20" (trim 3' reads with Phred quality < 20). The Uparse software (Edgar, 2013) was then used to relabel the reads, and Usearch version 10.0.240 (Edgar, 2018) was used to filter by quality (-fastq_filter -fastq_maxee 0.8), dereplicate the reads (-fastx_uniques -sizeout), sort by size (-sortbysize -minsize 2), cluster OTUs (-cluster_otus), map raw data over the OTUs (-usearch_global -strand plus -id 0.97). Uparse was then used to generate the list of OTUs and convert the UC map file to an OTUs table, and QIIME version 1 (Carporaso et al., 2010) was used to: assign taxonomy (--similarity 0.7), align the OTU sequences, filter the alignment, and make a phylogeny tree. Finally, Biom version 2.1.5 (McDonald et al., 2012) was used to: convert the biom table to json, add QIIME taxonomy metadata (--observation-header OTUID, taxonomy, confidence --sc-separated taxonomy --float-fields confidence) and summarize the OTUs table. Usearch version 10.0.240 includes chimera filters used during the cluster OTUs step (-cluster_otus). The two barcodes with mock communities were evaluated using the same steps in a different assay.

To perform the Alpha (α) and Beta (β) diversity analyses, QIIME was used to filter samples from the OTUs table (-n 1000); create a single rarefaction (-d 1000) to use in β diversity; calculate unweighted (--metrics unweighted_unifrac) and weighted (--metrics weighted_unifrac) β diversity; and produce multiple rarefactions (-m 10 -x 50000 -s 2000) for use in calculating α diversity. The α diversity individual-based rarefaction curves were obtained by plotting the Chao1, a measure based on operational taxonomic unit (OTU) (Bai et al., 2017). The *ggplot2* package of R software was used to generate plots of α and β diversity, as well as perform principal coordinate analysis (PCoA) and statistical comparisons of family abundance based on the QIIME results. Wilcox Test with Bonferroni correction was used for statistical comparisons. Only families representing ≥1% in at least one barcode were included in the analysis.

4.4. RESULTS AND DISCUSSION

4.4.1. Reactor performance

Table 1 shows an overview of the physicochemical parameters measured during the experimental period. The raw wastewater was also analyzed, which revealed a mean pH of 7.0 a VFA of 424 mg L^{-1} , a BOD of 502 mg L^{-1}, and a COD of 1195 mg L^{-1}. In addition, its high biodegradable fraction was verified through the COD/BOD ratio, which averaged 2.39.

The results of this study allow to state that the pilot-scale reactors were functionally stable at both 20°C and 37°C and that the ideal pH range in anaerobic digestion is close to neutral (6.8–7.2). Out of this range, the rate of methanogenesis can be greatly reduced at values below 6.6, and at high alkaline pH, microbial granules can disintegrate and lead to failure of the process (Franke-Whittle et al, 2014; Ward et al, 2008). The reactors at 20°C showed average pH values between 7.65 and 7.98, while at 37°C the average values fluctuated from 7.93 to 8.05. Despite their higher pH values, the reactors had stable operation, similar to that found in other studies with anaerobic reactors (Alcántara-Hernández et al., 2017; Franke-Whittle et al, 2014). In addition, these values indicate that the reactors were producing methane, since methanogenesis stabilizes pH in the higher range (7.2–8.2) (Abdelgadir et al., 2014).

VFA is another important parameter to measure, since its accumulation may cause toxicity to methanogenic microorganisms and has been regarded as sign of process failure (Vanwonterhem et al., 2015; Rodrigues et al, 2014; Ward et al, 2008). In the replicated reactors in this study, VFA remained low (average values less than 280 mg L^{-1}), indicating that the microorganisms were consuming the acids and converting them to methane (CH_4). Although IA/PA ratios are recommended to be below 0.3, some authors (Rodrigues et al, 2014; Foresti, 1994) report that stability may occur at values different from 0.3, and that it is prudent to examine each particular case. Even though there were statistically significant differences in IA/PA ratio between the temperatures at days 10 and 60, they were all lower than 0.37, which is still considered stable according to standards described in the literature (Rodrigues et al., 2014; Martín-Gonzáles et al.,

2013; Ripley et al., 1986). This suggests that anaerobic reactors can achieve satisfactory performances in terms of process stability when treating effluents from the fish processing industry at both studied temperatures.

To better visualize the efficiency of biodegradable and non-biodegradable organic matter removal, boxplots were created showing BOD and COD removal at 20°C and 37°C throughout the experimental period (Figure 1). Evaluating the values singularly, the maximum amount of BOD and COD removal were 72% and 50%, respectively, both occurring at 37°C on day 50. When comparing the average organic matter removal at 20°C and 37°C, taking into account all collection time points, it was observed that at 37°C BOD and COD removal rates were 2.40% and 10.46% higher, respectively, than at 20°C. Moreover, BOD removal was superior than COD removal at both temperatures.

The reactors started to present statistical differences in COD removal from day 50, with the 37°C reactors showing better results. At day 60, despite having a lower COD than day 50, removal continued to be higher in reactors at 37°C. The higher efficiency at higher temperatures can be explained by the hydrolysis coefficient, which describes the rate of organic matter degradation and is consistent with an increase of the kinetics of the reaction according to the Arrhenius equation (Ho et al., 2014). This result is also in agreement with similar studies of anaerobic reactors treating different types of effluents, which revealed that temperature is an important factor that has a positive impact on hydrolysis and is responsible for complex patterns of variation (Hai et al., 2014; Ho et al., 2014; Ju et al, 2014).

BOD removal was also higher in the reactors at 37°C. However, there were no statistical differences between these and those incubated at 20°C. The best removal efficiencies, 60.15% at 20°C and 61.75% at 37°C, were obtained on day 60. Thus, although reactors at both temperatures show good efficiencies, other organic loads can be applied to evaluate if the effectiveness of anaerobic reactors would be compromised, requiring the adequation of the working temperature.

4.4.2. Sequencing parameters result check

High-throughput sequencing of the sludge samples produced 4,760,822 reads with length mean of 248 bp which mapped to 36 phyla, 69 classes, 99 orders, 164 families, 119 genera and 41 species. The read lengths were compatible with amplicons fully spanning the V4 region (Tremblay et al., 2015). Each barcode was present in an average of 78,339±49,905 reads. Furthermore, to evaluate the sequencing run and validate the quantitative results, two mocks were analyzed, which found OTU sequence reads in proportion to the expected abundance reported by the manufacturer (BEI Resources).

4.4.3. α and β diversity

The α diversity curves reveal that the barcodes tended to have a horizontal plateau, which indicated that sequence coverage was sufficient to reliably describe the total diversity present in the samples. A series of t-tests were used to compare α diversity between samples. The mean chao1 α diversity was significantly higher (p = 2.137e-05) at 20°C than at 37°C (Figure 2).

To evaluate β diversity, which reflects the similarities and differences in the composition of microbial communities, the weighted UniFrac distances between the OTUs in the samples were compared. Principal Component 1, 2 and 3 (PC1, PC2, and PC3) represented 66,34%, 14,26% and 4,29%, respectively, of the variation in community structure between the samples. The distance showed how similar the samples are in terms of microbial community composition (Figure 3). PCoA analysis of the differences between the sludge samples showed that they clustered according to temperature. The results also showed that compared to the communities in 37ºC reactors, those in 20ºC reactors were more closely grouped and taxonomically similar to each other.

4.4.4. Community selection by temperature

To evaluate the dynamics of microbial communities, OTU abundance at the phyla and family levels were determined for each of the temperatures at the six sampling times. Figure 4 shows a comparison between the predominant phyla at 20°C and 37°C throughout the experimental period, showing the changes occurring in the same community at different temperatures and collection times.

Mean archaeal abundances remained below 1% of the OTUs throughout the experiment and were grouped with other non-significant microorganisms. As for the bacterial communities, the main representative phyla differed at each temperature, and their proportions changed over time.

At the beginning of the experiment the phyla *Synergistetes* and *Proteobacteria* predominated in the 20°C reactors. The phylum *Synergistetes* was common throughout the experiment reaching its maximum abundance in T5, at 52.45% of OTUs. On the other hand, despite reaching a peak of 41.04% of the OTUs during T2, the prevalence of the *Proteobacteria* decreased from T3 to only 14.12% in T5 and was surpassed by the phylum *Firmicutes*. As the start of the experiment, *Firmicutes* showed an abundance of 9.41% and increased in abundance throughout the experiment, finishing at 22.94% of the OTUs by T6, becoming one of the dominant phyla. Another common phylum was *Bacteroidetes*, accounting for between 5.58% (at T2) and 14.48% (at T4).

At 37°C the phylum *Bacteroidetes* predominated throughout the experimental period, ranging between 47.86% at T2 (47.86%) and 38.10% at T5. The abundance of the *Synergistetes*, *Proteobacteria* and *Firmicutes* phyla decreased throughout the

experimental period, having higher values in T1 and smaller values in T6. On the other hand, the *Chlorobi* and *Spirochaetes* phyla grew considerably from T3, increasing from less than 1% at T1 and T2 to 6.63% and 19.56% of the OTUs at T6, respectively.

There were also differences in the abundance and community dynamics of taxonomic families at both temperatures. Representative sequenced families selected at 20°C and 37°C are shown in Figure 5, demonstrating the main ones at each of the analyzed temperatures.

Some microorganisms were not identified to the family level, while others had comprised less than 1% of the OTUs and were grouped into a single category. Mean archaeal abundance remained below 1% of the OTUs throughout the experiment, and so are grouped with other non-significant microorganisms. When evaluating these communities, it was observed that temperatures influenced archaeal abundance, as they were more prevalent in the reactors run at 37°C at all collection times. On average, they represented 0.04% of the identified OTUs in the 20°C reactors but 0.20% in the 37°C reactors. This decrease in the archaeal population mainly involved a reduction in *Methanobacterium*, the predominant representative of the *Archaea* in all reactors.

A pattern can be observed in the differences between microorganisms at the two temperatures. In the reactors incubated at 20°C, seventeen dominant groups accounting for more than 1% of OTUs, were detected in all samples, with a mean relative abundance of 61.46%. Among these, the family *Synergistaceae* was the most predominant throughout the experiment, peaking at day 50 (40.20%). The family *Moraxellaceae* had a sudden drop in their abundance over the six time points, which reduced by about 88%. The *Enterobacteriaceae* also had a large decrease, from 9.21% to 0% at day 50. Although having a lower abundance, the *Bacteroidaceae*, which, at day 10 comprised 2.56% of the OTUs, was also reduced to 0%. Moreover, some families such as *Comamonadaceae*, *Pseudomonadaceae* and *Veillonellaceae* increased slightly until day 30, before reducing in abundance. In contrast, the *Porphyromonadaceae* and *Ruminococcaceae* families increased in abundance, reaching peaks on day 40 and 60, respectively. The other families listed (*Christensenellaceae, Desulfovibrionaceae, Dethiosulfovibrionaceae, Francisellaceae, Legionellaceae, Mogibacteriaceae, Paraprevotellaceae* and *S24-7*), despite having more than 1% of OTUs, remained relatively rare and did not change during the experimental period.

The microorganisms grouped together as non-significant increased in abundance throughout the experiment representing on average 19.43% of the OTUs, while the unassigned microorganisms accounted for 0.18% throughout the experiment. A mean of 18.93% of OTUs were not identified to the family level. Investigating the order assigned to these OTUs, it was observed that the majority (45.99%) belonged to the *Synergistales*, which allows us to state that this was the most abundant group of microorganisms in the 20°C reactors. In addition, a significant number (40.70%) belonged to the *Clostridiales*, which includes significant minor families described at

this selected temperature, such as *Christensenellaceae*, *Mogibacteriaceae*, *Veillonellaceae* and *Ruminococcaceae*.

On the other hand, reactors incubated at 37°C had the great majority of OTUs not identified to the family level. Despite accounting for an average of 45.78% of the OTUs, they decreased in abundance throughout the experiment. Among those OTUs that could not be assigned to a family, the vast majority (71.50%) belonged to the order *Bacteroidales* with *Clostridiales* (25.75%) making up the majority of the remainder. The *Clostridiales* order did not contain any significant identified families, suggesting that these might be some functional microorganisms that remained unclassified.

The *Porphyromonadaceae* and *Rikenellaceae* families of the order *Bacteroidales* proved to be important to the process. The first grew in abundance over time with a peak at day 50 (7.02%), while the second showed a remarkable increase growing from 0% at day 1 to 15.34% at day 60. Similarly, the *Spirochaetaceae* family also became considerably more prevalent, from 0% to 19.16% on the final collection day. On the other hand, the families *Aminiphilaceae* and *Rhodocyclaceae* reduced in abundance over time. Both, however, maintained low abundance, with small peaks on days 30 and 50, respectively.

Similar to 20°C, at 37°C, *Synergistaceae* was the sequenced family representing the highest mean number of identified OTUs (14.88%), although they were considerably more abundant in the cooler reactors (33.05%). Moreover, it was more stable at 20°C, considering that the abundance of this family decreased from day 10 to 60 at 37°C. In addition, the non-significant microorganisms stayed stable throughout the collection period, representing 10.64% of the total at 37°C. Similar to that in reactors at 20°C, the number of unassigned microorganisms was low, accounting for an average of 0.07% of OTUs. To further reveal the dynamics of the communities, all the main identified microorganisms (OTUs above 1%) at the two temperatures and six time points, with all available taxonomic information, are listed in Supplementary Table S1.

Understanding the factors that shape the structure of archaeal and bacterial communities in anaerobic reactors could potentially enhance control of anaerobic digestion (Hai et al., 2014). The metagenomic assay in the present work revealed a low abundance of *Archaea* at both temperatures, similar to the findings of previous studies (Ju et al., 2014; Li et al., 2013). Although most *Methanobacterium* are described as able to grow in a wide range of temperatures (3°C to 50°C) (Battumur et al., 2016), in this work the mesophilic strains predominated. The hydrolytic phase could be the rate limiting step at lower temperatures, once it limits the energy gain from VFA degradation (McKeown et al, 2012).

Bacterial communities were common at all evaluated temperatures and collection times. The main phyla and families observed in this study were also reported at different abundances in previous studies using different substrates and anaerobic reactor configurations (Ouyang et al, 2018; Wojcieszak et al, 2017; Gunnigle et al, 2015; Guo

et al, 2015; Vanwonterghem et al, 2015). Furthermore, in the present work, temperature appeared to play a major role in the adaptation of microorganisms, since noticeable shifts in the bacterial community structure were seen throughout the experiment. Within each condition (20°C and 37°C) there were also changes in the composition and abundance of microorganisms over time. At lower temperatures, microorganisms have evolved sophisticated adaptation strategies to grow, which allows them to adapt to various conditions, including changes in proteins (to more flexible structural conformations) and increase of membrane fluidity through altering lipid membrane composition (McKeown et al, 2012).

Besides that, individuals with similar nutritional requirements will compete for nutrients, which can lead to selection for the better suited strain for an ecological niche (Hibbing et al, 2010). Considering that the initial populations were identical and there was no input from new populations at any time, the changes in composition could be related to the physiological needs for diverse nutrients by the various microbial groups, as it is reported that nutritional resources are a focal point of microbial competition (Hibbing et al, 2010). These needs may or may not have been supplied with the inoculated influent, since its chemical composition is extremely complex, probably favoring the survival and growth of bacteria with better fitness for this substrate. Therefore, although there were major changes in the microbial profile, the efficiency of the reactors remained similar. Thus, different populations may present a redundancy in the dynamics of the reactor, which maintains its operation even with the temperature change.

4.4.5. Bacterial dynamics

Sludge from anaerobic digestion contains highly complex microbial communities (i.e., fermentative acidogens, H_2 producing acetogens and methanogens). A mature and well-balanced community is vital for biological treatment success and better performance can be linked to differences in dominant bacterial populations (Shi et al, 2016; Guo et al, 2015).

Although they had different microbial profiles, the reactors were stable and had satisfactory BOD removal, demonstrating its potential for practical application. Nevertheless, the statistically superiority of 37°C reactors at COD removal may be related to fluctuations in bacterial communities. As with temperature, BOD and COD can be linked to bacterial community structure, as they provide carbon to heterotrophic bacteria, influencing their growth rate (Hai et al, 2014).

Among the families that stood out during the experimental period, *Synergistaceae* was one of the most representative. Members of this family could be identified to the genus level, the majority of which were *VadinCA02*. This genus and the *Synergistaceae* family in general are reported to be commonly present in anaerobic reactors, where their ability to degrade amino acids is an important characteristic of the group (He et al., 2018;

Meng et al., 2017; Si et al., 2016). The *VadinCA02* had high abundance in the 20°C reactors throughout the experiment. However, at 37°C it started to decline in prevalence from day 30, indicating a possible reduction in the hydrogenotrophic pathway for methane production at this temperature. This can occur because this genus commonly interacts syntrophically with methanogenic microorganisms by hydrogen (H_2) transfer, optimizing the performance of the *Archaea* hydrogenotrophic pathway. In this experiment, the archaeal community was dominated by the hydrogenotrophic *Methanobacteriaceae* (H_2 utilizing microorganism), indicating that the methane production process occurred through methanogenic microorganisms using H_2 and carbon dioxide (CO_2) at both studied temperatures, which has also been observed in previous studies (He et al., 2018, Meng et al., 2017, Wojcieszak et al., 2017).

The *Firmicutes* phylum were identified only up to the order level (*Clostridiales*) at 37°C and had opposite results at the studied temperatures, increasing in abundance in the reactors at 20°C and decreasing considerably in the reactors at 37°C. This phylum encompasses a group of syntrophic bacteria that can degrade various substrates and produce VFA. This activity is related to hydrolytic processes, providing evidence of greater hydrolysis activity in reactors subjected to lower temperatures (Ouyang et al, 2018).

At 37°C, the *Bacteroidetes* phylum showed a high abundance over the six time points. Nevertheless, its prevalence fell throughout the experiment. These OTUs were also only identified to order level (*Bacteroidales*). In contrast, it was present at low abundance in the reactors at 20°C throughout the experimental period. The microorganisms belonging to this phylum are fermentative and have the ability to degrade polysaccharides. They are also described as producers of VFA and as acetate suppliers for methanogens, accelerating methane production and COD removal. This feature may be related to the best COD removal occurring on day 50 at 37°C, when the abundance of these microorganisms was highest (Ozbayram et al., 2018; Han et al., 2017; Wojcieszak et al, 2017; Si et al., 2016; Wang et al., 2016).

Another interesting family at 37°C was the *Rhodocyclaceae*, which began with a significant proportion of OTUs and decreased abruptly. They are considered to be phosphate accumulating organisms, which can increase phosphate removal performance, and are also nitrate reducers, thus their absence may have resulted in a reduction in denitrification and a possible increase of phosphate (Kong et al., 2017). On the other hand, the *Rikenellaceae* and *Spirochaetaceae* families became considerably more common during the experimental period. Contributing to acidogenesis, *Rikenellaceae* are fermentative microorganisms capable of degrading polysaccharides and are described as VFA and hydrogen producers (Ozbayram et al., 2018; Han et al., 2017; Wojcieszak, 2017; Meng et al., 2017). In addition, *Spirochaetaceae*, which are also frequently found in anaerobic reactors, have been described as fermenters and acetate oxidizers, with hydrogen and carbon dioxide being the main final products (Si et al., 2016; Lee et al., 2015).

The growth of microorganisms belonging to the *Rikenellaceae* and *Spirochaetaceae* families promotes the presence of acidogenic microorganisms at 37°C. As fermentative microorganisms are the first to act in the substrate degradation, they also benefit the most energetically, justifying their remarkable prevalence. Furthermore, since acidogenic bacteria have high growth rates, it is very important that the material to be degraded is readily hydrolyzed, so that this step does not limit the process.

The main observed families presented different dynamics, which may be related to different phases of the anaerobic digestion process, as shown in Figure 6. Despite the efficiency in organic matter removal demonstrated by reactors at both temperatures, the observed microbial profiles at 20°C and 37°C were different throughout the experiment, suggesting a modulation of these populations based on temperature. However, it must be emphasized that altering the conditions - such as the applied organic load and the experimental time - should produce different results, and future studies are needed to understand how the communities would behave under other specific conditions.

4.5. CONCLUSION

During the experimental period, it was observed that the different temperatures did not change the reactors performance in a significant way. However, the microbial profile of the communities, as assessed by metagenomics, was influenced by this parameter. These results demonstrate that anaerobic reactors can be used both in winter and summer in tropical countries for the treatment of fish processing wastewater in the studied conditions. However, further studies over long periods and with different organic loads are necessary to verify whether there would still be differences in reactor performance and microbial communities.

4.6. REFERENCES

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TABLES

Table 1. Overview of reactor effluent physicochemical parameters.

Supplementary Table S1. Significant microorganisms and their taxonomic levels found throughout the experimental period at 20°C and 37°C.

FIGURE LEGENDS

Figure 1. Percentage of BOD and COD removal at 20°C and 37°C throughout the experiment. The y-axis shows the percentage removed, and x-axis, the six collection time points during the experiment.

Figure 2. α-Diversity rarefaction curves for the 16S rRNA V4 region of bacterial and archaeal reads on Chao1 comparing the temperatures at the six collection times.

Figure 3. Weighted UniFrac principal coordinate analysis (PCoA) obtained from the β-diversity calculation in QIIME, showing dissimilarities in microbial composition. The colored dots show that the sludge samples collected at different times, clustered by temperature (20°C and 37°C).

Figure 4. Representative sequenced phyla clustered by temperatures (20°C and 37°C) and collection times, including others (significance level less than 1%) and unassigned microorganisms.

Figure 5. Representative sequenced families clustered by temperatures (20°C and 37°C), including OTUs that could not be identified to the family level and unassigned microorganisms. The y-axis shows the OTUs percentage, and the x-axis, the six collection times.

Figure 6. Main families observed in the two studied temperatures (20°C and 37°C) and their relationship with the phases of the anaerobic digestion process.

Table 1. Overview of reactor effluent physicochemical parameters.

Day	20°C (mean±sd)	37°C (mean±sd)	P value
	pH		
10	7.88±0.02	7.93±0.03	0.75700
20	7.65±0.03	7.93±0.03	0.14348
30	7.83±0.02	8.00±0.04	0.33486
40	7.80±0.02	7.98±0.01	0.15630
50	7.83±0.02	7.95±0.04	0.47068
60	7.98±0.02	8.05±0.03	0.60372
	IA/PA		
10	0.21±0.16	0.15±0.10	0.02592*
20	0.28±0.12	0.26±0.04	0.26879
30	0.28±0.18	0.28±0.10	0.93268
40	0.26±0.11	0.29±0.04	0.21394
50	0.28±0.09	0.26±0.09	0.43010
60	0.37±0.03	0.27±0.14	0.00929*
	VFA mg L^{-1}		
10	297±0.13	351±0.13	0.12123
20	330±0.13	273± 0.15	0.09650
30	264±0.10	264±0.24	1
40	237±0.13	222±0.21	0.61031
50	189±0.17	171±0.24	0.51320
60	177±0.15	162±0.18	0.47150
	BOD mg L^{-1}		
10	270±0.17	295±0.35	0.67450
20	266±0.10	240±0.28	0.51261
30	206±0.10	186±0.03	0.15915
40	219±0.07	189±0.14	0.11179
50	201±0.15	188±0.09	0.47313
60	200±0.02	192±0.05	0.22768
	COD mg L^{-1}		
10	977±0.09	886±0.23	0.45330
20	1056±0.11	911±0.18	0.20676
30	870±0.11	801±0.15	0.41454
40	913±0.12	782±0.09	0.10570
50	872±0.09	680± 0.20	0.01219*
60	926±0.05	802± 0.07	0.01995*

IA/PA: alkalinity ratio; VFA: volatile fatty acids; BOD: biochemical oxygen demand; COD: chemical oxygen demand.

P value: comparison with the null hypothesis of equal means.

Supplementary Table S1. Significant microorganisms and their taxonomic levels found throughout the experimental period at 20°C and 37°C.

T1 – 20°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*		2,06
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			2,16
Firmicutes	*Clostridia*	*Clostridiales*				5,88
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*		11,96
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*		1,57
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*		7,91
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*			8,77
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		27,91
Synergistetes	*Synergistia*	*Synergistales*				6,76
<1% reads						25,02

T2 – 20°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			1,73
Firmicutes	*Clostridia*	*Clostridiales*				6,24
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*A.lwoffii*	3,60
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*P.fragi*	2,15
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*		9,83
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*		2,35
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*			9,54
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		13,80
Synergistetes	*Synergistia*	*Synergistales*				3,64
<1% reads						47,14

T3 – 20°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*		2,54
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			2,00
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				1,94
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*			1,25
Firmicutes	*Clostridia*	*Clostridiales*				6,33
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*	*A.lwoffii*	1,97
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*		1,97
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*		2,55
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*		1,08
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Hydrogenophaga*		1,56
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*			4,50
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		2,51
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*vadinCA02*		35,37
Synergistetes	*Synergistia*	*Synergistales*				8,26
<1% reads						26,20

T4 – 20°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*		1,61
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*		2,90
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*		1,11
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			2,99
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*S24-7*			1,87
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				1,34
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*VadinHB04*		1,09
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*			3,22
Firmicutes	*Clostridia*	*Clostridiales*				9,39
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*		1,08
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*		1,49
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*			2,16

Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		6,64
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		24,64
Synergistetes	*Synergistia*	*Synergistales*				7,52
<1% reads						30,96

T5 – 20°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*		2,52
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			3,32
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				1,45
Firmicutes	*Clostridia*	*Clostridiales*	*Mogibacteriaceae*	*Anaerovorax*		1,43
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*			3,75
Firmicutes	*Clostridia*	*Clostridiales*				9,93
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		40,12
Synergistetes	*Synergistia*	*Synergistales*				9,68
<1% reads						27,80

T6 – 20°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*	*Paludibacter*		2,04
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			3,36
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				2,10
Firmicutes	*Clostridia*	*Clostridiales*	*Mogibacteriaceae*	*Anaerovorax*		1,74
Firmicutes	*Clostridia*	*Clostridiales*	*Christensenellaceae*			1,36
Firmicutes	*Clostridia*	*Clostridiales*	*Ruminococcaceae*			3,81
Firmicutes	*Clostridia*	*Clostridiales*	*Veillonellaceae*			1,71
Firmicutes	*Clostridia*	*Clostridiales*				8,93
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*		1,43
Proteobacteria	*Gammaproteobacteria*	*Legionellales*	*Francisellaceae*	*Francisella*		1,07
Synergistetes	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*PD-UASB-13*		1,12
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		30,67

Synergistetes	*Synergistia*	*Synergistales*				6,83
<1% reads						33,84

T1 – 37°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				33,76
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			4,59
Firmicutes	*Clostridia*	*Clostridiales*				20,78
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*			1,07
Proteobacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*			0,72
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*			4,81
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*		2,36
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Azospirillum*		0,50
Proteobacteria	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfomicrobiaceae*	*Desulfomicrobium*		0,72
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Azovibrio*		0,29
Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*		1,16
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		7,05
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		14,40
<1% reads						7,82

T2 – 37°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				40,20
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			5,69
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*		0,27
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*S24-7*			0,44
Firmicutes	*Clostridia*	*Clostridiales*				13,98
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Azovibrio*		1,00
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*			1,81
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*			0,89
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*		1,42
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*		0,87

Proteobacteria	*Gammaproteobacteria*	*Pseudomonadales*	*Pseudomonadaceae*	*Pseudomonas*	*P.fragi*	0,29
Proteobacteria	*Gammaproteobacteria*					0,40
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*		0,26
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		2,24
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		16,65
<1% reads						13,61

T3 – 37°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				35,39
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Blvii28*		3,52
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			5,18
Chlorobi	*SJA-28*					1,28
Firmicutes	*Clostridia*	*Clostridiales*				8,67
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*			0,46
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Azospirillum*		2,17
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*		0,26
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Azovibrio*		0,45
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*			0,34
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*		1,08
Proteobacteria	*Gammaproteobacteria*					0,26
Proteobacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*			0,80
Proteobacteria	*Betaproteobacteria*	*Thiobacterales*				0,63
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*		3,99
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		1,37
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		21,73
<1% reads						12,44

T4 – 37°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				30,75
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Blvii28*		7,82

Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*		6,50
Chlorobi	*SJA-28*				4,29
Firmicutes	*Clostridia*	*Clostridiales*			7,47
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*		0,40
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Thauera*	0,41
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Azovibrio*	0,65
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Beijerinckiaceae*		0,27
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*	2,10
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Rhodospirillaceae*	*Azospirillum*	0,80
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*		1,04
Proteobacteria	*Betaproteobacteria*	*Thiobacterales*			0,27
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*	10,63
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*	0,85
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*	13,40
<1% reads					12,37

T5 – 37°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				19,10
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Blvii28*		11,71
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			6,96
Chlorobi	*SJA-28*					4,42
Firmicutes	*Clostridia*	*Clostridiales*				7,58
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*			0,27
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*			0,41
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*		3,01
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Beijerinckiaceae*			0,37
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*			0,79
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*			0,72
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*		*15,39*
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		*1,48*
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		*12,98*

Synergistetes	*Synergistia*	*Synergistales*				*0,40*
TPD-58						*0,34*
<1% reads						14,09

T6 – 37°C

Phylum	Class	Order	Family	Genus	Species	% reads
Bacteroidetes	*Bacteroidia*	*Bacteroidales*				18,11
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Rikenellaceae*	*Blvii28*		15,31
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			6,77
Bacteroidetes	*Bacteroidia*	*Bacteroidales*	*S24-7*			0,29
Chlorobi	*SJA-28*					6,63
Firmicutes	*Clostridia*	*Clostridiales*				5,37
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*	*Novosphingobium*		2,48
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*			0,37
Proteobacteria	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*			0,57
Proteobacteria	*Betaproteobacteria*	*Rhodocyclales*	*Rhodocyclaceae*	*Azovibrio*		0,34
Proteobacteria	*Alphaproteobacteria*	*Sphingomonadales*	*Sphingomonadaceae*			0,92
Spirochaetes	*Spirochaetes*	*Spirochaetales*	*Spirochaetaceae*	*Treponema*		19,16
Synergistetes	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		8,94
Synergistetes	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		0,72
Synergistetes	*Synergistia*	*Synergistales*	*Anaerobaculaceae*	*Anaerobaculum*		0,30
TPD-58						0,26
<1% reads						13,48

Figure 1. Percentage of BOD and COD removal at 20°C and 37°C throughout the experiment. The y-axis shows the percentage removed, and x-axis, the six collection time points during the experiment.

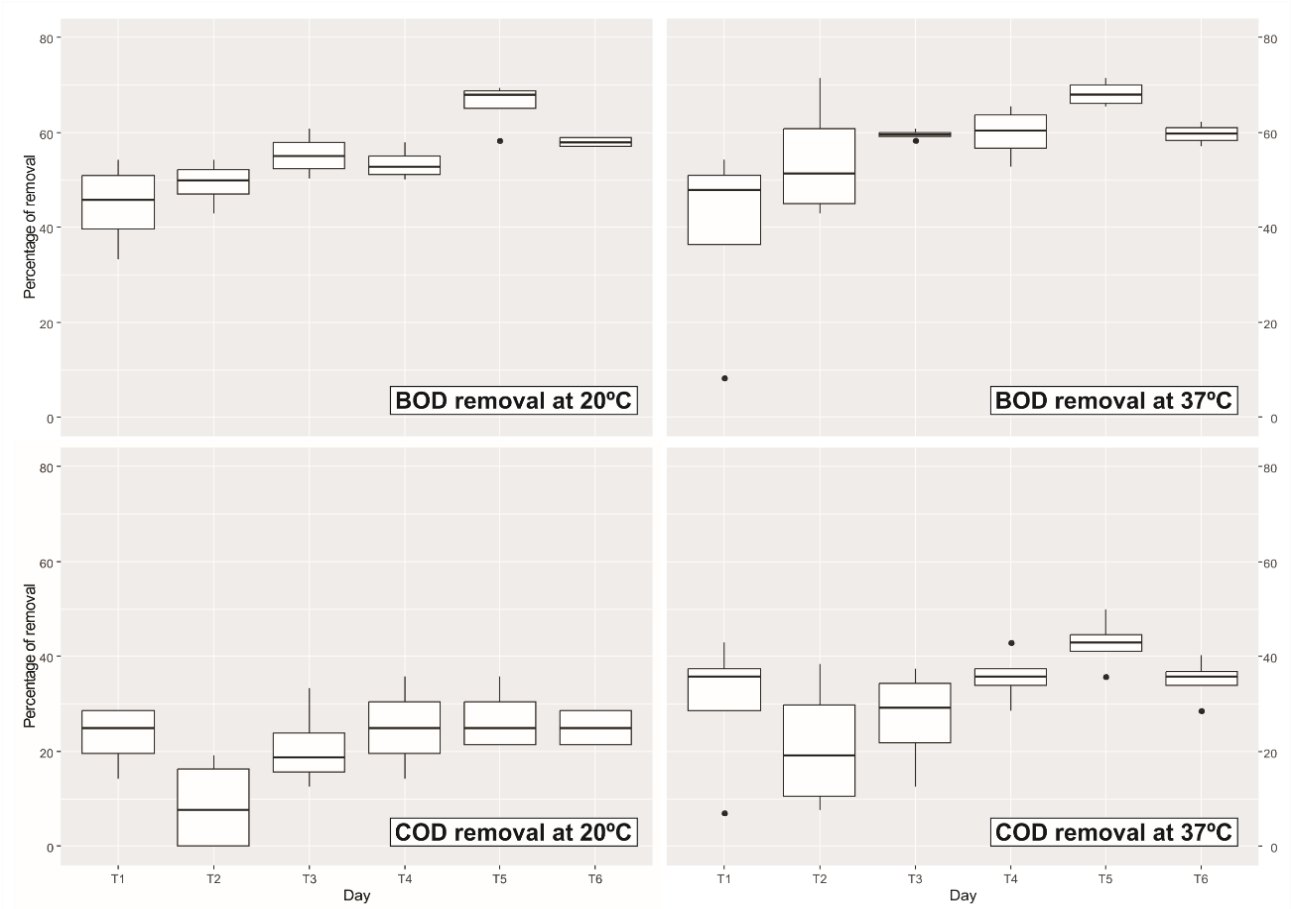


Figure 2. α-Diversity rarefaction curves for the 16S rRNA V4 region of bacterial and archaeal reads on Chao1 comparing the temperatures at the six collection times.

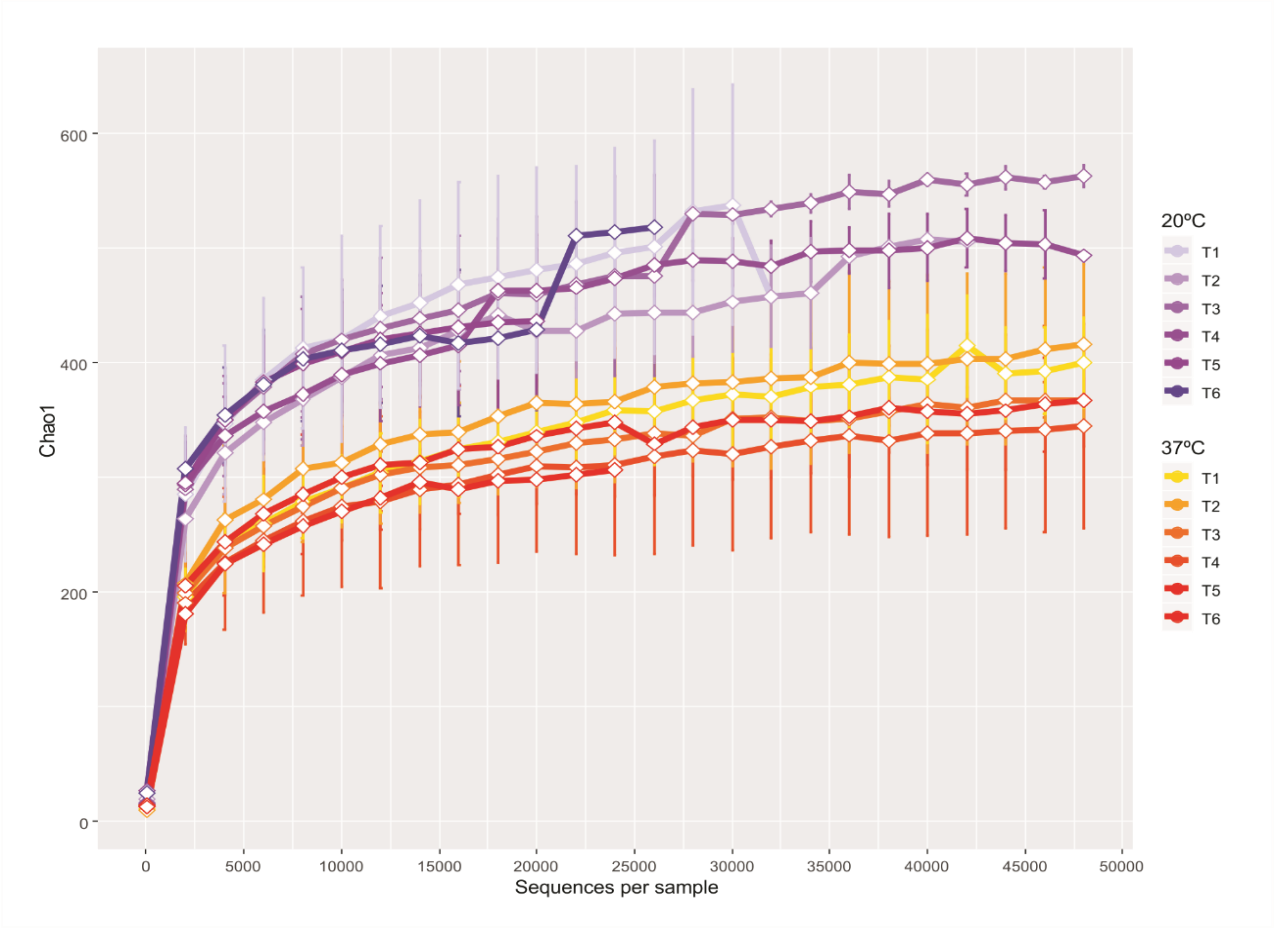


Figure 3. Weighted UniFrac principal coordinate analysis (PCoA) obtained from the β-diversity calculation in QIIME, showing dissimilarities in microbial composition. The colored dots show that the sludge samples collected at different times, clustered by temperature (20°C and 37°C).

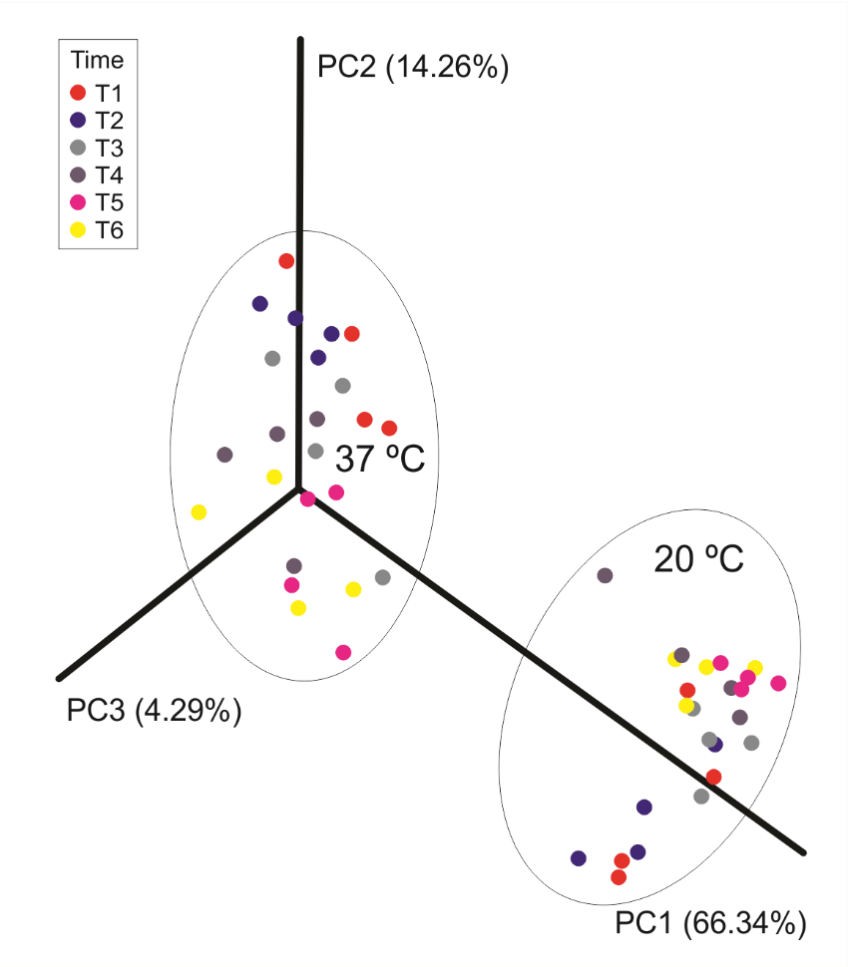


Figure 4. Representative sequenced phyla clustered by temperatures (20°C and 37°C) and collection times, including others (significance level less than 1%) and unassigned microorganisms.

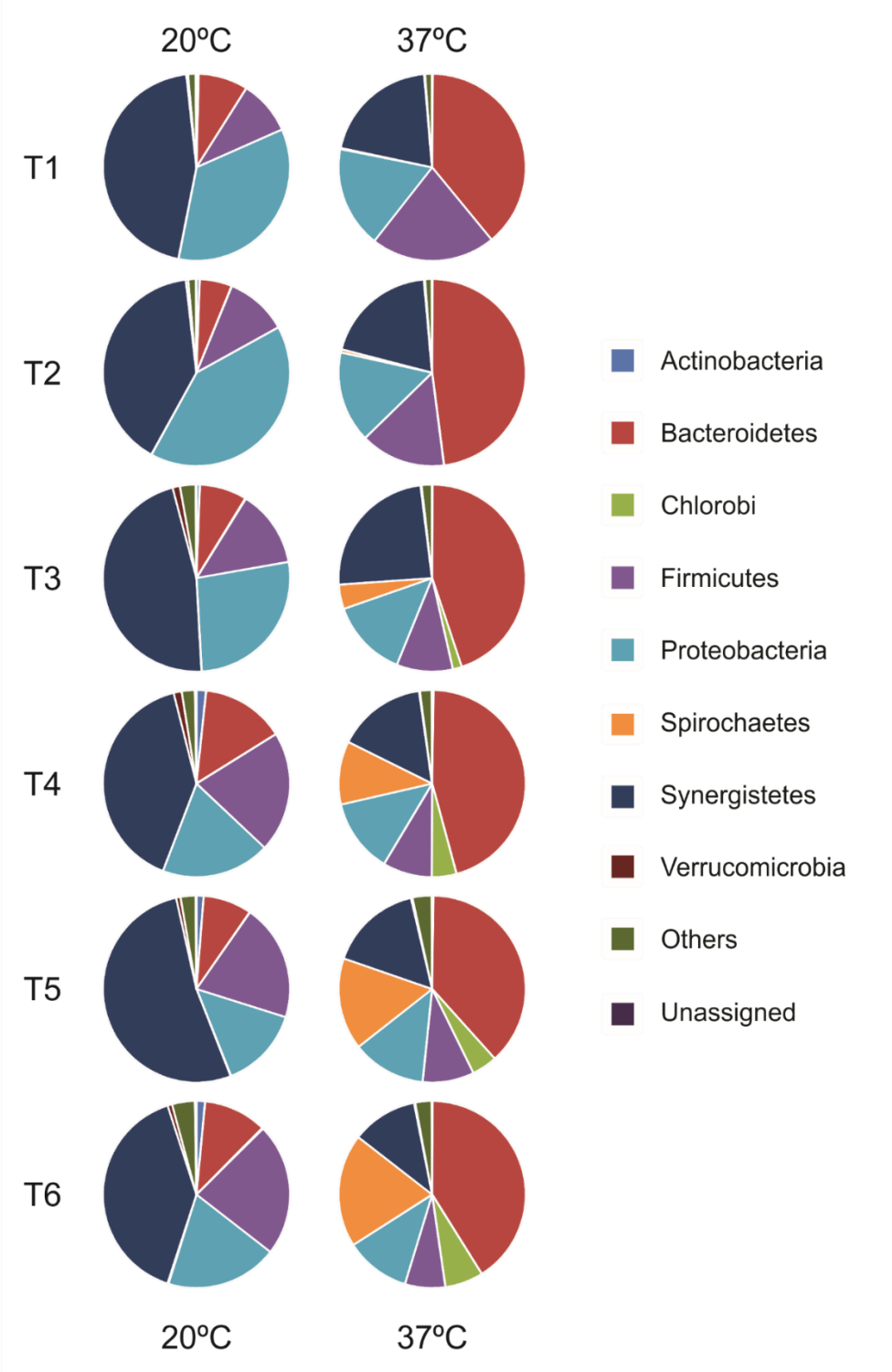


Figure 5. Representative sequenced families clustered by temperatures (20°C and 37°C), including OTUs that could not be identified to the family level and unassigned microorganisms. The y-axis shows the OTUs percentage, and the x-axis, the six collection times.

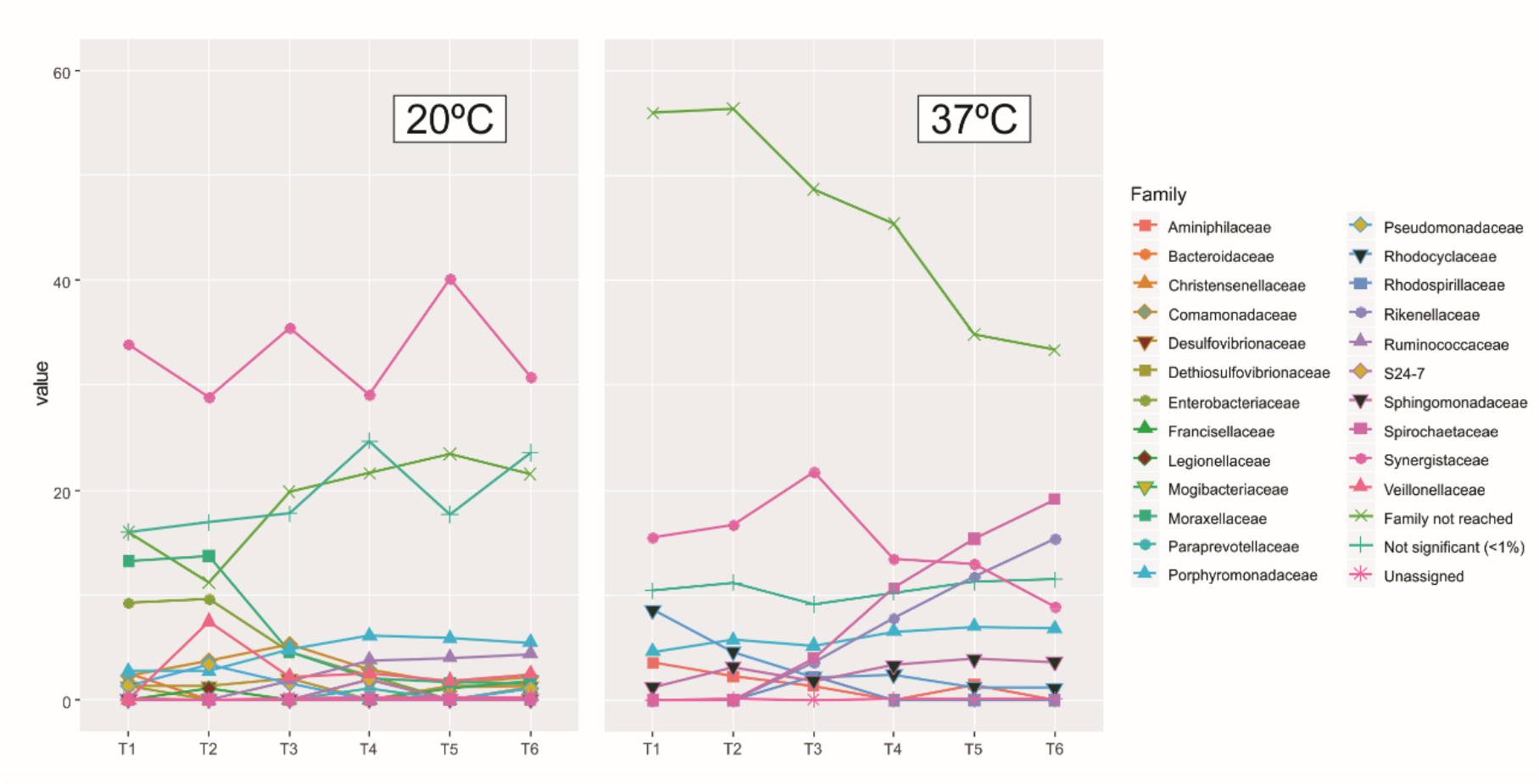
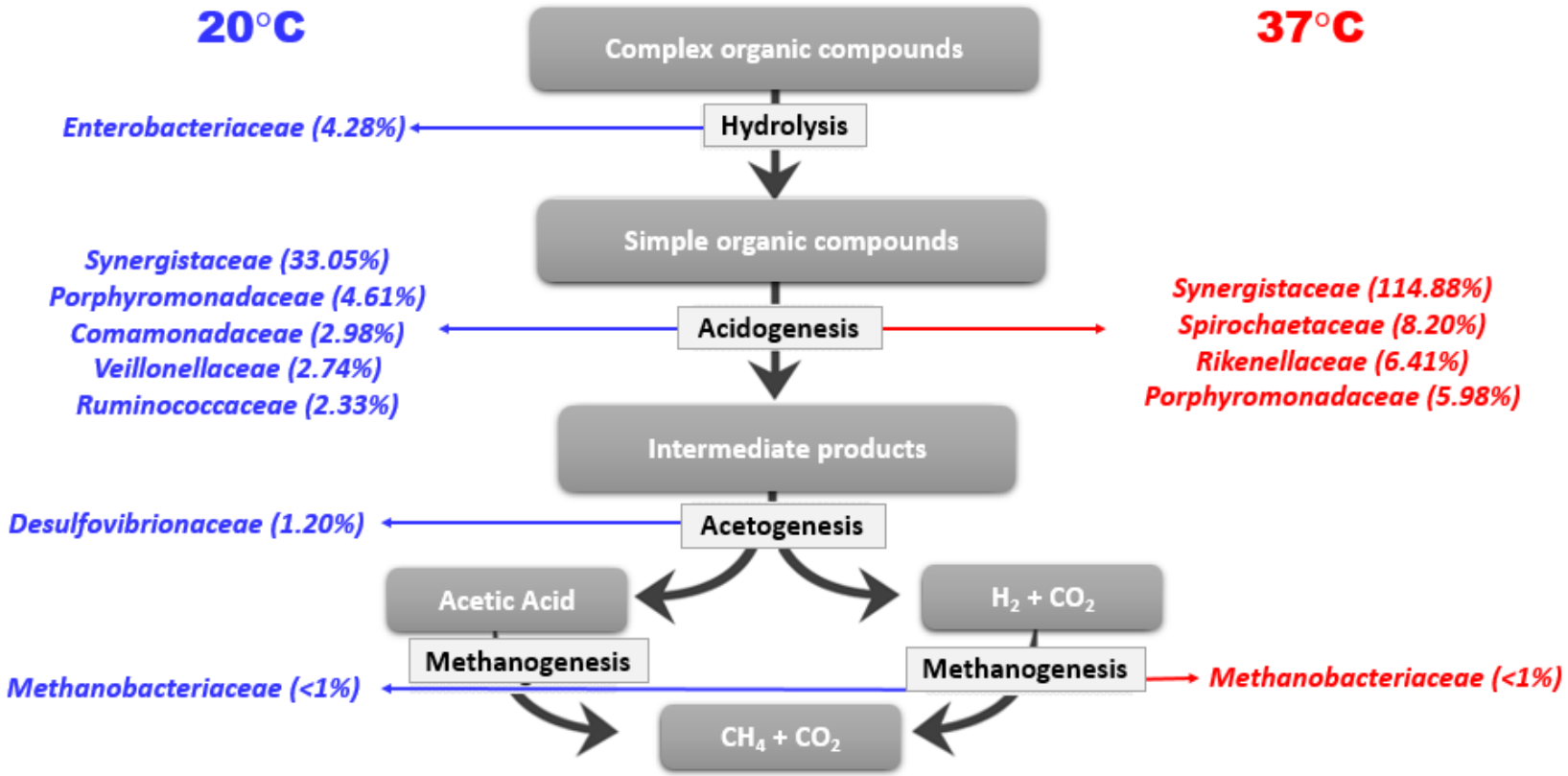


Figure 6. Main families observed in the two studied temperatures (20°C and 37°C) and their relationship with the phases of the anaerobic digestion process.



5. CHAPTER 3. METAGENOMIC ANALYSIS OF SLUDGE FROM ANAEROBIC REPLICATED REACTORS TREATING HIGH ORGANIC LOAD FISH PROCESSING WASTEWATER

5.1. ABSTRACT

This study aimed to investigate the effect of different temperatures (20°C and 37°C) associated with high organic loads in anaerobic reactors . The evaluation of the stability and microbial community structure were conducted by physicochemical analysis and a metagenomic assay. The 37°C reactors showed VFA accumulation, which lead to superior organic matter removal at 20°C. The main involved families in both temperatures were *Ectothiorhodospiraceae*, *Syntrophorhabdaceae*, *Dethiosulfovibrionaceae* and *Synergistaceae*, appearing with different abundances. However, the *Anaerobaculaceae* family, present only at 37°C, was suggested as closely linked to high VFA levels in these reactors. The results provide insights about the understanding of the complex biological communities which conduct the dynamic anaerobic digestion processes.

Keywords: fish slaughterhouse, microbial community, Ion Torrent sequencing.

5.2. INTRODUCTION

Aquaculture and fisheries activities play a significant role in the economy worldwide, as its constitute a source of food of a large part of population and a huge employment sector (Li, 2018; Sunny and Mathai, 2013). However, in its operation, the fish industry consumes considerable amounts of water, consequently generating effluents with characteristics that require specific treatment technologies (Sunny and Mathai, 2013; Achour et al., 2000).

The fish processing effluents are described as rich in organic matter, nevertheless, depending on the processed raw material and production process, these effluents may present important variations in their composition (Chowdhury et al., 2010; Najafpour et al., 2006; Palenzuela-Rollon et al., 2002; Achour et al., 2000). Aiming at; sustainable production, it is essential that these effluents are widely studied to a better understanding of the best approaches in their treatment (Chowdhury et al., 2010).

Biological processes have been suggested for the treatment of fish industry effluents with different organic loads (Li, 2018; Chowdhury et al., 2010; Najafpour et al., 2006; Palenzuela-Rollon et al., 2002; Achour et al., 2000). In the previous chapter, effluents from a Tilapia processing industry were treated in pilot-scale anaerobic reactors with an organic load rate (OLR) of 0.08 kg COD/m^3d and exposed to different temperatures (20°C and 37°C) for 60 days. Physicochemical and metagenomic assays revealed that although the microbial community was considerably altered, the organic matter removal was similar in both conditions.

The anaerobic digestion process can be influenced directly by a series of operational parameters, such as hydraulic retention time (HRT), temperature and OLR (Keating et al., 2018; Li, 2018; Song et al., 2017; Vanwonterghem et al., 2015). Organic overloads are directly related to effluent concentration, in addition, the structure and diversity of microbial community can be affected according to the applied load, influencing the success of the treatment (Pérez-Pérez et al., 2018; Song et al., 2017).

Aiming a greater understanding of the OLR influence on anaerobic reactors treating fish processing wastewater, this study simulated similar conditions to those described by the previous chapter, however, a higher organic load was applied to evaluate, together with the temperatures, the effects of the organic load on the stability and profile of the microbial communities involved in the process.

5.3. MATERIAL AND METHODS

5.3.1. Experimental design

Six laboratory scale anaerobic reactors with a working volume of 2.1 liters were operated for 60 days semi-continuously, in a daily process of feeding and wasting. Reactors 1, 2, and 3 were biological replicates submitted to 20ºC, while reactors 4, 5 and 6 were biological replicates submitted to 37ºC. The start-up and operation process were similar to that described in the previous chapter. However, in this experiment a not sifted effluent was used, with OLR of 0.24 Kg DQO/m^3.day. Wastewater and sludge were collected during the experimental period with an interval of 10 days, being denominated as T0 (day 0), T1 (day 10), T2 (day 20), T3 (day 30), T4 (day 40), T5 (day 50) and T6 (day 60).

5.3.2. Wastewater physicochemical parameters

The performed analyses were: pH, intermediate alkalinity (IA), partial alkalinity (PA), alkalinity ratio (IA/PA), volatile fatty acids (VFA), total chemical oxygen demand (COD) and total biochemical oxygen demand (BOD). Wastewater sampling was carried out simultaneously to sludge collection. The pH value was measured using pH meter (PHS-3D, Sanxin, Shanghai). Alkalinity was measured by titration method, carried out according to the methodology described by Jenkins et al., (1983) and Ripley et al. (1986). In turn, COD and BOD were carried out according to the Standard Methods for the Examination of Water and Wastewater (APHA et al., 2012). The analyses statistical significance was performed using unpaired T test and R Program (R Core Team, 2015).

5.3.3. Sludge DNA extraction, PCR and next generation sequencing

DNA extractions of the 42 samples were performed using QIAamp Fast DNA Stool Mini Kit (Qiagen, USA) according to the manufacturer's instructions. After extraction, the V4 region of the bacterial and archaeal16S rRNA genes were amplified using fusion primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-

GGACTACHVGGGTWTCTAAT-3′) (Bokulich et al., 2013; Bokulich et al., 2012). This method includes 2 pairs of customized primers, 1 reverse primer and 96 fusion primers with barcodes for V4 region (Ion Amplicon Library Preparation - Fusion Method, Publication Number 4468326, Revision C).

To PCR reactions, manufacturer's recommendations were followed (Ion Amplicon Library Preparation - Fusion Method, Publication Number 4468326, Revision C). PCR products were confirmed using QiAxcel Advanced System (Qiagen) and purified with Agencourt® AMPure XP Reagent (Beckman Coulter, USA). Each amplicon concentration was measured using Qubit®2.0 Fluorometer and Qubit® dsDNA HS Assay Kit (Life Technologies) being pooled in equimolar amounts proportion to produce a composite sample with a final concentration of 26pM.

Sequencing was performed using the Ion Personal Genome Machine (PGM)™ Hi-Q™ View OT2 Kit (Life Technologies). Sample emulsion PCR, emulsion breaking, and enrichment were performed according to the manufacturer's instructions. Briefly, an input concentration of one DNA template copy/Ion Sphere Particle (ISP) was added to the emulsion PCR master mix, and the emulsion was generated using an OT2 (Life Technologies). Subsequently, the ISPs were recovered, and Dynabeads MyOne Streptavidin C1 beads (Life Technologies) were used to enrich for template-positive ISPs. The sample was prepared for sequencing using the Ion PGM™ Hi-Q™ View Sequencing Kit (Life Technologies). Each composite sample was loaded onto an Ion 318 chip v2 and sequenced on the Ion Torrent PGM system for 850 flows. Two synthetic 16S microbial communities (Mock Communities, HM-782D and HM-783D; BEI Resources, USA) of species with known genomes were used to evaluate the quality of metagenomic method.

For the treatment of the sequences obtained in the sequencing process, the data were analyzed as follows: Fastq file with raw data of all barcodes (with the exception of mock communities) was used in OTUs classification pipeline derived from 16S profiling data analysis pipeline of Brazilian Microbiome Project (Pyrlo et al., 2014). Firstly, the raw data was filtered using an *in-house* script (available at: https://github.com/aquacen/fast_sample) with parameters: "-n 100" (test all reads), "-s 160" (include only reads >= 160 bp), "-b 310" (to trim reads >= 310 bp), "-l 0" (no left clip), and "-q 20" (trim 3' reads with Phred quality < 20). After, Uparse software (Edgar, 2013) was used to relabel reads, and Usearch version 10.0.240 (Edgar, 2018) was used to: filter by quality (-fastq_filter -fastq_maxee 0.8), dereplication reads (-fastx_uniques -sizeout), sort by size (-sortbysize -minsize 2), cluster OTUs (-cluster_otus), map raw data over OTUs (-usearch_global -strand plus -id 0.97). Then, Uparse was performed to generate OTUs list and convert UC map file to OTUs table, and the QIIME version 1 (Carporaso et al., 2010) was used to performs: assign taxonomy (--similarity 0.7), align OTUs sequences, filter alignment, and make phylogeny tree. Finally, the Biom software version 2.1.5 (McDonald et al., 2012) was used to: convert biom table do json, add metadata of QIIME assign taxonomy (--observation-header OTUID,taxonomy,confidence --sc-separated taxonomy --float-fields

confidence) and summarize OTUs table. The Usearch version 10.0.240 includes chimera filters in cluster OTUs step (-cluster_otus). Two barcodes with mock communities were evaluated using the same steps in different assay. Statistics and charts of Alpha (α) and Beta (β) diversity were generated in the Web Server Microbiome Analyst (Dhariwal et al., 2017), with the following filters: minimal count=4; prevalence in sample=10%; low variance percentage to remove=5% and rarefying to the minimum library size The differences in the overall community composition and structure among all groups were visualized using the non-metric multidimensional scaling (NMDS) ordination plots of Bray–Curtis distance matrices.

5.4. RESULTS AND DISCUSSION

5.4.1. Operational stability and organic matter removal

Results of physicochemical parameter obtained in the experimental period are showed in Table 1. The raw wastewater was also analyzed, which revealed a pH average of 6.61 and 345 mg L^{-1} of VFA. BOD and COD means were 1226 mg L^{-1} and 3405 mg L^{-1}, respectively. This effluent has higher organic load values when comparing to those described in the previous chapter, founding 502 mg L^{-1} of BOD, and 1195 mg L^{-1} of COD.

The pH values were close to neutrality at both conditions, however, at T3 20°C reactors were discretely more alkaline. The methanogens growth rate is greatly reduced below 6.6, whereas an excessively alkaline pH can lead to microbial granules disintegration and hence process failure (Ward et al., 2008). Although pH rates between 7.2 and 8.2 indicates stabilization in gas production (Abdelgadir et al., 2014), in anaerobic reactors monitoring the alkalinity values may be more accurate in determining stability, since pH variation implies the consumption of a large amount of alkalinity, which reduces the buffer capacity of the medium (Pereira et al., 2013; Ward et al., 2008). Evaluating the IA/PA ratios, it can be observed that the reactors at 37°C were out of recommended values (up to 0.3) from T5 (Rodrigues et al, 2014; Martín-González et al. al., 2013). VFAs also showed statistical differences from T2, being superior at 37°C reactors. In T3, values higher than 500 mg L^{-1} were detected at 37°C, which remained high until the end of the experiment.

BOD and COD values showed a tendency to decrease during the experimental period in both temperatures, showing statistical differences between 20°C and 37°C ($p = <0.005$) from T5 and T3, respectively. To visualize the global organic matter removal efficiencies, BOD and COD percent indices were analyzed and are shown in Figure 1. The maximum values of BOD and COD removal were respectively 70% (T5 and T6) and 67,37% (T5), both occurring at 20°C. Another important point to be highlighted is that 20°C reactors were more efficient than reactors at 37°C all over the experiment, showing better performances. When comparing the averages of organic matter removal taking into account all collection times, it was observed that at 20°C reactors were

17.47% superior at BOD removal and 19.58%% at COD removal. Thus, despite the superiority of the 20°C reactors, the reactors at 37°C were also efficient, even with the accumulation of VFA due to the increase in the OLR.

5.4.2. Microbial community structure and diversity

The high-throughput data from sequencing of sludge samples resulted in 42 phyla, 67 classes, 102 orders, 142 families, 201 genera and 40 species from 3,231,358 reads. To evaluate the sequencing run and validate the quantitative results, the two used mocks showed OTUs sequenced identification according to the expected abundance reported by the manufacturer (BEI Resources). Chao1 individual-based rarefaction curves (Figure 2) indicated that microbial communities were well sampled and allows diversity comparisons, confirmed by the Good's coverage values (>99% in all samples). The diversity of the microbial community (Simpson index) at family level was closely related to temperature. Reactors at 20°C have values closer to 1 comparing to those at 37°C (p = 0.00092), indicating that this temperature have low relative diversity (Figure 3). Principal Coordinates Analysis from Bray–Curtis distances showed that samples were clustered according to temperatures, being more similar and closer to each other at 20°C (Figure 4).

In relation to detected communities, analyses were made to identify microorganism's abundance at phyla and family levels, which are demonstrated in Figures 5 and 6, respectively. The archaeal abundance means remained below 1% of the reads at all collection times, being grouped with other non-significant microorganisms. In the present work, temperature and OLR appeared to play a major role in the adaptation of the communities, since noticeable shifts in the bacterial community structure were seen throughout the experiment. In addition, it could be observed that within each condition there were also changes in the composition and abundance of microorganisms over time. Considering the reactors at 20°C, at T0 there was a domain of *Proteobacteria* (47.97%) and *Synergistetes* (31,23%) phyla. However, this value changed throughout the experiment and in T6 the phylum *Synergistetes* became the predominant, presenting an abundance of 34.10%, against 27.88% of the *Proteobacteria* phylum. *Firmicutes* and *Bacteroidetes* also presented growth, showing higher abundances in T6 (18.70% and 12.45%, respectively). At 37°C, although in T0 there were a similar division of the communities with the 20°C reactors, the phylum *Proteobacteria* falls abruptly, going from 39.32% in T0 to 12.56% in T6, different from what occurs in the cooler reactors. The *Proteobacteria* gives place to *Synergistetes* phylum, which becomes dominant, presenting abundance of 28.17% in T0 and 52.29% in T6. *Firmicutes* and *Bacteroidetes* have their maximum values in T1 (28.09%) and T3 (13.60%) respectively, with a slight posterior decrease.

The main detected phyla and families in this study were also reported at different abundances in previous studies using different substrates and anaerobic reactor configurations. Gunnigle et al. (2015) evaluated laboratory-scale anaerobic reactors at

different temperatures. At 37°C they detected a domain of the *Proteobacteria* phylum (61%), which decreased to 19% at 15°C. Bacteroidetes accounted for 10% of the OTUs at 37°C and increased to 16% at 15°C. In addition, *Firmicutes* were found to be more prevalent at 15°C when compared to 37°C. Differently, evaluating a full-scale mesophilic anaerobic reactor, Qin et a. (2019) found as the most predominant the phyla *Chloroflexi* (18.0%), *Proteobacteria* (12.5%), *Bacteroidetes* (9.5%) and *Firmicutes* (8.2%). Evaluating two anaerobic reactors under an overloading phase, Braz et al. (2019) found similar compositions in different proportions, where over 70% of bacterial community belonged to *Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Chloroflexi* phyla. In its turn, in the previous chapter, where the same temperatures and lower applied organic loads were evaluated, in addition to the observed phyla in this work (*Bacteroidetes*, *Firmicutes*, *Proteobacteria* and *Synergistetes*), the *Chlorobi* and *Spirochaetes* phyla were significant only in reactors at 37°C.

Regarding families, it could be observed that there was also a differentiation through the temperatures and studied times. Most of the detected families have already been mentioned in previous studies related to anaerobic processes treating different types of effluents (ElNaker et al., 2018; Delforno et al., 2017; Wojcieszak et al., 2017). The predominant archaeal family of was *Methanobacteriaceae*, but as this phylum showed very low values (0.29% at 20°C and 0.42% at 37°C), these microorganisms were grouped with other non-significant (reads with values lower than 1%). In its turn, considering bacteria, there were different patterns at both evaluated temperatures. Altogether, nineteen families with abundances greater than 1% were obtained at 20°C and twenty-two at 37°C. At 20°C, the *Ectothiorhodospiraceae* family starts with the greatest abundance, however, their values fall considerably from 18.41% in T0 to 6.55% in T6. The families *Syntrophorhabdaceae* and *Enterobacteriaceae* also presented falls during the experimental period, presenting smaller abundances in the end of the experiment. The *Moraxellaceae* family presents low abundance in T0, however, it rises abruptly, presenting a peak of 17.53% in T3 with subsequent fall, presenting an abundance of 6.95% in T6. The family *Dethiosulfovibrionaceae* showed an increase in their abundance, which raised from 9.76% in T0 to 13.64% in T6, however, the family with the greatest abundant growth was *Synergistaceae*, increasing from 7.46% in T0 to 18. 07% in T6. Despite the average abundance of 6.92%, the *Veillonellaceae* family does not show great variation, maintaining similar values at all collection times. The same occurs with the other families represented (*Aminiphilaceae*, *Comamonadaceae*, *Desulfovibrionaceae*, *Fusobacteriaceae*, *Pirellulaceae*, *Porphyromonadaceae*, *Rhodobacteraceae*, *Ruminococcaceae*, *S24-7*, *Sphingomonadaceae* and *TTA_B6*), which, although values higher than 1%, presented smaller abundances.

In contrast, at 37°C one main family showed prominence, and there were the *Synergistaceae* family. In T0, this group did not present the greatest abundance (9.97%) but grew considerably, with a peak in T5 (30.82%) and 28.36% in T6. In the beginning of the experimental period, the families *Dethiosulfovibrionaceae* and *Ectothiorhodospiraceae* had the highest abundances (12.91% and 14.92%, respectively), however, both showed a decrease throughout the experiment. The family

Anaerobaculaceae presented significant growth, rising from 0.07% to 11.57% of abundance. In turn, the other represented families (*Acetobacteraceae*, *Aminiphilaceae*, *Anaerolinaceae*, *Clostridiaceae*, *Desulfomicrobiaceae*, *Desulfovibrionaceae*, *Enterobacteriaceae*, *Mogibacteriaceae*, *Pirellulaceae*, *Porphyromonadaceae*, *Rhodobacteraceae*, *Rhodocyclaceae*, *Sphingomonadaceae*, *Syntrophaceae*, *Syntrophorhabdaceae*, *TTA_B6*, *Veillonellaceae*, *Xanthomonadaceae*) remained stable and with lower abundances. Besides that, tables were also elaborated to show all the main sequenced microorganisms (reads above 1%) at both temperatures and collection times, with all taxonomic levels obtained through the metagenomic assay (Supplementary Tables S1 and S2).

5.4.3. Associations between, temperature, organic load rate and microbiome

In anaerobic digesters with low buffering capacity, pH, alkalinity and VFA are reliable indicators for process imbalance. Nevertheless, even in stressed process, pH changes may be unimportant in highly buffered systems. In these cases, VFA can be considered more reliable for process monitoring (Franke-Whittle et al., 2014). In this study it can be observed that VFA concentrations reflected a reduction in the removal efficiency of both BOD and COD in the reactors at 37°C from T3, indicating the influence of this parameter in the process. High OLR may cause an imbalance in anaerobic digestion, as overloading can lead to a VFA production and accumulation, acidifying the medium. These acids reflect a kinetic uncoupling between acid producers and consumers, being one of the most common reasons for operational failure (Braz et al., 2019; Vanwonterhem et al., 2015; Franke-Whittle et al., 2014; Solli et al., 2014; Martín-González et al. al., 2013; Pereira et al., 2013). In relation to the removal of organic matter, different from that shown in the previous chapter, the reactors at 20°C showed better results when compared to those at 37°C at all collection times. Despite considerable values of BOD and COD removal at both temperatures, reactors at 20°C were statistically superior from T5 and T3, respectively. It may be related to the lower concentrations of VFA that these reactors were subjected to.

Temperature is usually associated with higher efficiency rates due to increased hydrolysis coefficient and kinetic reactions, once enhance the contact between sludge and solids (Abdelgadir et al., 2014; Ho et al., 2014). However, in the present study, mesophilic temperatures associated with a higher organic load lead to a VFA concentration, which influence on reactor performance. Similar studies showed that in anaerobic digestion high load rates may influence on VFA accumulation, indicating that biotransformation of substrate to biogas is related to influent concentration (Ding et al., 2017; Solli et al., 2014; Massouri et al., 2013).

Considering the shift in bacterial communities, during organic overloading disturbances in its behavior may be complex due to the high number of species and their functional redundancy (Braz et al., 2019). As shown in Figure 7, the main observed families may be related to different phases of the anaerobic digestion process and may occur on a smaller or larger scale in each step. Amid microorganisms that stood out due to their

growth or decay, *Ectothiorhodospiraceae* is a gram-negative anaerobic growing family with high diverse physiology. Some members use nitrate and other oxidized nitrogen compounds as electron acceptors, as well performs autotrophic nitrite oxidation. Their 64% and 80% decay in the reactors at 20°C and 37°C (respectively) can be explained by their alkaline pH requirement for their optimum growth, since the reactors at 20°C present higher pH values (Oren, 2014; Leguijt et al., 1993).

Syntrophorhabdaceae, widely distributed in anaerobic environments, also showed decay at both temperatures, with values reduced in 75% at 20°C and 97% at 37°C. Some members of this family have been suggested to be effective in the anaerobic degradation of phenol (commonly present compound in industrial disinfectants) to acetate. Their abundance may be regulated by optimal hydrogen partial pressure, acting in syntrophy with hydrogen concentration reducers such as *Dethiosulfovibrionaceae,* which grow by fermentation of amino acids to produce H_2S when sulfur is present (Chen et al., 2009; Qiu et al., 2008; Surkov et al., 2001; Magot et al., 1997). The smaller reductions in the abundance of *Syntrophorhabdaceae* at 20°C may then be related to the increase (39%) in the abundance of *Dethiosulfovibrionaceae.* The augment occurred only at this temperature, and its values dropped exactly 39% at 37°C.

Another important family was *Anaerobaculaceae*, which showed a remarkable increase only at 37°C, rising from 0.07 to 11.57, a growth of more than 16.000%. Confirming what has been described by Regueiro et al. (2016), VFA provoked an increase in the *Anaerobaculaceae* family, appointing that this group was closely linked to the VFA levels in the 37°C reactors, since this temperature showed higher acids rates.

In its turn, *Synergistaceae* family presented a growth at both temperatures (142% and 184% at 20°C and 37°C, respectively), but the final abundance was higher at 37°C reactors (28% against 18%). In agreement to that found in the previous chapter, *Synergistaceae* was the most representative family in both temperatures, being *VadinCA02* the predominant genus. *Synergistaceae* have the ability to degrade amino acids into VFA, and along with *Dethiosulfovibrionaceae,* contribute to acidogenesis and acetogenesis via syntrophic relationships with methanogens, for example by hydrogen transfer. Thus, it can improve *Archaea* performance through hydrogenotrophic pathway. In fact, although *archaeal* community was low in this study, it was dominated by hydrogenotrophic *Methanobacteriaceae,* which indicates that adaptation process is toward methanogens that utilize $H_2 + CO_2$ (Wojcieszak et al., 2017; Ferguson et al., 2016; Militon et al., 2015). However, this result differs from expected, since previous knowledge assumed that two-thirds of methane are obtained from the acetoclastic methanogenesis and one-third from hydrogenotrophic methanogenesis. Methanosaeta, which metabolize acetate as their only source of energy, appeared at all times in an average concentration of 0.1% (Meegoda et al., 2018; Manyi-Loh et al., 2013).

Notably, the methane formation groups a specialized community who depend on each other to survive and maintain their metabolic activities. As well, this species requires specific physicochemical and nutritional conditions for their survival and multiplication.

Microorganisms obtained from different environments with specific needs and subjected to controlled external factors (such as temperature) tend to vary, even though they continue to perform the phases of anaerobic digestion in a greater or lesser extent. Therefore, the microorganism's interactions that role the anaerobic digestion are incredibly complex, and these relationships strongly influences reactors performances (Manyi-Loh et al., 2013).

5.5. CONCLUSION

Compare 20°C and 37°C reactors treating high organic load fish processing wastewater lead to some insights in the observed anaerobic digestion dynamics. An accumulation of VFA at 37°C favored a superiority of 20°C reactors in the removal of organic matter, in addition, communities' profiles presented differences in relation to their structure. These results suggest that several microorganisms become predominant as the consequence of the increase in VFA production is induced by high OLR. Besides operational parameters, the microbial community structure is shown to be useful for the monitoring and understanding of the anaerobic process.

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TABLES

Table 1. Physicochemical analysis results of reactors effluents.

Supplementary Table S1. Detected microorganisms and their taxonomic levels found at 20°C separated by collection times.

Supplementary Table S2. Detected microorganisms and their taxonomic levels found at 37°C separated by collection times.

FIGURE LEGENDS

Figure 1. Percentage of BOD and COD removal at 20°C and 37°C throughout the experimental days. The y-axis reveals the percentages of removal and x-axis represents the collection times during the experiment.

Figure 2: Chao1 individual-based rarefaction curves for the 16S rRNA V4 region of bacterial and archaeal reads comparing studied temperatures.

Figure 3: Simpson diversity index of samples at 20°C and 37°C.

Figure 4: Principal Coordinates Analysis plot derived from Bray–Curtis distances between samples.

Figure 5. Representative sequenced phyla clustered by temperatures (20°C and 37°C) and collection times, including others (significance level inferior than 1%) and unassigned microorganisms.

Figure 6. Representative sequenced families clustered by temperatures (20°C and 37°C), including OTUs that did not reach the family level and unassigned microorganisms. The y-axis represents the reads percentage and the x-axis the collection times.

Figure 7. Relationship of the main families observed in the two studied temperatures (20°C and 37°C) with the anaerobic digestion process steps.

Table 1. Physicochemical analysis results of reactors effluents.

Day	20ºC (mean±sd)	37ºC (mean±sd)	P value
	pH		
0	7,88±0,01	7,78±0,00	0,11487
10	7,82±0,01	7,77±0,00	0,51135
20	7,81±0,00	7,77±0,00	0,20219
30	7,81±0,00	7,73±0,00	0,01678*
40	7,84±0,00	7,74±0,00	0,01324*
50	7,89±0,01	7,71±0,00	0,00224*
60	7,84±0,01	7,67±0,01	0,01788*
	IA/PA		
0	0,27±0,04	0,29±0,02	0,06677
10	0,28±0,00	0,30±0,03	0,02572
20	0,26±0,04	0,33±0,02	0,00039*
30	0,26±0,04	0,35±0,06	0,00219*
40	0,27±0,06	0,36±0,04	0,00196*
50	0,29±0,03	0,40±0,02	0,00018*
60	0,32±0,02	0,40±0,02	0,00078*
	VFA mg L^{-1}		
0	96±0,13	108±0,11	0,28786
10	160±0,22	220±0,16	0,10119
20	220±0,16	300±0,00	0,01613*
30	376±0,13	588±0,08	0,00576*
40	376±0,05	552±0,08	0,00291*
50	368±0,07	520±0,10	0,00922*
60	372±0,03	540±0,06	0,00102*
	BOD mg L^{-1}		
0	800±0,04	820±0,04	0,51852
10	740±0,05	780±0,08	0,37390
20	660±0,09	700±0,05	0,37390
30	600±0,10	660±0,09	0,28786
40	520±0,13	640±0,11	0,10119
50	440±0,16	640±0,11	0,02411*
60	440±0,16	720±0,00	0,00219*
	COD mg L^{-1}		
0	1986±0,03	1964±0,02	0,64333
10	1700±0,03	1850±0,02	0,01324
20	1717±0,02	1815±0,03	0,06701
30	1434±0,04	1942±0,03	0,00025*

40	1291±0,02	1778±0,02	0,00005*
50	1206±0,05	1969±0,02	0,00005*
60	1207±0,02	1868±0,03	0,00003*

IA: intermediate alkalinity; PA: partial alkalinity; IA/PA: alkalinity ratio; VFA: volatile fatty acids; BOD: biochemical oxygen demand; COD: chemical oxygen demand.
P value: referring the test of the null hypothesis of equality of means.
* P value <0,005.

Supplementary Table S1. Detected microorganisms and their taxonomic levels found at 20°C separated by collection times.

KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	T0	T1	T2	T3	T4	T5	T6
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*	*B. acidifaciens*	0,03	0,00	0,03	0,04	0,32	0,06	1,12
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Bacteroidaceae*	*Bacteroides*		0,04	0,03	0,05	0,15	0,49	0,12	1,16
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Paraprevotellaceae*	*Prevotella*		0,00	0,01	0,08	0,20	0,52	0,26	1,72
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*S24-7*			0,01	0,05	0,16	0,22	0,66	0,23	2,27
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*				1,32	1,32	1,77	1,71	2,29	2,56	3,08
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Ruminococcaceae*			0,44	0,48	0,72	0,79	1,16	1,82	3,25
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*			6,34	4,60	5,16	4,89	3,77	4,69	5,33
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*	*VadinHB04*		0,81	0,73	1,82	1,97	1,74	1,84	1,85
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*				4,92	2,56	1,76	2,50	2,37	2,37	3,23
Bacteria	*Fusobacteria*	*Fusobacteriia*	*Fusobacteriales*	*Fusobacteriaceae*	*U114*		1,96	2,33	1,77	2,04	2,04	2,01	1,43
Bacteria	*OP8*						1,46	0,57	0,33	0,36	0,32	0,26	0,16
Bacteria	*Planctomycetes*	*Planctomycetia*	*Pirellulales*	*Pirellulaceae*			1,79	2,02	1,77	1,49	1,23	1,15	0,92
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacterales*	*Caulobacteraceae*	*Caulobacter*	*C. henricii*	0,05	0,31	1,10	0,12	0,12	0,20	0,04
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*			1,72	1,30	0,80	0,73	0,57	0,55	0,39
Bacteria	*Proteobacteria*	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Comamonas*		1,22	2,42	1,62	1,31	1,70	1,44	1,20
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfomicrobiaceae*	*Desulfomicrobium*		1,28	0,61	0,50	0,68	0,91	1,09	0,75
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Desulfovibrio*		1,39	1,09	1,77	2,06	1,92	2,10	2,57
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophorhabdaceae*			8,17	6,40	4,52	3,86	3,02	3,03	1,99
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	*Ectothiorhodospiraceae*			18,41	17,27	14,68	12,73	8,95	8,67	6,55
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*		2,97	0,95	0,43	0,32	0,35	0,22	0,48
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*			1,46	0,16	0,13	0,13	0,20	0,11	0,33
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonadales*	*Moraxellaceae*	*Acinetobacter*		4,90	8,91	9,35	17,53	16,79	13,42	6,95
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		1,89	0,96	1,04	1,10	0,99	1,04	1,01
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*HA73*		3,52	3,55	3,81	4,17	3,95	4,08	3,83
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*PD-UASB-13*		6,15	6,99	8,78	8,44	7,75	8,95	9,27
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		6,54	12,95	16,02	12,24	14,62	16,93	15,97

Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*		0,92	1,31	1,59	1,62	1,78	1,98	2,10
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*TTA_B6*	*E6*	1,63	2,14	1,43	1,26	1,36	1,35	0,83
Unassigned						0,24	0,10	0,06	0,07	0,04	0,05	0,04
Not significant (<1%)						18,43	17,87	16,97	15,24	18,05	17,43	20,17

Supplementary Table S2. Detected microorganisms and their taxonomic levels found at 37°C separated by collection times.

KINGDOM	PHYLUM	CLASS	ORDER	FAMILY	GENUS	SPECIES	T0	T1	T2	T3	T4	T5	T6
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*	*Porphyromonadaceae*			0,86	2,57	3,50	3,24	2,27	2,19	2,92
Bacteria	*Bacteroidetes*	*Bacteroidia*	*Bacteroidales*				1,03	3,69	4,03	9,58	8,50	7,45	7,52
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Clostridiaceae*	*Caloramator*		0,00	0,04	0,34	0,37	0,73	1,11	1,46
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Mogibacteriaceae*	*Anaerovorax*		0,03	1,45	0,59	0,41	0,16	0,20	0,07
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*	*Veillonellaceae*			4,40	1,60	1,69	1,39	0,96	1,30	3,31
Bacteria	*Firmicutes*	*Clostridia*	*Clostridiales*				8,81	21,39	18,52	20,78	16,59	15,28	12,66
Bacteria	*OP8*						1,46	0,42	0,30	0,17	0,23	0,10	0,06
Bacteria	*Planctomycetes*	*Planctomycetia*	*Pirellulales*	*Pirellulaceae*			1,63	0,51	0,31	0,35	0,27	0,26	0,22
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*			2,27	0,66	0,68	0,63	0,80	0,69	0,52
Bacteria	*Proteobacteria*	*Alphaproteobacteria*	*Rhodospirillales*	*Acetobacteraceae*			1,37	0,07	0,05	0,08	0,05	0,03	0,05
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*	*Bilophila*		0,12	2,36	2,55	1,99	0,93	0,60	0,63
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfomicrobiaceae*	*Desulfomicrobium*		1,52	1,27	0,12	0,08	0,07	0,24	0,19
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrionales*	*Desulfovibrionaceae*			0,79	1,13	1,35	1,34	1,36	1,46	1,57
Bacteria	*Proteobacteria*	*Deltaproteobacteria*	*Syntrophobacterales*	*Syntrophorhabdaceae*			3,81	0,75	0,25	0,18	0,11	0,09	0,11
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Chromatiales*	*Ectothiorhodospiraceae*			14,92	6,02	4,55	4,42	2,73	3,03	2,87
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*		1,47	1,16	0,50	0,97	0,25	0,17	0,20
Bacteria	*Proteobacteria*	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*			0,53	5,46	1,94	1,54	0,58	0,32	0,86
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Aminiphilaceae*	*Aminiphilus*		3,03	3,92	4,07	4,28	3,56	3,29	3,58
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Anaerobaculaceae*	*Anaerobaculum*		0,07	0,74	5,90	7,30	9,36	9,76	11,57
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*Aminobacterium*		0,07	0,71	5,15	4,82	4,21	3,35	2,55
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*HA73*		3,68	2,68	2,56	2,96	2,63	2,56	2,22
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Dethiosulfovibrionaceae*	*PD-UASB-13*		9,06	3,85	3,55	4,07	3,54	3,01	3,01
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*Synergistaceae*	*VadinCA02*		9,18	23,04	23,13	14,32	26,64	30,10	27,84
Bacteria	*Synergistetes*	*Synergistia*	*Synergistales*	*TTA_B6*	*E6*		1,84	1,06	1,12	0,95	1,25	0,78	0,88
Unassigned							0,20	0,06	0,03	0,03	0,03	0,02	0,03
Not significant (<1%)							27,86	13,41	13,24	13,74	12,18	12,60	13,09

Figure 1. Percentage of BOD and COD removal at 20°C and 37°C throughout the experimental days. The y-axis reveals the percentages of removal and x-axis represents the collection times during the experiment.

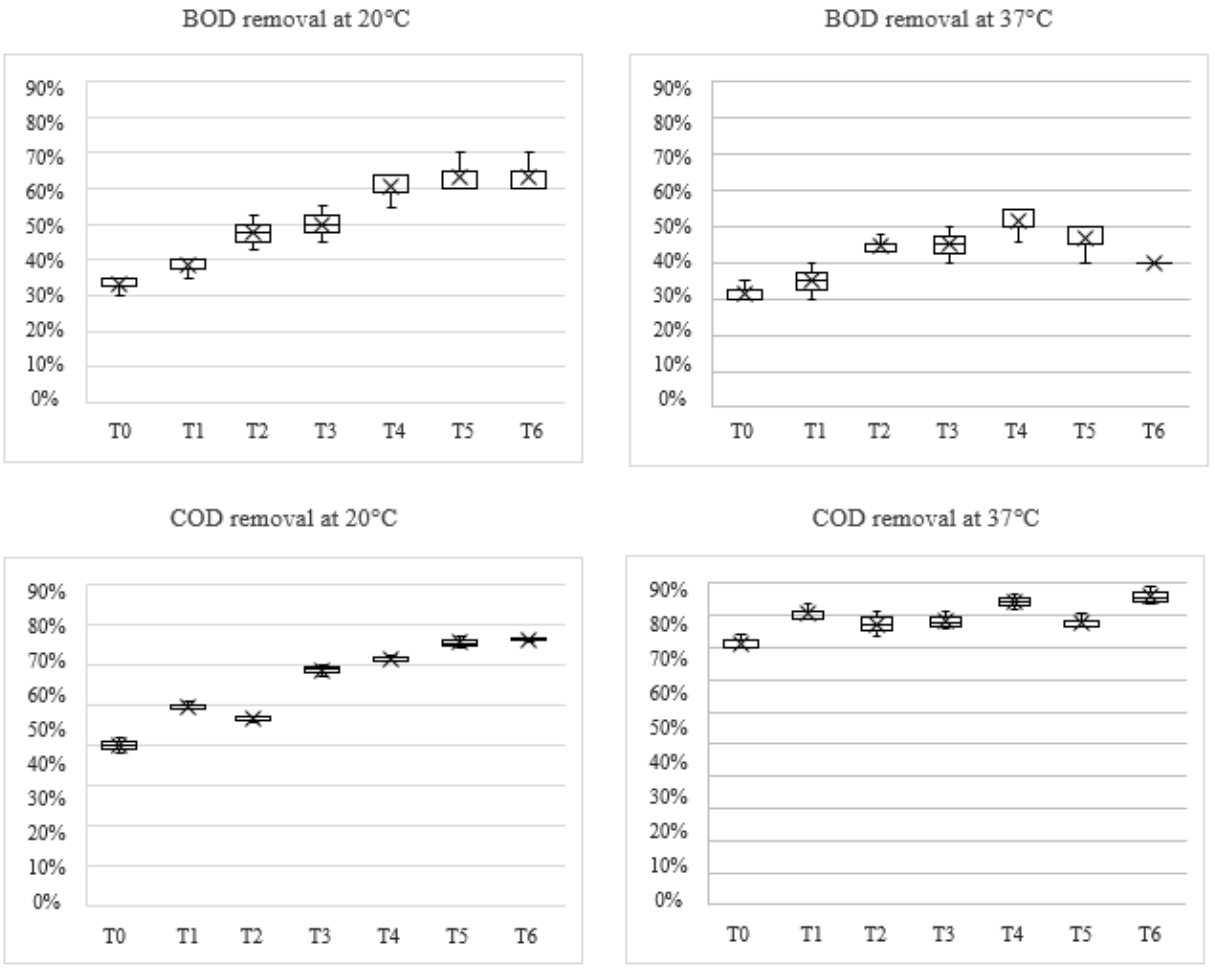


Figure 2: Chao1 individual-based rarefaction curves for the 16S rRNA V4 region of bacterial and archaeal reads comparing studied temperatures.

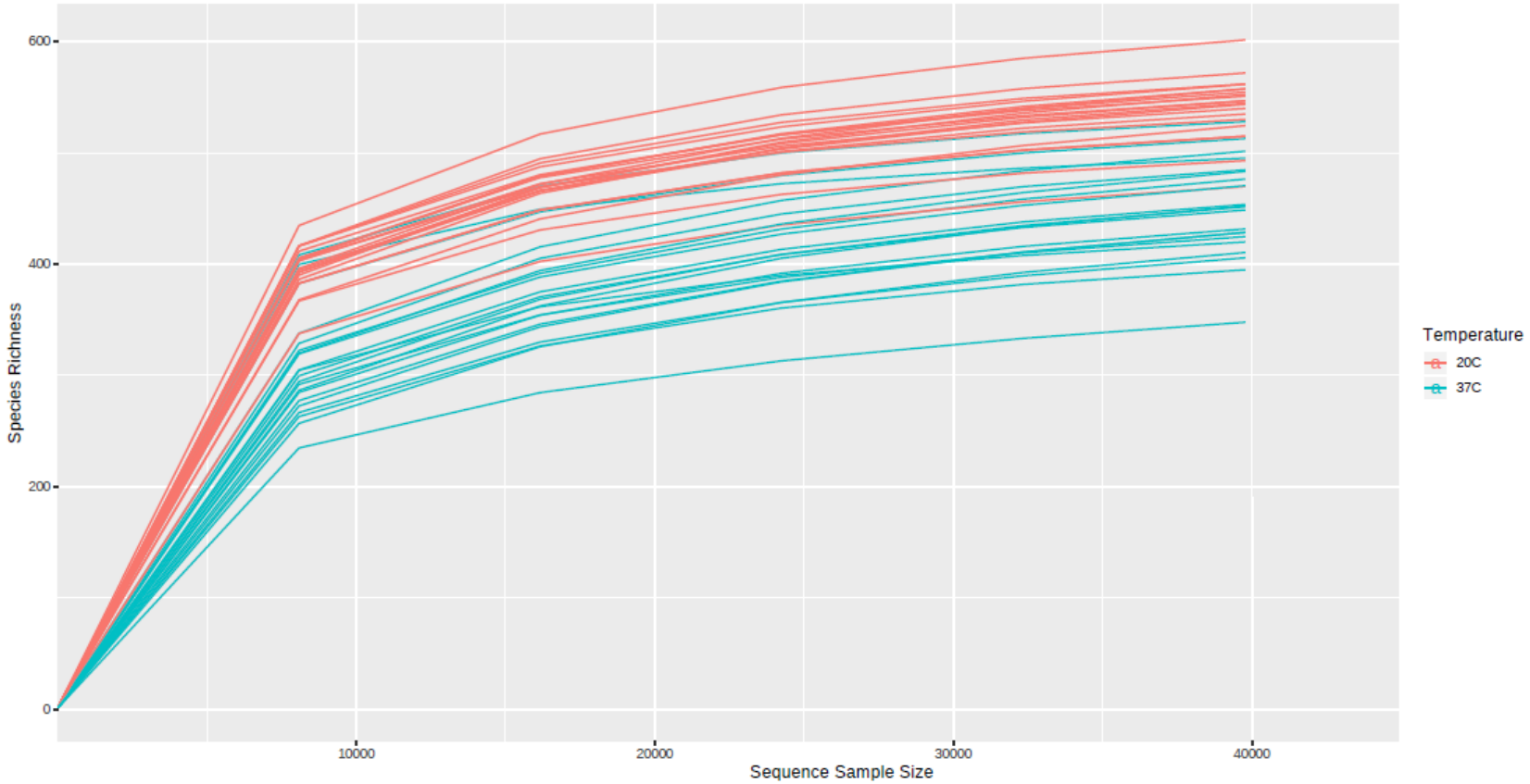


Figure 3: Simpson diversity index of samples at 20°C and 37°C.

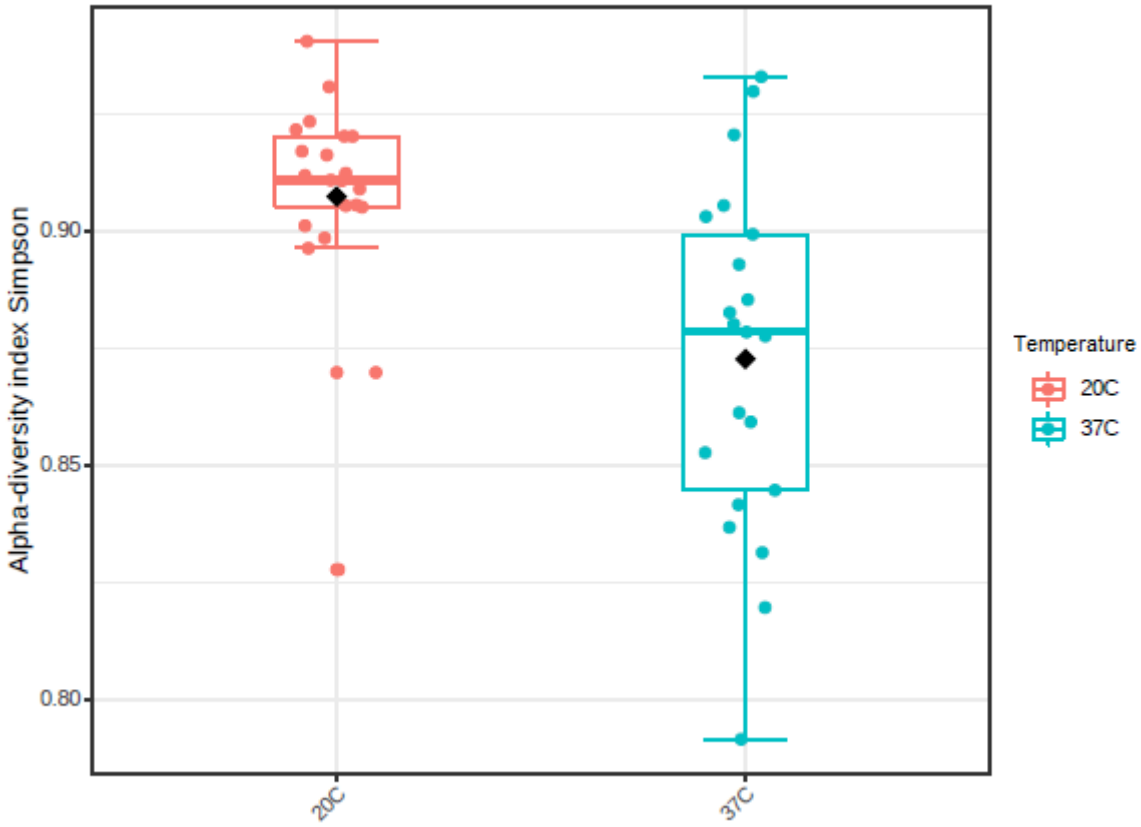


Figure 4: Principal Coordinates Analysis plot derived from Bray–Curtis distances between samples.

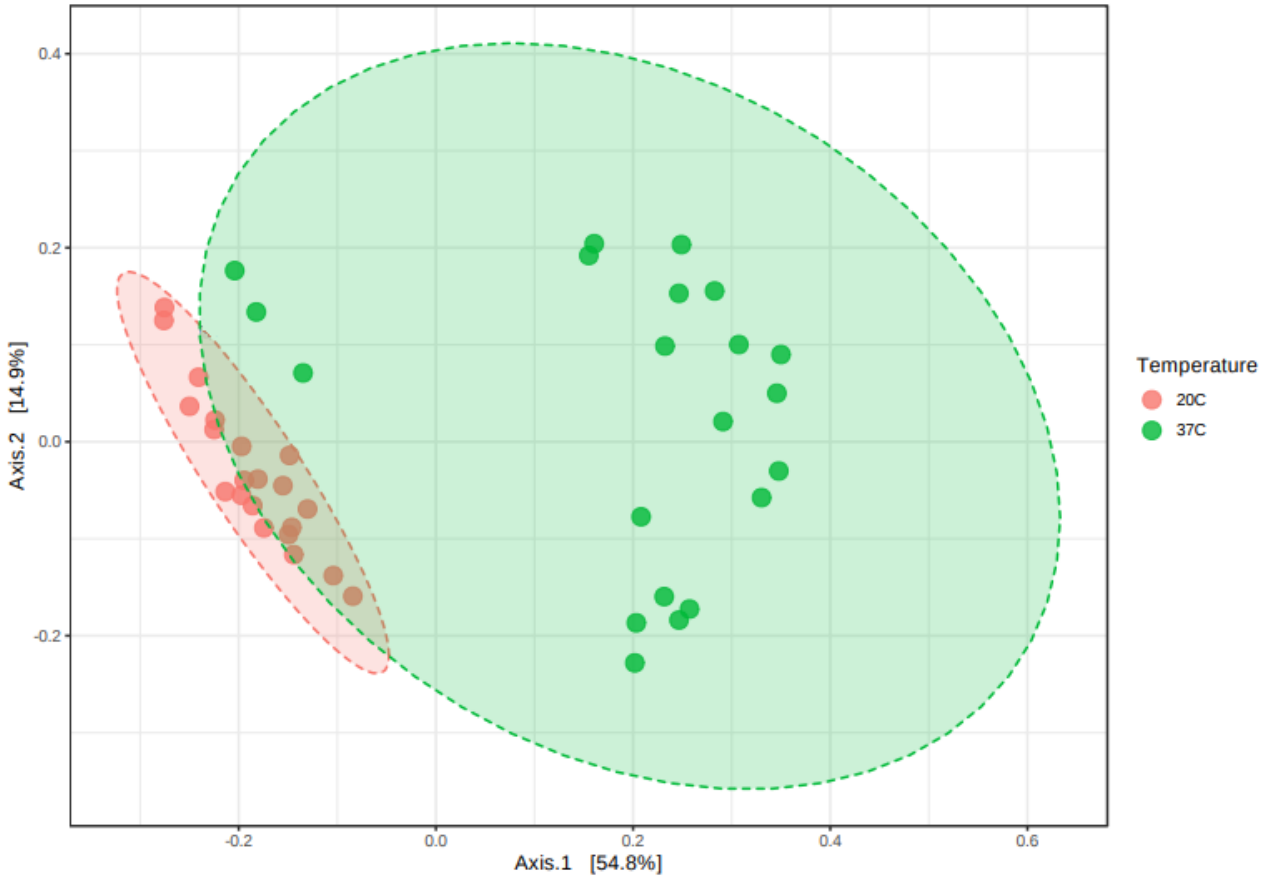


Figure 5. Representative sequenced phyla clustered by temperatures (20°C and 37°C) and collection times, including others (significance level inferior than 1%) and unassigned microorganisms.

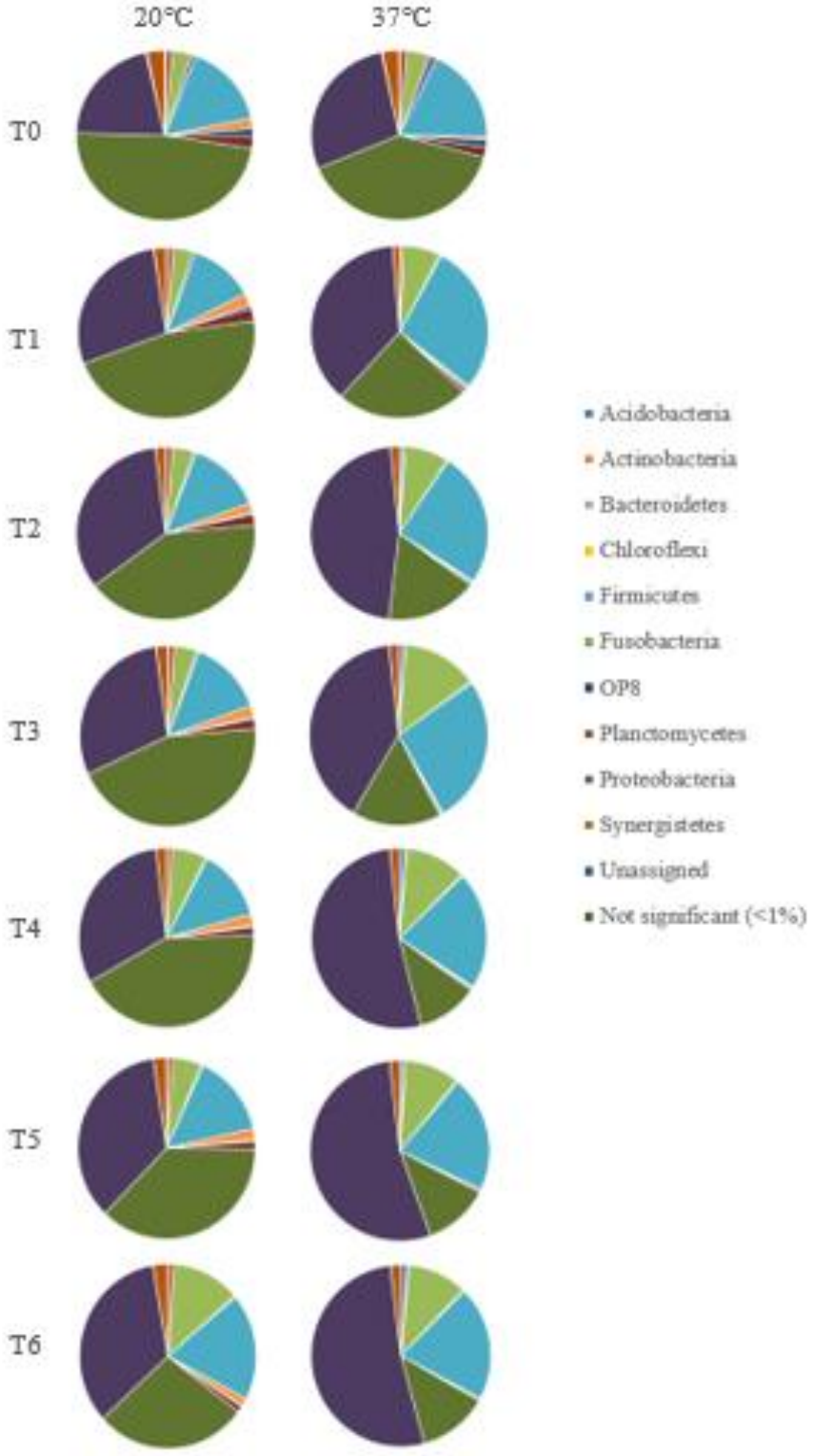


Figure 6. Representative sequenced families clustered by temperatures (20°C and 37°C), including OTUs that did not reach the family level and unassigned microorganisms. The y-axis represents the reads percentage and the x-axis the collection times.

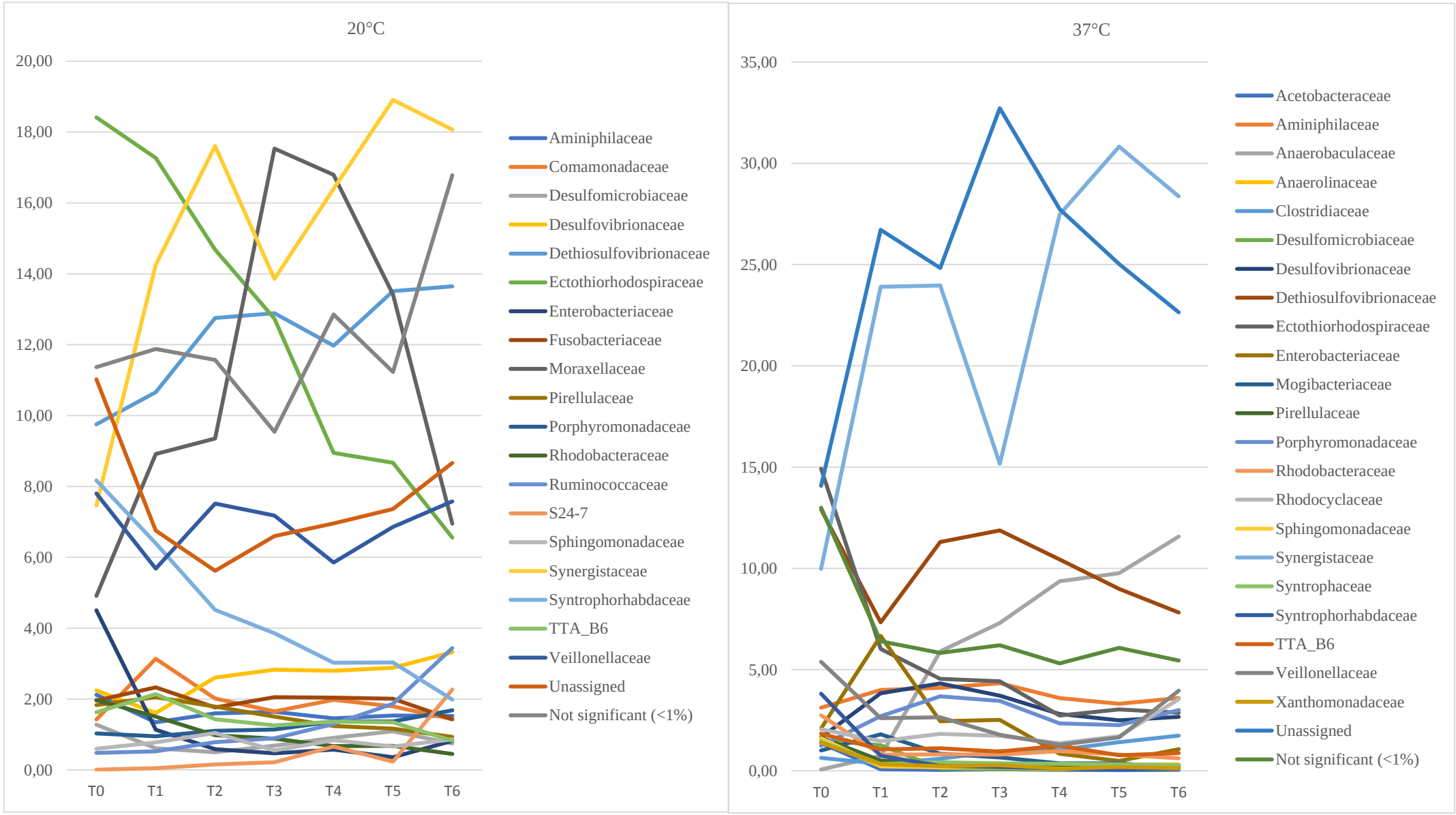
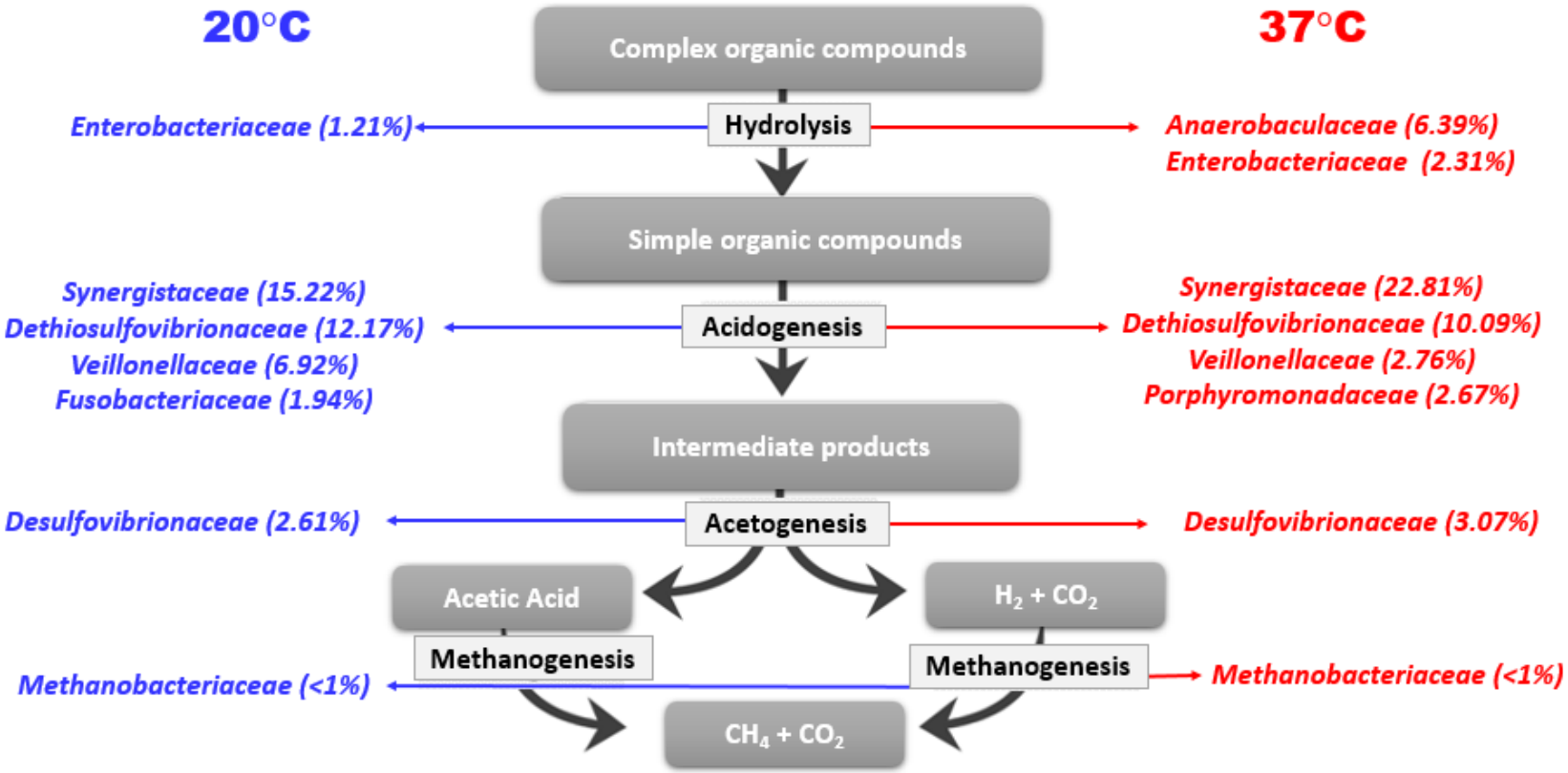


Figure 7. Relationship of the main families observed in the two studied temperatures (20°C and 37°C) with the anaerobic digestion process steps.



6. FINAL CONSIDERATIONS

In Brazil, there is a large number of small scale producers and fish slaughterhouses, through which market demand must be supplied. The availability of low cost and high efficiency wastewater treatment technologies can help to maintain this sector and its competitiveness. The physical and chemical characteristics of effluents from breeding and processing industries of animal products are highly variable, requiring specific studies for each system. Recent research has suggested the use of anaerobic biological processes for the treatment of fish processing wastewater, since it reduce biomass wastes and mitigates a wide spectrum of environmental undesirables. Previous work has attempted to explore the microbial community ecology of anaerobic digestion through the use of 16S rRNA gene high-throughput sequencing, however, this is the first metagenomic study of sludge from anaerobic reactors treating effluents from this industry.

Given the importance of the theme due to the increased production of fish effluents over the last decades, understanding the dynamics of microbial communities in anaerobic digestion is crucial for the optimization of this process. The comprehension of key microorganism's management may favor not only the identification of anaerobic microbiomes, but the establishment of microbial indicators and development of biotechnological methods to improve anaerobic systems. A greater understanding of the dynamics of these communities broadens the knowledge about biochemical reactions and can bring real perspectives on maximizing efficiency and reducing process costs.

This study contributes to the elucidation about anaerobic community's dynamics in fish processing wastewater treatment systems, evidencing that reactor stability and the microbial community may vary according to factors that influence anaerobic digestion, such as temperature and OLR. Pilot-scale anaerobic reactors proved to be efficient for the removal of organic matter at both 20°C and 37°C, demonstrating its applicability to treat fish processing wastewater in a country with a wide range of temperatures throughout the year, such as Brazil. The microbial communities fluctuated at both temperatures, however, even with changes in temperatures and applied loads, the microbiota has shown to balance and reactor efficiency was maintained.

The found results may allow a continuing searching for new data, and in turn, lead to a greater understanding of the anaerobic processes. It is recommended the study of other types of fish effluents at different physicochemical conditions, mainly of those from species that may have influence the national market. Besides that, encompassing metaproteomic and metabolomic approaches as other molecular tools can bring more response and great impact on this area. In coming years, the expectation is that a greater number of studies are carried out and published on the biological treatment of effluents from the fish processing industry, together with superior anaerobic reactors configurations, deeper sequencing, more comprehensive databases and the continuing improvement of next generation sequencing methods.