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POPULATION DYNAMICS OF METHANOGENIC ARCHAEAS IN CO-DIGESTION SYSTEMS

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Dissertação apresentada ao Programa de Pós-graduação em Ecologia Aplicada ao Manejo e Conservação dos Recursos Naturais, da Universidade Federal de Juiz de Fora como parte dos requisitos necessários à obtenção do título de Mestre em Ecologia aplicada ao Manejo e Conservação dos Recursos Naturais.

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Dedico este trabalho à minha família! Aos meus pais Heloisa e Mário,
meu irmão Gabriel e ao Leo, meu amor.

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E assim, depois de muito esperar, num dia como outro qualquer, decidi triunfar. Decidi não esperar as oportunidades e sim, eu mesmo buscá-las. Decidi ver cada problema como uma oportunidade de encontrar uma solução. Decidi ver cada deserto como uma possibilidade de encontrar um oásis. Decidi ver cada noite como um mistério a resolver. Decidi ver cada dia como uma nova oportunidade de ser feliz. Naquele dia descobri que meu único rival não era mais que minhas próprias limitações e que enfrentá-las era a única e melhor forma de as superar. Naquele dia, descobri que eu não era o melhor e que talvez eu nunca tivesse sido. Deixei de me importar com quem ganha ou perde. Agora me importa simplesmente saber melhor o que fazer. Aprendi que o difícil não é chegar lá em cima, e sim deixar de subir. Aprendi que o melhor triunfo é poder chamar alguém de 'amigo'. Descobri que o amor é mais que um simples estado de enamoramento, 'o amor é uma filosofia de vida'. Naquele dia, deixei de ser um reflexo dos meus escassos triunfos passados e passei a ser uma tênue luz no presente. Aprendi que de nada serve ser luz se não iluminar o caminho dos demais. Naquele dia, decidi trocar tantas coisas.... Naquele dia, aprendi que os sonhos existem para tornarem-se realidade. E desde aquele dia já não durmo para descansar... simplesmente durmo para sonhar. (Walt Disney)

RESUMO

A co-digestão anaeróbica é uma ferramenta útil para aperfeiçoar a produção de biogás e pode ser desenvolvida a partir do uso de diversos tipos de resíduos, em especial resíduos recalcitrantes com alto poder de contaminação, como os resíduos industriais. O soro de ricota é um resíduo poluente devido a sua elevada concentração de lactose. O lodo residual de cervejaria é um resíduo igualmente perigoso ao meio ambiente, apresentando alta carga orgânica. Acredita-se que a associação destes resíduos da indústria alimentícia com o dejeto bovino, possa contribuir para melhorar a produção de biogás. Assim, objetivou-se analisar a dinâmica populacional de arqueas metanogênicas em sistemas de co-digestão operados sob diferentes concentrações de resíduos industriais em associação com dejeto bovino. Foi realizada a extração de DNA de amostras de afluente e efluente. Para a análise da comunidade arqueal foi realizada a partir do sequenciamento da região V4 do gene 16s rRNA. Os gêneros que mais se destacaram na co-digestão de soro de ricota foram *Methanosaeta* e *Methanosarcina*. Já os gêneros mais abundantes durante a co-digestão do lodo de cervejaria foram *Methanosaeta*, *Methanocorpusculum* e *Methanobrevibacter*. Os resultados mostram que a co-digestão de soro de ricota com dejeto bovino é admissível em um sistema operando com até de 80% do co-substrato, em contrapartida para o lodo de cervejaria só é possível a utilização de até 20% do co-substrato.

Palavras-chave: Biogás. Metagenômica. Ecologia Microbiana. Soro de Ricota. Lodo de Estação de Tratamento de Cervejaria.

ABSTRACT

Anaerobic co-digestion is a useful tool to improve the biogas production and can be developed from the use of several types of waste, especially recalcitrant waste with high contamination power, such as industrial waste. Ricotta cheese whey is a polluting residue due to its high lactose concentration. Residual sludge of brewery is a waste equally dangerous to the environment, with a high organic load. It is believe that the association of these residues from the food industry with bovine manure can contribute to improving biogas production. The objective was to analyze the population dynamics of methanogenic archaea operated in co-digestion systems under different concentrations of industrial waste in association with bovine manure. DNA extraction from influent and effluent samples was perform. To analysis the archaeal community, it was performed the sequencing of the V4 region of the 16s rRNA gene. The genera highlighted in the co-digestion of ricotta cheese whey were *Methanosaeta* and *Methanosarcina*. The most abundant genera during the co-digestion of residual sludge of brewery were *Methanosaeta*, *Methanocorpusculum,* and *Methanobrevibacter*. The results show that the co-digestion of ricotta cheese whey with bovine manure is permissible in a system operating with up to 80% of the co-substrate, in contrast to the residual sludge of brewery, it is only possible to use up to 20% of the co-substrate.

Keywords: Biogas. Metagenomics. Microbial Ecology. Ricotta Cheese Whey. Residual Sludge of Brewery.

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LISTA DE ABREVIATURAS E SIGLAS

$(Ca(OH)_2)$	Calcium Hydroxide
AB	Anaerobic Biodigestion
AcoD	Anaerobic co-digestion
BM	Bovine Manure
CH_4	Methane
CO_2	Carbon Dioxide
DBO	Demanda Bioquímica de Oxigênio
DQO	Demanda Química de Oxigênio
EMBRAPA	Empresa Brasileira de Pesquisa Agropecuária
H_2	Hydrogen
HRT	Hydraulic Retention Time
NH_4	Ammoniacal Nitrogen
OTU	Operational Taxonomic Units
PCA	Principal Component Analysis
RCW	Ricotta Cheese Whey
RML	Rumen Microbiology Laboratory
RSB	Residual Sludge of Brewery
WWTP	Wastewater Treatment Plant

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1 INTRODUCTION

The anaerobic biodigestion (AB) is a natural and complex process that occurs spontaneously in the environment and in the digestive system of ruminants. The AB is a metabolic process with multiple syntrophic relationships that occur between microorganisms to degrade and transform the biomass into carbon dioxide (CO_2) and methane (CH_4) (AMARAL et al., 2019; SCHINK, 2002).

The degradation of organic matter is divided in four phases (hydrolysis, acidogenesis, acetogenesis and methanogenesis), and in each one there is the activity of specific microorganisms. The methanogenesis takes place in strict anaerobic conditions, and is a crucial step to methane (CH_4) production (RUI et al., 2015), and the methanogenic archaea are the key microorganisms of this step.

In general, archaea distinguish from bacteria due to differences in the lipid composition of their cell membrane (GROSS, 2018; TOURTE et al., 2020). Furthermore, there are important differences between representatives of the archaea domain, once the mechanism of methanogenesis takes place by three different pathways, depending on the substrate used to CH_4 production (NGUYEN et al., 2019).

The acetoclastic methanogenesis occurs from the reduction of acetate in CH_4, on the other hand, the CH_4 production during the hydrogenotrophic methanogenesis occurs with the reduction of H_2 and CO_2. The third pathway of methane production, the methylotrophic path, occurs from the reduction of methylated compounds (methanol and methylamine) to CH_4 and CO_2 (NGUYEN et al., 2019).

The identification of archaea in environmental samples is possible through metagenomic analysis (NESME et al., 2016). As a technique independent of cultivation, the metagenomic allows the identification of the microbiological diversity from complex environmental samples (BUKIN et al., 2019; KUMAR et al., 2015). This is possible by the investigation of the hypervariable regions (V1 to V9) of the 16s rRNA of prokaryotes.

Studies of microbial ecology associated with engineering techniques allows simulating the natural environmental conditions of anaerobic biodigestion systems within controlled systems, such as biodigesters. The biodigesters are widely used for the degradation of various organic materials, in order to produce clean energy (biogas), and a highly nutritious and natural fertilizer, the biofertilizer (GARCIA JÚNIOR et al., 2016).

Therefore, the treatment of organic waste by the anaerobic biodigestion process, besides, contribute to the stabilization of the substrate, can also contribute to the production of renewable energy (ABUBAKER et al., 2012; DE VRIEZE et al., 2017; LATHA et al., 2019; MATA-ALVAREZ et al., 2014).

The animal waste, in particular the swine and bovine waste are widely used in the anaerobic biodigestion systems (ABDESHAHIAN et al., 2016). The use of animal waste in biodigesters cooperate with the preservation of the soil and water bodies, avoiding their disposal into the environment, once these waste are characterized by a high concentration of nutrients and of microbial load (ABDESHAHIAN et al., 2016; SANTOS E NOGUEIRA, 2012).

The extensive use of bovine manure (BM) in biodigesters in Brazil has a direct relationship with the diffusion of cattle farming in the country. It is estimated that the number of cattle in Brazil is close to 213.5 million head currently (IBGE, 2018), with a large part of these animals destined to the dairy sector.

Although the widespread use of swine and bovine manure in mono-digestion systems, several authors point out to instabilities in this process and, mainly, to the reduce yield of biogas production due to the low C/N ratio of the waste (DE VRIES et al., 2012; KOVACIC et al., 2019; PIÑAS et al., 2018; WU et al., 2010). However, the anaerobic co-digestion (AcoD) process appears as a possible solution to the problems found during the mono-digestion treatment.

The AcoD is a simultaneous and homogeneous biodigestion of two or more complementary substrates to produce biogas rich in methane, while reduce the supply of organic material in the environment (SIDDIQUE and WAHID, 2018). This practice is recommended especially for recalcitrant substrates (SZAJA and MONTUSIEWICZ, 2019), as well as industrial waste.

An appropriate choice of co-substrates, their mixing proportions and the analysis of operational parameters of the process are important points to consider while design a co-digestion system, once the association of these factors will determine the dynamics of microorganisms in the system and, consequently, the efficiency of the process (AMARAL et al., 2019; HAGOS et al., 2017).

Therefore, it is increasingly important to search methods that allow the treatment of these wastes through a sustainable and economical way. Among these methods, the anaerobic co-digestion stands out as an economic and environmentally attractive alternative.

However, several industrial sectors, produce tons of difficult degradation wastes (SZAJA and MONTUSIEWICZ, 2019), which makes essential to investigate their ability to act as co-substrates of anaerobic co-digestion systems. The food industry, for example, is an expanding sector that generates pollutant byproducts that need treatment before disposal. The Ricotta Cheese Whey (RCW) and the Residual Sludge of Brewery (RSB) are examples of byproducts with high polluting potential.

The RCW, also known as second-generation whey, is a byproduct of the dairy industry, resulting from the production of ricotta cheese. According to Sansonetti et al. (2009), this byproduct has an average composition of 0.15-0.22% of proteins; 4.8-5.0% of lactose; 1.0-1.3% of salts, and 0.20-0.25% of organic acids. Due to its composition, represents a product of low nutritional value, therefore, without potential for reuse, whether in the food or veterinary industry (CAROTA et al., 2017). Furthermore, the RCW has high values of BOD (50 g/L) and COD (80 g/L), which makes it a waste with highly polluting characteristics, therefore requiring the application of environmentally correct measures for it disposal in the environment (SANSONETTI et al., 2009).

The RSB is a byproduct generated in large quantities by brewery industries through Wastewater Treatment Plants (WWTPs) after the reuse of process leftovers such as spent grains, hops, and yeast (FILLAUDEAU et al., 2006). Usually the WWTPs of breweries perform aerobic processes to treat their wastes. This process bases on the biological oxidation of the organic material, where in its final stage there is the decantation of a thick mud with an unpleasant odor with a low economic benefit that must be disposed of in landfills (FILLAUDEAU et al., 2006; KANAGACHANDRAN and JAYARATNE, 2006; OKEYINKA et al., 2019).

The RSB properties can vary according to the type of beer produced and the operations carried out at the WWTP (LU et al., 2017). However, according to Stocks et al. (2002), this sludge has the following properties: 18.000 mg/L of suspended solids; 1.350 mg/L of dissolved solids; 770 mg/L of NH_3; BOD equal to 9.600 mg/L; COD equal to 21.800, and pH of 7.28. Based on these characteristics, it is possible to conclude that RSB is a highly polluting waste that requires a careful disposal. In general, the brewing industry wastes have a high organic load and, for this reason, are considered as potential environmental polluters (MAINTINGUER et al., 2017).

The association of different substrates during the AcoD promotes the system balance, increases the production of biomethane, and contributes to the environmental preservation

(HENARD et al., 2017; MATA-ALVAREZ et al., 2014). Therefore, both RCW and RSB are considered as candidates substrates for AcoD process.

The combination of these industrial wastes with bovine manure in AcoD may contribute to the production of biogas, awakening a new possibility for RCW and RSB disposal. The use molecular tools provides an insight into the dynamics of microorganisms present in AcoD systems, and can contribute to understand and model more efficient and productive biodigestion processes. Therefore, this work aimed to identify the population dynamics of methanogenic archaea during the co-digestion of industrial waste under different concentrations of co-substrates.

Based on this, the following hypotheses were tested:

1. The population dynamics of methanogenic archaea in anaerobic co-digestion process changes according to the co-substrate used.

2. The population dynamics of methanogenic archaea changes during the anaerobic co-digestion in systems operated at different concentrations of the same co-substrate.

2 MATERIALS AND METHODS

This section describes the entire methodological approach used in the research.

2.1 PILOT-SCALE BIODIGESTER

Seven biodigesters with a continuous and manual supply were used, with a total capacity of 60 L each. These biodigesters are constructed with PVC tubes and do not have an agitation system, similar to that described by Resende et al. (2015) (Fig. 1A). At the bottom of each biodigester, three sampling valves were installed. As this model has no heating system, the biodigesters were painted black to increase the absorption of solar radiation and maintain high temperatures inside. Each biodigester is coupled to a gasometer (Fig. 1B) made of PVC tubes where the biogas produced during biodigestion is stored.

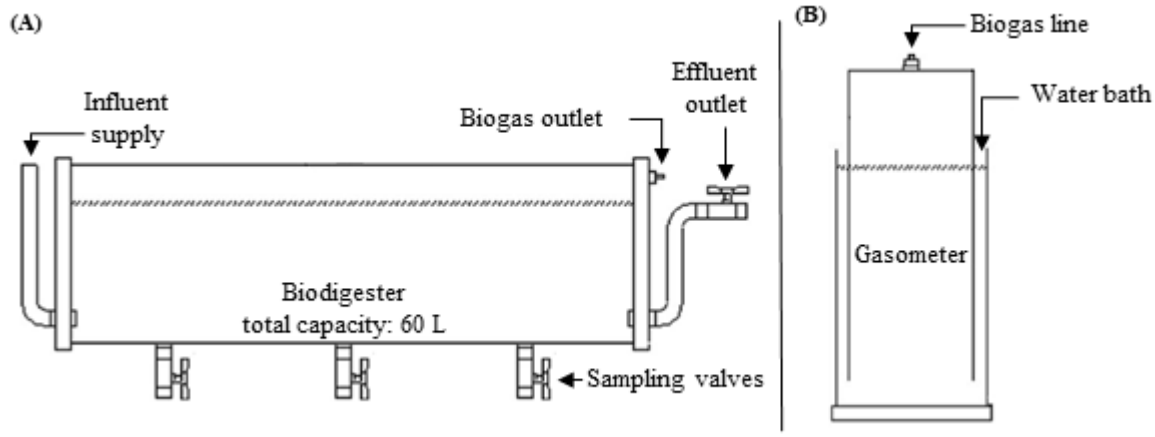

Fig. 1 (A) Anaerobic biodigester model; **(B)** Gasometer model.

Source: Elaborated by the author (2020).

2.2 SUBSTRATES

BM and wastewater were weekly collected in the production system of the José Henrique Bruschi experimental field of Embrapa Dairy Cattle, in Coronel Pacheco, Minas Gerais, Brazil. To obtain the liquid fraction of the BM, it was diluted with wastewater and sieved manually to achieve a concentration close to 6% of total solids (RESENDE et al., 2015). This material was stored at 4 °C for up to one week. Before use, each portion was removed from the refrigerator to reach room temperature (≈ 20 ºC).

A dairy company in the city of Juiz de Fora supplied the RCW. RCW samples were collected weekly, transported to the RML, homogenized, bottled, and frozen at -20 ºC. Before use, we defrosted each sample for 24 hours in a refrigerator, and then kept it at room temperature. The pH value was corrected with the addition of 59 mL of 4.24% limewater ($Ca(OH)_2$) for each liter of RCW, to obtain a pH close to neutrality (SANTANA et al., 2019).

An industrial brewery in Juiz de Fora supplied the RSB. After receiving the RSB, it was sieved and stored in a refrigerator at 4 ºC. Before use, each sample was removed from the refrigerator to reach room temperature.

2.3 EXPERIMENTAL DESIGN FOR ANALYSIS OF ARCHAEA DYNAMICS

The duration of the experiment was 165 days, starting in October 2018. It was divided into three phases called competent inoculum (phase 1), acclimatization (phase 2), and

anaerobic co-digestion (phase 3), respectively. The experimental design is present in the appendix.

During the competent inoculum phase, seven biodigesters were completely supplied with diluted BM, reaching their full capacity (60 L). Flame tests were carried out daily after the start of biogas production. The collection of biogas for chromatographic analysis was performed after the positive flame test for biogas production. The substrate remained in the biodigester until the biogas production in the system reached a concentration of at least 60% of CH4 (MENDONÇA et al., 2017). The Hydraulic Retention Time (HRT) for this phase was 15 days.

Subsequently, the acclimatization phase started. In this phase, one biodigester was selected as control and continued the supply exclusively with BM. Three biodigesters started the supply with the mixtures of RCW + BM and another three with mixtures of RSB + BM (Table 1). Biodigesters were identified according to the name and concentration of the co-substrate used in AcoD. The daily supply (2 L/ day) was performed according to the values of the mixtures indicated in Table 1. Simultaneously with the supply, the same volume of effluent (2 L) was removed, keeping the system operating at its maximum capacity. The HRT for this stage was 30 days, which was the time required for the full exchange of biodigester content.

The anaerobic co-digestion phase lasted 120 days. Daily supply continued respecting the concentrations indicated in Table 1. The control biodigester worked only with BM, so the term co-digestion does not apply to it.

Table 1
Characterization of supply mixtures and physicochemical characteristics.

Biodigester	Substrate characterization	Physicochemical characteristics	
		pH	Ammoniacal Nitrogen(mg / L NH_4)
BM	100% BM (Control)	6.20	134.67
RSB20	20% RSB + 80% BM	5.98	81.22
RSB40	40% RSB + 60% BM	5.89	145.04
RSB80	80% RSB + 20% BM	5.48	121.83
RCW20	20% RCW + 80% BM	6.09	133.44
RCW40	40% RCW + 60% BM	6.08	98.63
RCW80	80% RCW + 20% BM	6.01	98.63

2.4 ANALYTICAL TECHNIQUES

Ammoniacal nitrogen (NH_4) and pH analysis of effluents were performed every two weeks. The pH-meter Tec-3MP (Tecnal, Piracicaba, Brazil) was used to measure the pH values of effluents, and NH_4 was determined according to the Standard Methods Methodologies (APHA, 2018).

An analysis of the biogas concentration was performed weekly to measure the CH_4 concentrations in biogas samples. These procedures were conducted by the Chromatography Laboratory at Embrapa using the Agilent GC 7820A (Agilent Technologies, Santa Clara, USA), equipped with a separation system constituent of two columns, using hydrogen as the carrier gas.

2.5 COLLECT AND CONSERVATION OF SAMPLES

During the acclimatization phase, one influent sample was collected and after the CH_4 production (15 days of HRT), we collect the samples of effluent from the BM biodigester. All effluent samples were collected on sampling valves (Fig. 1A). Before the sample collected, sampling valves were open for some minutes to flush out the valves. Thereafter, the effluent samples for microbial analysis were collect in sterile glass bottles, and immediately sealed to maintain the anaerobic condition.

During the anaerobic co-digestion phase, influent samples were collected only for the biodigesters that operated in the co-digestion system (RSB20, RSB40, RSB80, RCW20, RCW40, and RCW80). In this phase, the collection of effluent samples occurred weekly until the end of the experiment.

After each collect, the samples were frozen at -80 °C. The samples were submitted to a freeze-drying process, which was conducted by Chromatography Laboratory using the Liotop model L120 freeze dryer (Liobras, São Carlos, Brazil). After freeze-drying, all samples were stored in Falcon 50 ml conical tubes.

2.6 SAMPLE SELECTION FOR MOLECULAR MICROBIOLOGY ANALYSIS

To characterize the acclimatization phase, samples of the influent and effluent from the control biodigester were selected. From the material collected in the sampling valves, sample pooling was made to obtain greater representativeness of the content of the

biodigesters. Therefore, each effluent collection had a single pooled sample representative of the biodigester instead of three samples. The preparation of sample pooling of effluent samples occurs from the lyophilized material.

It was weighed 70 mg of lyophilized effluent corresponding to the material collected in the V1 sampling valve and the same volume for the effluent of V2 and V3 valves. Then this material was homogenized and transferred to a microtube, representing a pooled sample with 210 mg of volume (Fig. 2A).

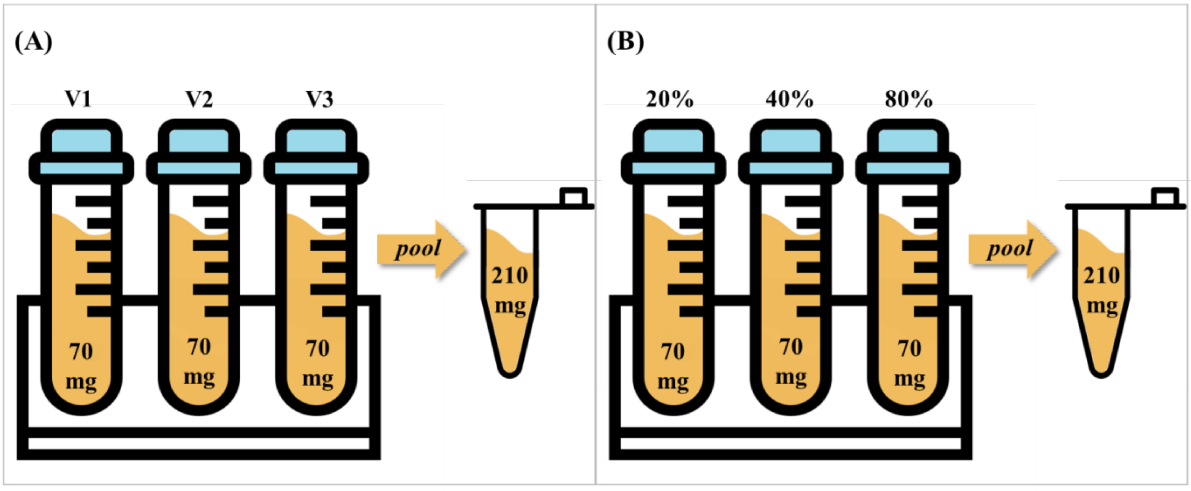

Fig. 2. Scheme of sample pooling preparation for DNA extraction. **(A)** Pooling of lyophilized effluents from 70 mg of the sample collected in each biodigester valve; **(B)** Pooling of lyophilized influents from 70 mg of each supply mixture sample.

Source: Elaborated by the author (2020).

A selection of samples was carried out in the anaerobic co-digestion phase, due to a financial infeasibility to conduce the molecular analysis of all collected material. For a better representation of this phase, the selection of at least one collection/month for each biodigester was prioritized. The effluent samples corresponding to the collections of days 1, 36, 78, 99, and 120 of the anaerobic co-digestion phase were chosen as representatives for molecular analysis. Sampling pooling of the selected effluent samples was also performed.

The characterization of the anaerobic co-digestion influents was also made through a lyophilized sample pooling. To characterize the RCW influents, were weighed 70 mg of the 20% RCW sample, 70 mg of the 40% RCW sample, and the same volume for the sample with 80% of RCW. Then this material was homogenized and transferred to a microtube,

representing a pooled sample with 210 mg volume (Fig. 2B). The same procedure was carried out for RSB influents.

2.7 DNA EXTRACTION

A pre-preparation of samples was made using enzyme lysis buffer (500 mM NaCl; 50 mM Tris-HCl, pH 8; 50 mM EDTA; 4% SDS) in association with mechanical lysis using the "Mini-Bead Beater 16" (BioSpec Products®, Bartlesville, OK, USA) with zirconia beads (0,1mm and 0,5 mm) (STEVENSON and WEIMER, 2007; YU and MORRISON, 2004). Subsequently, the samples were submitted to the QIAamp DNA stool mini kit protocol (QIAGEN, Heidelberg, Germany), following the manufacturer's recommendations. The determination of concentration and purity of DNA was performed in NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.8 METAGENOMIC ANALYSIS - SEQUENCING AND BIOINFORMATICS

The extracted DNA was sent to a service provider company responsible for executing the sequencing and bioinformatics steps. For amplification of the polymorphic region (V4) of the 16S rRNA gene, PCR was conducted in triplicate using the oligonucleotides indicated in Table 2. The bioinformatics analysis was performed on the QIIME2 platform, version 2019.7 (BOLYEN et al., 2019). The sequences were filtered by quality and grouped into Operational Taxonomic Units (OTUs) using 97% of the identity between them. The sequences were also compared with the Silva 132 database (GLÖCKNER et al., 2017) for taxonomic analysis. An OTU table selected by genus is available in the appendix.

Table 2
Primers used for amplification of the V4 region of the 16S rRNA gene.

Target	Primer	Primer Sequence (5'-3')	Target Region	Reference
Universal	515 F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTG CCAGCMGCCGCGGTAA	V4	(Turner et al., 1999)
Universal	806 R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGG ACTACHVGGGTWTCTAAT	V4	(Caporaso et al., 2012)

Subsequently, indexers were inserted into common adapters required for generating clusters and sequencing the samples. The indexing reaction was performed following the protocol of the Nextera XT Index Kit (Illumina, Inc., San Diego, CA, USA). Amplification reactions were conducted in Veriti™ Thermal Cycler (Applied Biosystems, Foster, CA, USA). The created libraries were submitted to the purification steps using Agencourt AMPure XP beads (Beckman Coulter, High Wycombe, UK), to remove small fragments of the total population of molecules and remains of primers.

After this step, the quantification was performed by Real-Time PCR using Kapa Library Quantification Kit Illumina GA Universal - KK4824 (Kapa Biosystems, Wilmington, MA, USA), according to manufacturer's recommendations. An equimolar DNA *pool* was generated by normalizing all samples to 3nM, to perform the sequencing, which was conducted using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) and MiSeq Reagent - V2 Micro 300 cycles Kit (Illumina, San Diego, CA, USA).

Bioinformatics analysis performed on QIIME2 platform version 2019.7 (BOLYEN et al., 2019). The sequences were filtered by quality and grouped into Operational Taxonomic Units (OTUs) using 97% of identity between them. The sequences were also compared with the *Silva 132* database (GLÖCKNER et al., 2017) for taxonomic analysis. An OTU table selected by genus is available in the appendix (Table A1).

2.9 STATISTICAL ANALYSIS

To investigate an association between the performance of the biodigesters and the composition of the archaeal microbiome, a Principal Component Analysis (PCA) was performed. The PCA tried to identify if the selected physicochemical variables (concentration of CH_4 and NH_4, and pH value) could justify the variation in the occurrence of OTUs in the biodigesters. This analysis was conducted on the statistical software JMP 14.0 (SAS Institute Inc., Cary, NC, USA).

3 RESULTS AND DISCUSSION

This section describes the results found from methanogenic and physicochemical analysis.

3.1 ARCHAEA ACCLIMATIZATION

To analyze the structure of the archaeal community, the taxonomic classification of sequences was performed to the genus level. In the influent samples, the most abundant genus was *Methanobrevibacter*, showing a high relative abundance for the three samples. The relative frequency of *Methanobrevibacter* in the influent samples of BM (Fig. 3A), RCW (Fig. 3C), and RSB (Fig. 3D) were 94.05%, 95.07%, and 92.30% respectively.

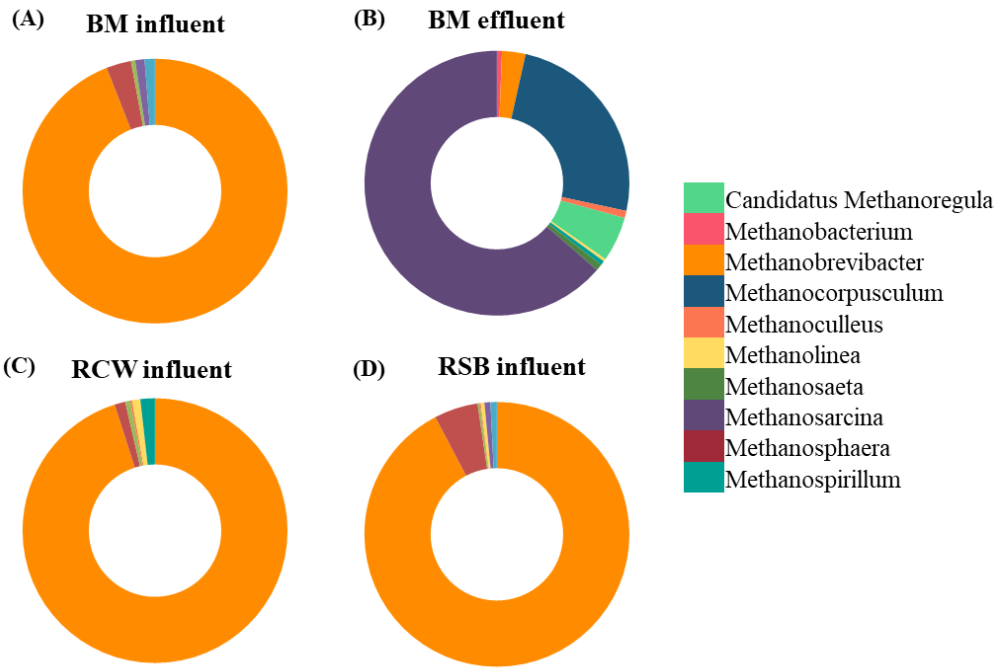

Fig. 3 Phylogenetic distribution of genera present in the competent inoculum and acclimatization phases. **(A)** Influent of the control biodigester; **(B)** Effluent from control biodigester after 15 days of retention; **(C)** Pool of influent samples of RCW biodigesters **(D)** Pool of influent samples of RSB biodigesters.

The abundance of *Methanobrevibacter* in BM supply samples is in accordance with previous studies that mention it as the most profuse hydrogenotrophic methanogen in bovine ruminal system (DANIELSSON et al., 2017; HENDERSON et al., 2015; LEAHY et al., 2013). Therefore, the high frequency of *Methanobrevibacter* in influent samples of RSB and RCW may be justified due to it being prepared with mixtures containing high amounts of bovine manure.

Senés-Guerrero et al. (2019) compared the abundance of this genus in rumen and biodigester samples, highlighting the taxon predominance in the rumen sample and its subsequent disappearance in the biodigester. Similarly, after 15 days of HRT from

acclimatization phase, the analysis of the BM effluent (Fig. 3B) had a reduction in the *Methanobrevibacter* frequency, from 94.05% in the initial sample to 2.87% in the digestate.

The reduction of this genus followed an increase of *Methanosarcina*, *Methanocorpusculum*, and *Candidatus Methanoregula* abundance. In influent samples, the genera *Methanosarcina* and *Methanocorpusculum* showed a relative abundance of 1.27% and 0.51% respectively, while the genus *Candidatus Methanoregula* was not identified. After 15 days of HRT, the relative abundance of the genera *Methanosarcina*, *Methanocorpusculum*, and *Candidatus Methanoregula* increased to 63.62%, 24.86%, and 5.49% respectively.

We assume that the hydrogenotrophic pathway was predominant during acclimatization phase. This is because the increase in the abundance of the facultative acetoclastic methanogen, *Methanosarcina* (HAO et al., 2015; VENKITESHWARAN et al., 2017), occurred concomitantly with the increase of two exclusively hydrogenotrophic genera, *Methanocorpusculum*, and *Candidatus Methanoregula* (LI et al., 2018; WANG et al., 2019).

According to Venkiteshwaran et al. (2017), *Methanosarcina* genus has less affinity to acetate, reinforcing the idea that the hydrogenotrophic pathway was the propellant of methanogens during the acclimatization phase. Furthermore, previous studies suggest that the hydrogenotrophic pathway is the main route for methane production from a biomass with bovine manure (YILDIRIM et al., 2017).

3.2 PHYSICOCHEMICAL ANALYSIS OF EFFLUENTS FROM FULL CO-DIGESTION PHASE

Here the results are divided according to the physicochemical analysis performed.

3.2.1 Methane Concentration

The methane concentration (% *v/v* CH_4) in biogas samples remained above 54% during the experiment, except for the RSB40 biodigester that declined the methane concentration from the 99th day of anaerobic co-digestion, and the RSB80 biodigester that declined it from the 36th day of anaerobic co-digestion and stopped biogas production on the 99th day (Fig. 4).

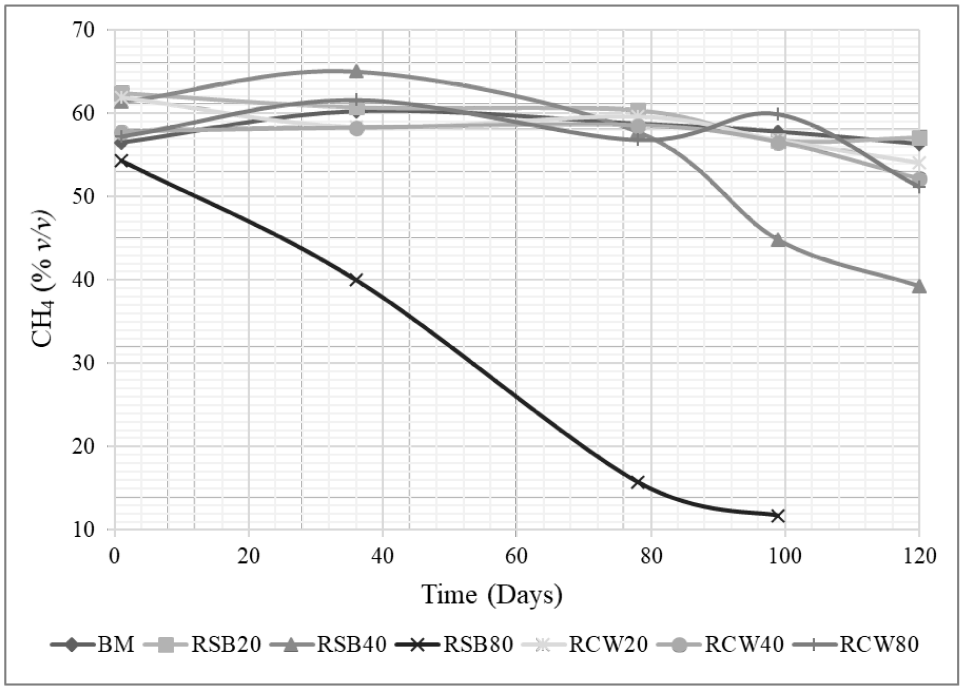

Fig. 4 Methane concentration (% *v/v* CH_4) in biogas during the AcoD phase.

The CH_4 average concentration in BM biodigester was 57.93% (± 1.66). These results are in accordance with authors who point out a variation between 55% and 70% in the CH_4 concentration in biogas samples generated from AB of bovine manure (MENDONÇA et al., 2017; NOOROLLAHI et al., 2015; RESENDE et al., 2015).

According to Chen et al. (2016) and Enitan et al. (2014), the methane concentration in biogas samples of the brewery residues of biodigestion can vary from 59% to 69% of CH_4. The RSB20 biodigester was the only one that showed results within this threshold with a CH_4 average production of 59.49% (± 2.46). The RSB40 and RSB80 biodigesters showed an average concentration of 53.67% (± 11.08) and 30.47% (± 20.21) respectively.

In biogas produced by biodigesters co-digested with RCW, this average was 58.15% (± 2.96) for RCW20, 56.66% (± 2.65) for RCW40 and 57.34% (± 3.99) for RCW80. These results are as expected, once the average value can vary between 50% and 65% of CH_4 in the cheese whey biodigestion (CARLINI et al., 2015; COMINO et al., 2012; IMENI et al., 2019).

3.2.2 pH Values

The pH did not show high variations and remained close to neutrality in all biodigesters, presenting an overall average of 7.1 (± 0.24) (Fig. 5). According to Lemmer et al. (2017), it is possible to achieve the optimal methane yield keeping the pH value in the

system between 6.8 and 7.2. From this result, it is possible to infer that the biodigesters showed favorable conditions for the survival of methanogenic archaea (YAO et al., 2017).

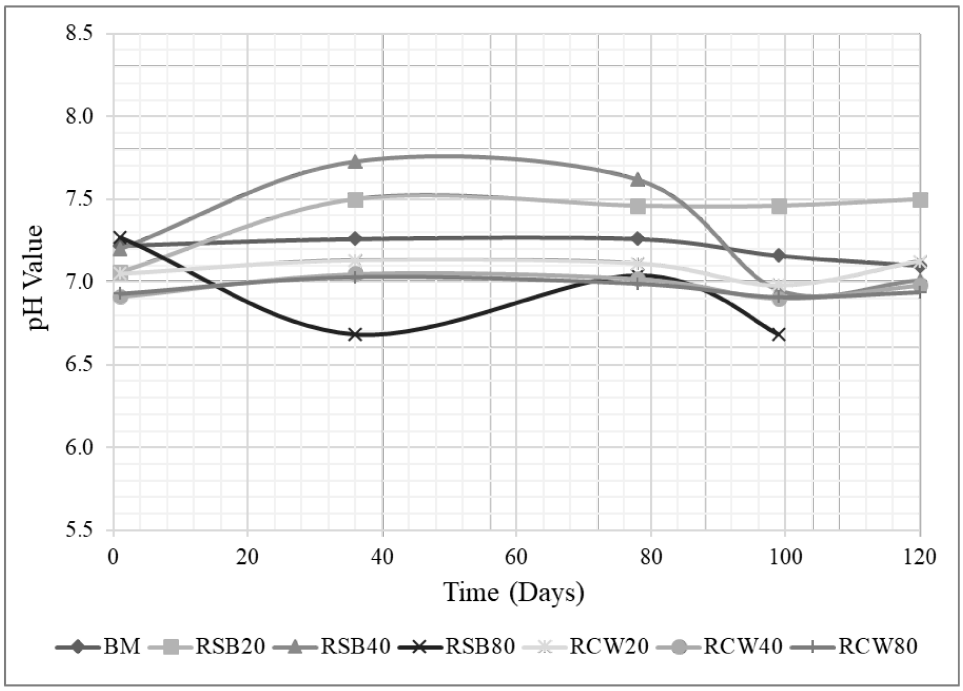

Fig. 5 pH values of effluents collect during the AcoD phase.

3.2.3 Ammoniacal Nitrogen Concentration

It is possible to observe in Fig. 6 a gradual increase in NH_4 concentration during the biodigestion of BM. The BM biodigester showed a minimum value of 128 and a maximum of 736 mg / L of NH_4, with an average of 493 mg / L of NH_4 (± 226). Mendonça et al. (2017) also reported this progressive increase in the concentration of NH_4 during the bovine manure biodigestion in a full-scale biodigester.

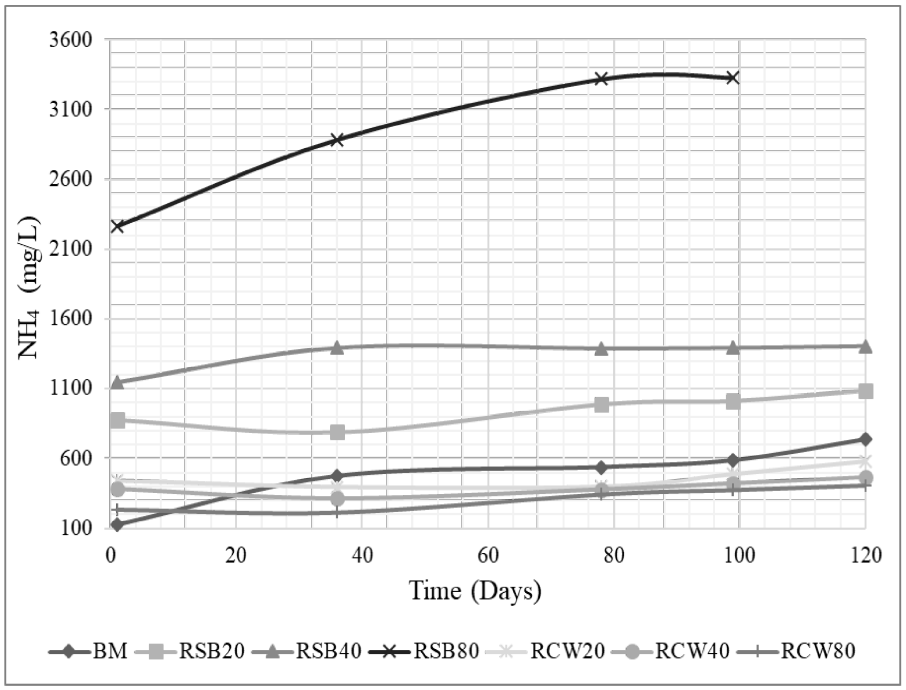

Fig. 6 Ammoniacal nitrogen concentration (mg/ L NH_4) in effluents collected during AcoD phase.

The RCW biodigesters showed close values for the ammoniacal nitrogen concentration of the digested. The averages of the ammoniacal nitrogen concentrations were 460 mg / L NH_4 (± 78) in RCW20, 394 mg / L NH_4 (± 58) in RCW40, and 315 mg / L NH_4 (± 85) in RCW80. Our results differ from those reported by Comino et al. (2012), during the co-digestion of cattle slurry and cheese whey in which were found values over 800 mg / L of NH_4. This may have a relation to the type of cheese whey used and to the addition of limewater to stabilize the RCW pH. We assume that the slight increase of NH_4 in BM and RCW biodigesters did not cause toxicity to the system since the increase of this compound did not affect methane production.

The RSB biodigesters showed a high concentration of ammoniacal nitrogen (> 700 mg / L NH_4) from the first digested collection. The average concentration of RSB20 was 949 mg / L NH_4 (± 116). The RSB40 and RSB80 biodigesters showed the highest concentrations of ammonia nitrogen, with average values of 1331 mg/L NH_4 (± 121) and 2952 mg/L NH_4 (± 501) respectively. It is possible that this considerable increase in the ammoniacal nitrogen concentration is related to the decline in the methane concentration of these biodigesters.

3.3 ARCHAEA IDENTIFICATION DURING ANAEROBIC CO-DIGESTION

The improvement in the generation of biogas rich in methane is the central point of AcoD processes, in which an appropriate selection of co-substrate and mixing ratio is necessary (ZHOU et al., 2012). Inappropriate choices can lead to an imbalance in the system, limiting the methane generation. The results show in Fig. 7 reveals the dynamics of methanogenic microbiota in different biodigesters during anaerobic co-digestion phase.

A high abundance of *Methanosarcina* genus is also observed in the BM samples during the initial anaerobic co-digestion phase with a relative abundance of 76% on the first day (Fig. 7A). However, there is a notable reduction in the frequency of this genus during biodigestion, from 61.55% on the 36th day to 14.41% on the 120th day. Simultaneously, there is a considerable increase of *Methanosaeta* from 5.68% on the 36th day to 42.11% on the 99th day, which registered its highest frequency.

Methanosarcina and *Methanosaeta* are responsible for producing a large part of the CH_4 generated during anaerobic digestion (NGUYEN et al., 2019). Individuals of *Methanosaeta* genus are mandatory acetoclastic methanogens, able to contribute to more than 60% of CH_4 generated through the oxidation of acetic acid (FERRARO et al., 2018; ZHAO et al., 2015).

This variation in the prevalence of *Methanosarcina* is consistent with the results of Song and Zhang (2015) observed during the co-digestion of a pretreated wheat straw with cattle manure, which shows a higher frequency of this group in the initial period of biodigestion and its subsequent decline. The expressive increase in the relative abundance of *Methanosaeta* may be indicative of its affinity for acetate, suggesting the acetoclastic pathway as the propulsive pathway of BM methanogenesis.

However, with the increase of *Methanosarcina*, there is a slow increase of some hydrogenotrophic genera (*Candidatus Methanoregula*, *Methanocospuscullum*, and *Methanospirillum*). The increase of these genera, concomitant with the increase of *Methanosarcina*, may be evidence of the coexistence of hydrogenotrophic and acetoclastic pathways (ZHANG et al., 2019). During the analysis of wetland samples, Zhang et al., (2019) found evidence that these two methanogenic pathways can coexist although one pathway may be more significant for methane production than the other one.

In this case, we have not been able to stipulate what was the exact propulsive pathway for methanogenesis from the 78th day on the BM biodigester. Although, we assume that acetogenesis may have prevailed, based on the high abundance of the *Methanosaeta* genus.

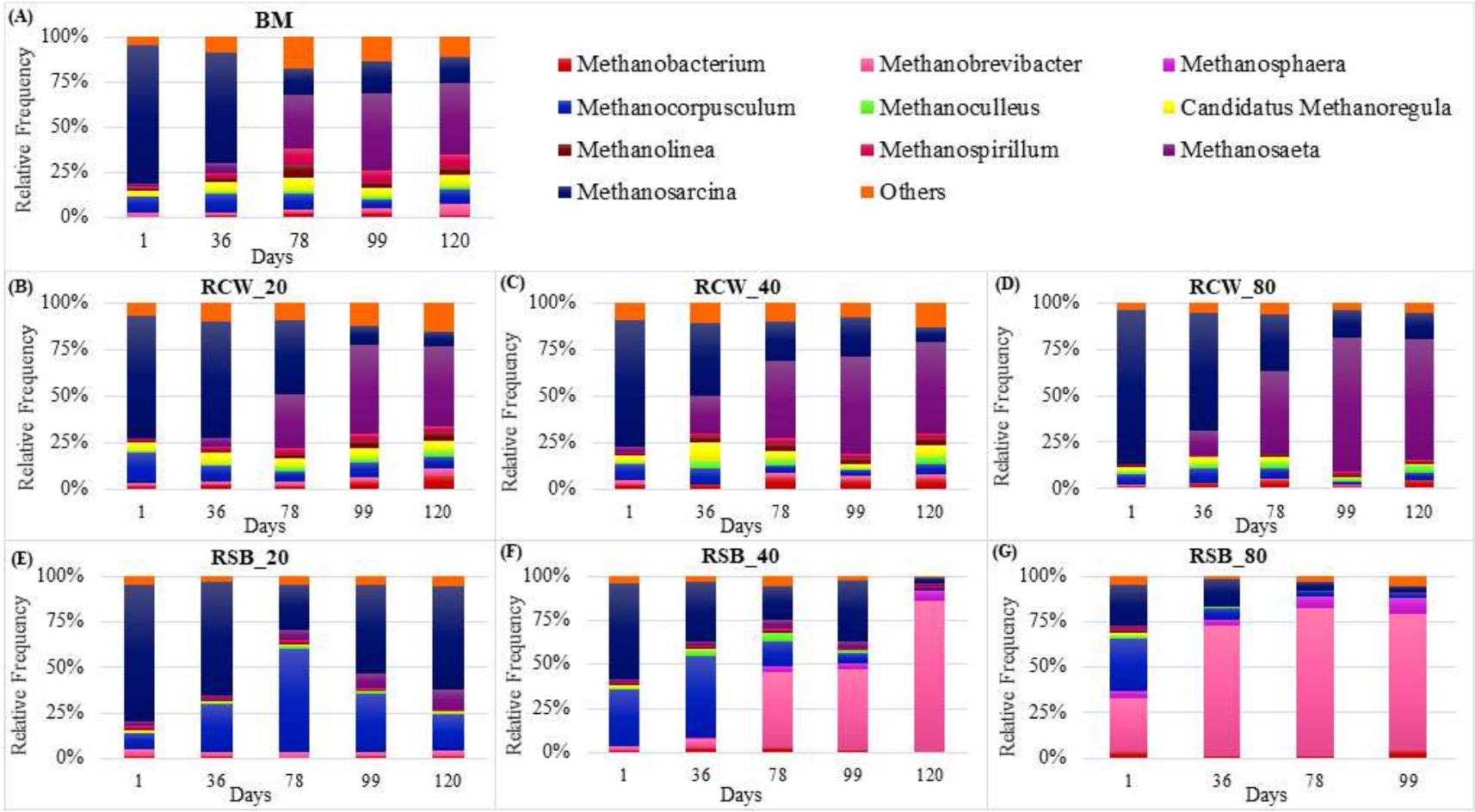

Fig. 7 Distribution and relative abundance of OTUs in effluent samples of AcoD phase. **(A)** Biodigester control supplied with bovine manure; **(B)** Biodigester supplied with 20% ricotta cheese whey; **(C)** Biodigester supplied with 40% ricotta cheese whey; **(D)** Biodigester supplied with 80% ricotta cheese whey; **(E)** Biodigester supplied with 20% of brewery sludge; **(F)** Biodigester supplied with 40% of brewery sludge; **(G)** Biodigester supplied with 80% of brewery sludge.

It was also noted that the RCW archaeal microbiota was quite similar to that found in the BM biodigester. The relative abundance of *Methanosarcina* on the 1st day of anaerobic co-digestion phase was 65.91% for RCW20, 66.04% for RCW40, and 82.75% for RCW80.

As in the BM biodigester, the RCW20 and RCW40 biodigesters presented an increase in the relative abundance of *Methanosaeta* along with a slight increase of hydrogenotrophic methanogens during co-digestion. This coexistence between acetoclastic and hydrogenotrophic methanogens again raises the question about which pathway promotes the methanogenesis in RCW20 (Fig. 7B) and RCW40 (Fig. 7C) co-digestion. Differently, the RCW80 (Fig. 7D) biodigester did not show a considerable increase in hydrogenotrophic methanogens. However, the genus *Methanosaeta* was dominant during anaerobic co-digestion, reaching 65.64% of relative abundance.

Chen and He (2015) demonstrated the prevalence of *Methanosaeta* over *Methanosarcina* in mesophilic biodigesters supplied with dairy residues under high organic loading rates. Saha et al. (2019) described similar results during the digestion of sludge obtained in a WWTP and augmented with a mixed waste of fruit, where the prevalence of *Methanosaeta* over *Methanosarcina* was observed. However, Chen et al., (2017), demonstrated that the prevalence of *Methanosaeta* could occur due to the competitiveness of this genus under high acetate concentrations.

Based on our results, we can assume that acetoclastic methanogenesis prevailed during the AcoD of ricotta cheese whey, due to the abundance of acetoclastic over methanogenic genera. A deep study of the bacterial metagenome that was found in this study may provide us with a more effective answer on this issue.

The biodigesters that used RSB as AcoD substrate differed from each other and the BM biodigester. *Methanosarcina* and *Methanocorpusculum* were the main representatives of the archaeal community in RSB20 (Fig. 7E), indicating that these two genera led the methane production. The co-occurrence of these two genera suggests that the biodigester RSB20 followed the hydrogenotrophic pathway for methane production.

A noticeable change in the dynamics of archaeal microbiota was observed in the RSB40 biodigester (Fig. 7F). The beginning of RSB40 AcoD is dominated by members of *Methanosarcina* and *Methanocorpusculum* genera with 54.80% and 31.97% of relative abundance respectively. Subsequently, there was a reduction in the abundance of these two genera and prevalence of *Methanobrevibacter* genus. *Methanobrevibacter* was also abundant in the RSB80 biodigester (Fig. 7G). Associated with the increase of this taxon, it is also possible to observe a slight increase of *Methanosphaera* genus, a hydrogenotrophic

methanogen equally abundant in the gastrointestinal tract of ruminants (HENDERSON et al., 2015; KONG et al., 2019). Principal component analysis (Fig. 8) revealed that these two genera, *Methanobrevibacter* and *Methanosphaera*, are strongly related to the increase of the NH_4 concentration.

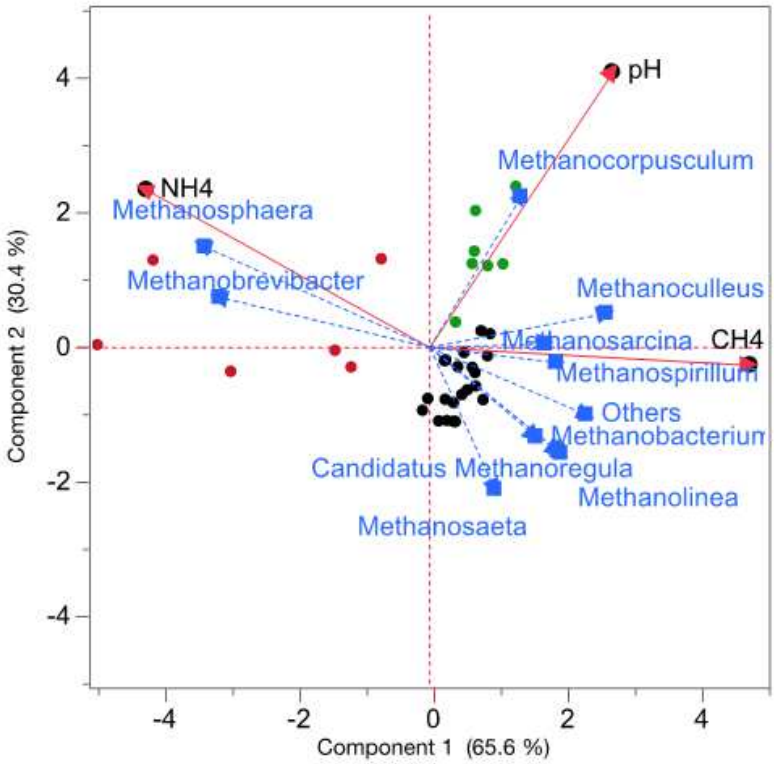

Fig. 8 Association between the physicochemical variables and the relative abundance of OTUs, classified at the genus level. The physicochemical variables included in the PCA plot are the values of CH_4 (% *v/v*), NH_4 concentration (mg/ L), and pH values.

Although ammoniacal nitrogen is crucial to microbial growth when in high concentration, it is considered an inhibitor of the system (WANG et al., 2016). The NH_4 increase is an important evidence to justify the discrepant occurrence of *Methanobrevibacter* in RSB40 and RSB80 samples, followed by a reduction in the relative abundance of the other genera. High concentrations of this compound have been associated with *Methanobrevibacter* survival in previous studies (BAYRAKDAR et al., 2017; MOLAEY et al., 2018).

Nevertheless, our results support Nguyen et al. (2019), who highlighted acetoclastic methanogens, the main responsible for methane production, as vulnerable to NH_4 increase. The predominance of hydrogenotrophic methanogen under high NH_4 concentration may be

considered as a response to the change from acetoclastic methanogenesis to hydrogenotrophic methanogenesis (ESQUIVEL-ELIZONDO et al., 2016).

We assume that the interruption in the production of biogas by the RSB80 biodigester before the end of the experiment is due to the increase of NH_4 in the system, once high ammoniacal nitrogen values can also prevent the growth of microbiota, affecting the AcoD process (MOSET et al., 2017; REILLY et al., 2016).

The PCA (Fig. 8) also revealed that the genus *Methanocorpusculum* has a connection with the pH value. Zhou et al. (2016) point *Methanocospusculum* as the main genus in laboratory-scale mesophilic reactors adjusted to pH 7. However, despite the PCA result, our study does not provide enough evidence to prove a relationship between the occurrence of *Methanocospusculum* and the pH variation. A Heatmap (Fig. 9) was created for a better visualization of the genera that dominated each biodigester.

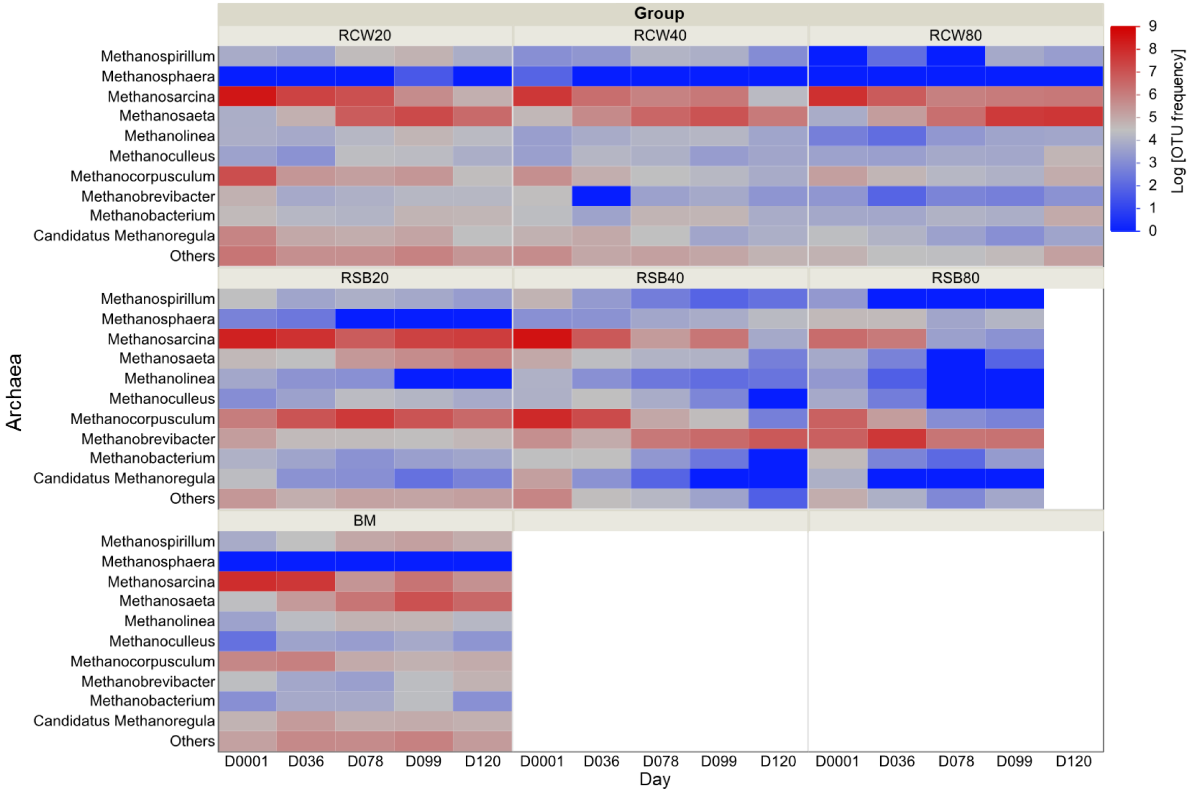

Fig. 9 Heatmap of genera and relative abundance (log scale) of archaea in different treatments. Red colors indicate higher abundance and blue colors lower abundance.

Therefore, it was observed that BM and RCW biodigestion is dominated by the genera *Methanosarcina* and *Methanosaeta*. RSB biodigestion, on the other hand, presents the genera *Methanosarcina*, *Methanocorpusculum*, and *Methanobrevibacter* as the propellants of methanogenesis.

4 CONCLUSION

It was observed that the biodigestion of bovine manure may assume the acetoclastic and hydrogenotrophic pathway and sometimes this path can coexist. The three treatments containing RCW showed good results in the production of CH_4, proving its efficiency in mixtures containing up to 80% of it. *Methanosarcina* and *Methanosaeta* genera are the most abundant in the RCW systems. The AcoD of the RSB is mostly conducted by hydrogenotrophic archaea, especially the *Methanocorpusculum* and *Methanobrevibacter* genera, and the facultative *Methanosarcina*, indicating that the hydrogenotrophic pathway dominated the CH_4 production. The RCW biodigesters have shown better results than the RSB biodigesters. The biodigester that worked with 20% of RSB showed a satisfactory result, indicating a possibility in the use of small proportions of this residue in the AcoD process.

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APPENDIX A

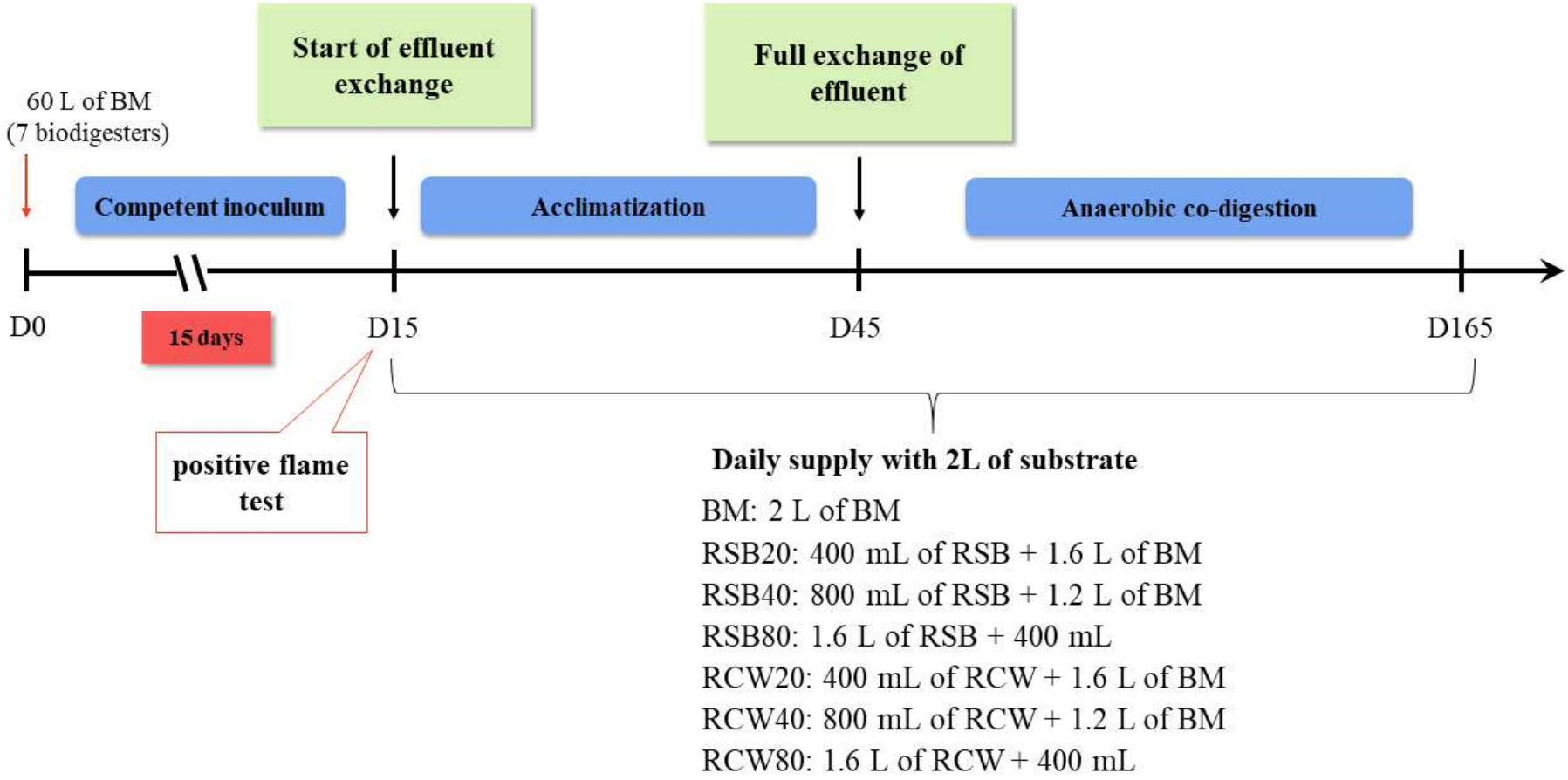

Fig. A 1 Experimental design of the project. The biodigesters were named according to the co-substrate concentration. *As the control-biodigester used only bovine manure, the term "anaerobic co-digestion" does not apply to it.

APPENDIX B

Table A 1 OTU table classified by genus level presenting the most abundant genera in influent and effluent samples. The column classified as "Others" contains OTUs that were not possible to be classify by gender or those with small numbers of representatives.

Sample ID	Methanobacterium	Methanobrevibacter	Methanosphaera	Methanocorpusculum	Methanoculleus	Candidatus Methanoregula	Methanolinea	Methanospirillum	Methanosaeta	Methanosarcina	Others
BM_IN	0	743	24	4	0	0	0	0	9	10	7
BM_E_F1	15	69	0	598	21	132	8	13	19	1530	172
BM_D001	22	83	0	349	9	121	38	49	85	3015	188
BM_D036	47	44	0	413	39	218	79	90	219	2374	334
BM_D078	47	35	0	150	33	136	123	161	541	250	320
BM_D099	81	81	0	127	47	149	115	191	1268	553	399
BM_D120	23	125	0	148	26	130	69	143	750	274	214
RSB_IN	0	1655	96	5	2	0	8	0	13	14	3
RSB20_D001	59	204	15	445	21	80	44	86	107	3953	236
RSB20_D036	40	103	11	1164	37	24	25	41	87	2704	136
RSB20_D078	24	100	0	2235	78	22	23	55	235	967	181
RSB20_D099	35	94	0	1183	65	9	0	45	309	1790	174
RSB20_D120	41	111	0	720	48	15	0	32	404	2052	196
RSB40_D001	86	288	23	3294	57	198	59	122	160	5647	370
RSB40_D036	91	146	24	1448	91	24	23	30	82	1051	95
RSB40_D078	28	501	43	161	51	6	11	13	62	214	69
RSB40_D099	11	694	50	97	17	0	8	6	60	518	39
RSB40_D120	0	1041	75	14	0	0	10	9	14	46	5
RSB80_D001	104	878	106	863	47	56	29	29	46	665	142
RSB80_D036	16	2335	104	205	13	0	5	0	15	471	56
RSB80_D078	7	537	42	21	0	0	0	0	0	36	18
RSB80_D099	31	571	65	15	0	0	0	0	6	24	44
RCW_IN	0	1234	17	0	0	0	8	3	13	23	18
RCW20_D001	104	128	0	1344	38	379	54	49	53	5196	539
RCW20_D036	69	47	0	242	24	159	47	40	131	1753	286
RCW20_D078	63	57	0	197	82	141	69	101	968	1309	290
RCW20_D099	119	70	4	241	78	176	115	125	1479	309	392
RCW20_D120	112	70	0	95	53	86	76	52	697	133	242
RCW40_D001	81	93	6	289	35	129	34	22	111	2359	308
RCW40_D036	39	0	0	137	68	154	49	27	323	632	164
RCW40_D078	120	37	0	87	56	90	63	63	773	397	190
RCW40_D099	115	46	0	71	32	42	68	55	1216	501	166
RCW40_D120	51	26	0	49	42	55	41	20	476	76	123
RCW80_D001	45	27	0	196	38	83	14	0	50	2767	124
RCW80_D036	44	6	0	117	35	62	8	8	205	999	86
RCW80_D078	60	16	0	69	46	37	28	0	613	409	84
RCW80_D099	53	14	0	59	44	22	40	46	2178	467	102
RCW80_D120	151	24	0	145	116	40	43	33	2377	498	194