Impact of anaerobic digestion and centrifugation/decanting processes in bacterial communities fractions

Short title: "Impact of anaerobic digestion in bacterial communities"

Ana Isabel Díaz[1], Paula Oulego[1], Sergio Collado[1], Adriana Laca[1], J. Manuel González[2] and Mario Díaz[1*]

[1]Department of Chemical and Environmental Engineering, University of Oviedo, C/ Julián Clavería, s/n, Oviedo, E-33006, Spain

[2] R&D, COGERSA SAU, C/ La Zoreda, s/n, Gijón, E-33697, Spain

[*]E-mail: mariodiaz@uniovi.es, Phone: +34 985 10 34 39, Fax: +34 985 10 34 34

ABSTRACT

Sewage sludge can be treated by anaerobic processes that frequently are followed by physical separation processes. In this work, a high-throughput sequencing technology, based on variation in the bacterial 16S rRNA gene, has been used to characterise the bacterial populations present in samples taken from different points of an industrial anaerobic digestion process fed with sewage sludge. Relative abundances of phyla and classes throughout the biological process and the subsequent separation steps were determined. Results revealed that the *Bacteroidetes, Firmicutes* and *Proteobacteria* phyla were the most representative. However, significant changes in relative abundance were detected along treatments, showing the influence of operational parameters on the distribution of microorganisms throughout the process. After anaerobic digestion, phylum *Firmicutes* doubled its relative abundance, which seems to indicate that the anaerobic conditions and the nutrients favoured its growth, in contrast to other phyla that almost disappeared. After centrifugation, *Proteobacteria* went preferentially to the solid phase, in contrast to *Firmicutes* which was the dominant phylum in the liquid phase. After decanting the liquid phase during 14 h, an important growth of *Proteobacteria*, *Spirochaetes* and *Tenericutes* was detected. At class level, only significantly changes were observed for *Proteobacteria* classes being *α-proteobacteria* dominant in the digestate, while *γ-proteobacteria* was the majority since this point to the final steps. To know the changes on the kind and abundance of microbial populations throughout the anaerobic and separation processes is very important to understand how the facilities design and operation conditions can influence over the efficiencies of next biological treatments.

KEYWORDS

16S rRNA gene; anaerobic digestion; PGM sequencing; bacterial community; separation processes; sewage sludge.

INTRODUCTION

The anaerobic processes take place in four stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis. The microorganisms of two biological domains, Bacteria and Archaea, carry out interdependent and complex biological reactions during the process. Bacteria microorganisms produce enzymes which hydrolyze polymers to monomers. These are subsequently converted by acidogenic and acetogenic bacteria to H_2 and volatile fatty acids (1). The Archaea domain is mainly involved in the last stage, forming methane by the reduction of CO_2 or by the decarboxylation of acetate. Microorganims within this domain can also utilize a limited number of other substrates, such as methanol, methylamines and formate, to produce methane (2).

Anaerobic digestion is the most widely employed method for sewage sludge treatment, and since the last years, it is an attractive technology for processing various organic wastes produced in urban, industrial, and agricultural settings (3). In this process, a large fraction of the organic matter, which is degraded by a complex community of microorganisms, is broken down into carbon dioxide (CO_2) and methane (CH_4). Such decomposition occurs in the absence of oxygen and two main final products can be distinguished: digestate and biogas, which is considered a product of high added value to be used as biofuel in a sustainable and environmentally friendly way (4). After the anaerobic digestion, the digestate can be treated to remove liquid fraction and simultaneously concentrate nutrients, so a solid–liquid phase separation is usually carried out prior to any further post-treatment. The solid fraction mixed with vegetable wastes, is mainly used in composting processes to be employed as a fertilizer (5).

Regarding the liquid fraction, depending on substrates used and final disposal, a biological post-treatment could be necessary to reduce its toxicity and nitrogen content.

Microorganisms play a main role in anaerobic digestion treatments, so it is essential to characterize the microbiota at each stage of the process in order to detecting the core functional groups responsible of anaerobic degradation and key for the further post-treatment. The substrate characteristics and operational parameters are determining factors for the stability of the microbial communities.

Previous studies describing the microbial communities employing methods such as terminal restriction fragment length polymorphism (T-RFLP), denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), or Sanger sequencing of clone libraries (3, 6).

Just fear years ago, the next generation of sequencing (NGS) based on 16S rRNA gene sequencing has been applied to characterize anaerobic digestion processes. This technology allows the generation of thousands to millions of short sequencing reads for massive studies of genes and a higher taxonomic resolution (7). Most of such works investigated only a few hypervariable regions, mainly V3, V4 and V5, so the phylogenetic and taxonomic information obtained was limited. In this study, 7 of the 9 hypervariable regions of the 16S rRNA gene have been sequenced to obtain extensive phylogenetic information by using the Ion Torrent PGM system of mass sequencing.

The microbiota present in anaerobic processes has been studied by several authors as Chen et al (8) which studied the microbiota present in a anaerobic digestion of pharmaceutical wastewater and Zhou et al (9) which showed the variation of the microbial community in thermophilic anaerobic digestion of pig manure mixed with different ratios of rice straw.

However, as far as we know, the evolution of the microbial population throughout the anaerobic digestion of sludge and the subsequent separation processes, i.e. centrifugation

and decanting, have not been previously studied. Obviously, changes in microbiota distribution, especially in their relative abundance and activity, are ultimately reflected in the reactor performance and the efficiency of following biological treatments, where the bacteria also play an important role.

In particular, the aims of this work were: i) To characterize the bacterial population in the anaerobic digestion process and compare the results with the microbiota present in raw material. ii) To study the distribution of the bacterial communities between the separated fractions to determine the effect of the separation processes as centrifugation and decanting.

MATERIALS AND METHODS

Plant operation parameters and sample description

The samples used in this study were taken from the anaerobic digestion plant sited the waste treatment centre, COGERSA (Asturias, Spain).

Anaerobic digestion process was conducted with mixed sludge, sludge (primary and activated sludge) that has been dehydrated by centrifugation or press filter. Previously to the digestion, the sewage sludge was received in two concrete pits, each one with an operational capacity of 175 m^3. The sludge was pumped from these pits to a mixer, where the sludge is mixed with recirculated process water, in a ratio of 1: 1.2 (v/v), respectively. This raw mixture was stirred for 60 minutes. This treatment was carried out in order to disintegrate the biodegradable waste for improving the subsequent digestion process and removing the non-biodegradable contaminants, such as the heavy fraction (stones, large bones, glass, batteries and metal objects) and the light one (textiles, wood, plastic sheets and thread, etc.). The mixture was sent to the Grit Removal System (GRS) consisting of three hydro-cyclones connected in cascade for removing any sand or impurities that might

remain in the suspension. All impurities removed were sent to the reject container. Finally, it was stored in a 1000 m^3 capacity tank to enable continuous feeding to the digester.

The anaerobic digestion process was carried out in two anaerobic completely stirred tanks with a total capacity of 6000 m^3, under mesophilic conditions (36 ± 0.6ºC). The digester was heated up by means of external heat exchangers. Proper mixing system inside the digester was achieved by injecting part of the produced biogas through an air-cooled rotary vane compressor. The plant produces around 50000 m^3/week of biogas with 64% of CH_4.

After anaerobic digestion process, biogas and digestate was obtained. The biogas was extracted and leaded to the general biogas network of the landfill. The digestate was separated in solid and liquid phase using an industrial centrifuge at 3000 rpm. The percentage of solid and liquid phase obtained was around 20% and 80% of the incoming digestate, respectively. For this process, it was necessary the addition of a polyelectrolyte (Chemipol CZ-600) in a concentration of 0.35% (v/v). The solid phase was sent to a composting plant and the liquid phase was decanted for 45 hours in an open tank with a volume of 80 m^3, before being sent to the biological subsequent treatment. Occasionally, some biodegradable-organic effluents from other treatments carried out in the waste treatment centre are spilled into the decanter. The volume is always lower than 10% of the incoming water flow. A flow diagram of the treatment plant is shown in Figure 1.

FIGURE 1

Sample processing and DNA extraction

Different samples were collected in the anaerobic digestion plant in order to be analysed microbiologically. Such samples were the following: the feed to the anaerobic digester

(S1), the digestate (S2), the solid fraction (S3) and the liquid fraction (S4) obtained after centrifugation and the liquor obtained after decanting (S5). Detailed information for each sample is shown in Table 1.

TABLE 1

In all cases, 160 ml of each of the samples were centrifuged for 20 minutes at 13000g. The supernatant was discarded, and the solid fraction was preserved for DNA extraction. DNA extraction was performed with the Power Biofilm DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA), which is specific for sludge samples. The extraction was carried out with 0.25 g of wet sludge (solid fraction) according to the manufacturer's instructions. The only modification was the addition of 200 µl of solution BF3 due to the excessive colour of the samples (recommended in the protocol of the kit).

Extracted DNA was concentrated using Concentrator Plus Vacufuge® (Eppendorf, Germany) and the concentrations were determined through the BioPhotometer Plus (Eppendorf, Germany), ensuring that the amount of DNA was high enough to continue the process.

DNA amplification and purification

For DNA amplification, Ion 16S Metagenomics Kit (Ion Torrent, Life Technologies) was employed. This kit simultaneously examines 7 of the 9 hypervariable regions in the bacterial 16S rRNA gene, using one primer for the V2-4-8 regions and another primer for V3-6 and V7-9. This method has a wide range of uses, including the characterization of bacterial populations, taxonomical analysis, and species identification.

The DNA samples and the primers with their respective linkers were amplified by PCR reaction, which was performed in several steps: i) initial heating at 95 °C for 10 minutes, ii) 25 cycles of denaturation at 95 °C for 30 seconds, iii) alignment at 58 °C for 30

seconds, iv) extension at 72 °C for 30 minutes seconds, and v) elongation at 72 ° C for 7 minutes. Next, a preservation step at 4 °C for 20 minutes was carried out. The resulting products were purified using the Agencourt AMPure XP Kit (Beckman Coulter, USA) and finally the 16S rRNA amplicons were quantified through Qubit® 2.0 Fluorometer using dsDNA HS Assay Kit (Invitrogen, USA).

Library construction and sequence analysis

Once the DNA was purified, the sequencing step was performed. First, the library was prepared using the Ion Plus Fragment Library Kit (AB Library Builder™). During this process, the fragmentation of the DNA obtained in the purification phase was carried out in order to obtain smaller fragments of up to 150 base pairs (bp). For the library construction, each fragment of the DNA obtained was coupled to a marker or barcode and two adapters. Each library corresponds to a different collection of DNA fragments to be sequenced and is unique to each sample. Construction of the library was conducted using the PGM™ Hi-Q™ OT2 Kit. Subsequently, the samples were sequenced using the PGM™ Hi-Q™ Sequencing Ion Kit and the Ion 318™ Chip Kit v2, which has a minimum capacity of 4 million readings.

The final results obtained were analysed with Life Technologies Ion Reporter™ Software, which provided the final sequencing results. The restriction criteria applied, was as follows: i) read length filter: 150 bp, ii) minimum alignment coverage: 90%, iii) read abundance filter: 10, iv) genus cut off (level of coincidence to determine gender): 97%, and v) species cut off (level of coincidence to determine species): 99%.

RESULTS AND DISCUSSION

PGM sequencing and the amplification of hypervariable regions of 16S rRNA allowed us to obtain a detailed taxonomic bacterial classification. Twenty-one different phyla were

identified in the samples. The classification of the microorganisms up to specie level is shown in Fig.S1 to Fig.S5. Taxonomic classification is represented by Krona plot, which depicts different bacteria taxonomic levels in concentric circles, from subspecies in the outermost circle to the bacteria kingdom in the innermost circle. The relative abundance of bacterial phyla was estimated in each sample as the percentage of mapped reads.

The phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* were the more representative in all the samples. Nelson et al (10) and Sundberg et al (11) found in samples taken from anaerobic processes the cited phyla and also *Chloroflexi* and *Actinobacteria*.

In this work, *Actinobacteria*, and *Chloroflexi* were detected, but with relative abundances below 15% and 1%, respectively, in contrast to data reported in other anaerobic digestion processes (12, 13).

Finally, other phyla as *Synergistetes* and *Thermodesulfobacteria* were also detected in all analysed samples, although in low concentrations.

Anaerobic digestion process

The mixture fed to the digester (S1) and the digestate (S2) were analysed. Fig.2.A, presents the relative abundance of the different phyla in the initial mixture (S1) fed to the anaerobic digester.

FIGURE 2

In sample S1, that contains the pretreated sludge mixed with the recirculation, *Firmicutes* was the majority phylum representing 41% of the total abundance. Its presence has been reported by several authors as one of the most abundant phyla in anaerobic digestion processes (14, 15, 16). During the hydrolysis, the complex molecules are broken down into simpler ones. This phylum is related to the production of extracellular enzymes with cellulases, lipases or proteases (17). Therefore, a high relative abundance of this phylum

205 in S1 is an advantage for the subsequent anaerobic digestion process. The recirculation
206 of the clarified during the sludge treatment is an important aspect that can influence
207 significantly in the relative abundance of each phylum in the anaerobic reactor. In this
208 case, *Firmicutes* is present in the recirculation (S4, see Fig.3B), contributing to its high
209 relative abundance in S1. Zamanzadeh et al (12) studied the microbial communities
210 present in mesophilic and thermophilic anaerobic digesters, with and without
211 recirculation. The study showed that phylum *Firmicutes* was dominant in processes with
212 recirculation and the phylum *Chloroflexi* was dominant in processes without
213 recirculation. This last phylum was identified in this work with a relative abundance lower
214 than 1% in all samples.
215 The phyla *Bacteroidetes, Proteobacteria* and *Spirochaetes*, which are associated with
216 fermentative metabolism of macromolecular organic compounds, represent 19%, 15%
217 and 15% of the total abundance in S1, respectively (18).
218 The phylum *Bacteroidetes* is composed of gram-negative bacteria which are specialist for
219 the degradation of high molecular weight organic matter to acetic and propionic acid (19).
220 This phylum has been frequently detected in anaerobic reactors loaded with sludge,
221 vegetal biomass or mixed organic residues (20, 21).
222 *Proteobacteria* and *Spirochaetes* phyla contain anaerobic and aerobic bacteria, and they
223 are associated with hydrolysis, acidogenesis and acetogenesis reactions in anaerobic
224 digestion processes (22). Within *Proteobacteria* phylum, order *Syntrophobacterales* was
225 detected. Some bacteria belonging to this order are known for its syntrophic acetogenesis
226 activity, especially due to its propionate-oxidizing capacity. Syntrophic acetogenesis is a
227 crucial step to maintain stable the anaerobic digestion because high concentrations of
228 propionate inhibit methanogenesis (2). Also, genus *Paracoccus*, *Hyphomicrobium*,

229 *Comamonas* and *Nitrosomonas* were detected. These bacteria are also characterized for
230 its nitrifying and denitrifying activity, which may greatly contribute to the nitrogen
231 removal.
232 Other phyla as *Tenericutes, Actinobacteria* and *Synergistetes* do not exceed 5% of the
233 total abundance which is in accordance to results obtained in other studies for similar
234 samples (15, 23).
235 Fig.2B, presents the relative abundance of the different phyla in the digestate after
236 anaerobic digestion (S2). Results showed that an important variation occurs in the
237 microbiota during the process. The phylum *Firmicutes* increases severely reaching 83%
238 of total abundance. On the opposite, *Bacteroidetes* and *Proteobacteria* decreased
239 drastically below 10%. In the case of *Spirochaetes*, almost the disappearance of the
240 phylum occurred during the anaerobic process.
241 The majority of microorganisms who belong to *Firmicutes* phylum are mesophilics, so
242 its growth was favoured at 36ºC. Its predominance in the S2 sample showed a correct
243 functioning of the digester. The presence of *Bacteroidetes* phylum is also closely related
244 with operating temperature, decreasing their relative abundance with the increase in
245 operating temperature (24). *Proteobacteria* and *Spirochaetes* were reduced in the
246 digestate because of the absence of oxygen during the anaerobic process (1).
247 With respect to the classes, five different classes were detected within the phylum
248 *Proteobacteria* in S1 and S2: *δ-proteobacteria, ε-proteobacteria, α-proteobacteria, β-*
249 *proteobacteria*, and *γ-proteobacteria* (see Fig.2)
250 After anaerobic digestion, in the digestate (S2), the *α-proteobacteria* class significantly
251 increased their relative abundance reaching values higher than 50%, while *β-*
252 *proteobacteria* and *γ-proteobacteria* classes decreased. The *α-proteobacteria class*

253 include mainly fermentative anaerobic bacteria, while *β-proteobacteria* and *γ-*
254 *proteobacteria* classes contain aerobic bacteria, so the anaerobic conditions limited the
255 viability of these microorganisms. Previous studies described *α-proteobacteria* as
256 predominant class in mesophilic digesters while *γ-proteobacteria* was dominant in
257 thermophilic digesters (11). Pervin et al (25) detected *β-proteobacteria* as dominant class
258 in a thermophilic reactor, especially genus *Comamonas* that can metabolise a wide variety
259 of organic acids.
260 For *Firmicutes* and *Bacteroidetes* phyla, no significant changes were detected in the
261 distribution of the classes after anaerobic treatment (Fig.2B).
262 Within *Firmicutes* phyla in S1 sample, *Clostridia* was the most abundant class,
263 represented mainly for the *Clostridial* order. Bacteria of the genus *Clostridium* are strict
264 anaerobes, typical in biogas plants, which have the capacity of producing hydrogen (26).
265 Many species of *Clostridia* class had been defined as typical anaerobic cellulolytic
266 bacteria. Areas of strong degradation in cellulose structures with these bacteria occupying
267 these depressions had been reported (27). Michalke et al (28) have reported that
268 microorganisms belonging to *Clostridia* class and sulphate-reducing bacteria are
269 producers of trimethylarsines and small amounts of arsine in sewage sludge digestion.
270 These compounds are considered with environmental risks because of their toxicity. Guo
271 et al (29) have described that this class performed the acidogenic process at the second
272 stage and produced VFA, CO_2 and H_2.
273 Jaenicke et al (30) carried out metagenomic studies during the anaerobic digestion of a
274 mixture of maize silage, green rye and chicken manure. Results showed that *Clostridia*
275 populations were in syntrophic association with hydrogenotrophic methanogens. For this

reason, the presence of this class supposes an advantage to maintain efficient biogas production.

For the digestate (S2), the *Bacilli* and *Clostridia* classes represented 19% and 80% respectively; increasing their relative abundance compared with other classes as *Erysipelotrichi*, *Erysipelotrichia* and *Negativicutes* which have hydrolytic properties. The class *Bacilli*, was mainly represented by the order *Lactobacillales*, where the family of the *Carnobacteriaceae*, which contains bacterial species for decomposing fat and carbohydrate, supposed more than 90% (31).

For *Bacteroidetes* phylum, the class *Bacteroidia* was the most representative in both samples (S1 and S2), previously reported as the most abundant in mesophilic reactors. This class play an important role in hydrolyzing and fermenting organic materials and producing organic acids, CO_2 and H_2 during the anaerobic digestion process (29). The relative abundance found for other classes as *Flavobacteria* and *Sphingobacteria* were lower in comparison with other studies that reported these classes as dominant (32).

Separation process I: Centrifugation

The data obtained for the solid phase (S3) and the clarified (S4) resulting after the centrifugation process are shown in Fig.3.

FIGURE 3

With respect to the centrifuge feed (S2), in the solid phase (S3), the abundance of the phylum *Firmicutes* decreased to a value of 57%. On the opposite, the phylum *Proteobacteria* increased its abundance reaching a value of 31%. Therefore, it can be concluded that bacteria belonging to the phylum *Proteobacteria* have a greater tendency to be removed by centrifugation. Yi et al (33) studied the effect of total solids concentration on microbial communities involved anaerobic digestion processes of food

300 waste and, in agreement with our results, reported an increase in *Proteobacteria*
301 abundance for higher solid concentrations.
302 After the centrifugation process, sample S3 is mixed with vegetable wastes and sent to a
303 composting process. *Proteobacteria* and *Firmicutes*, between others, are able to
304 synthesize enzymes that catalyse the hydrolysis of plant polymers (34). For this reason,
305 the high abundance of these phyla in S3 is an advantage for the subsequent composting
306 process.
307 In the clarified (S4) important variations were not observed compared to the digestate
308 (S2). It should be noted that the majority of *Firmicutes* microorganisms from the digestate
309 were preserved in the liquid fraction after the centrifugation process.
310 Gao et al (35) studied the microbial populations in a bioreactor fed with sludge mixed
311 with sewage from cat food and they reported high relative abundance of the phylum
312 *Firmicutes* in the bioreactor suspension, as occurs here in the clarified (S4). This fact,
313 together with the recirculation of sample S4 during the sewage sludge treatment,
314 contributes to a correct operation of the anaerobic reactor.
315 With respect to the classes, in the solid phase (S3), *γ-proteobacteria* represented the 90%
316 of relative abundance, within *Proteobacteria* phylum, being the predominant families
317 *Pseudomonadaceae* and *Xanthomonadaceae*. This class showed a greater ability to be
318 removed by centrifugation than the *β-proteobacteria* and the *α-proteobacteria* classes
319 whose abundances were higher in the clarified (S4). This fact may be due to a lower
320 interaction of the *β-proteobacteria* and the *α-proteobacteria* with the polyelectrolyte
321 added for the centrifugation process and a lower tendency to the form flocs.
322 De Gannes et al (36) studied microbiota diversity in composts employing 454-
323 pyrosequencing, and they observed that *γ-proteobacteria* was especially abundant in the

mesophilic stage, whereas during thermophilic and mature stages *α-proteobacteria* and *γ-proteobacteria* classes were more abundant. The high relative abundance of *γ-proteobacteria* class in S3 sample, may be important in favour of the initial phase of composting.

With respect to *Firmicutes* phylum, 6 different classes were detected in solid phase and clarified samples (S3 and S4), i.e. *Bacilli, Clostridia, Erysipelotrichi, Erysipelotrichia, Negativicutes* and *Thermolithobacteria* (Fig.3).

Significant differences in the relative abundances were not observed compared with digestate. The class *Clostridia* was again the most representative and, as previously commented, these bacteria are known to metabolize relatively recalcitrant materials such as cellulose and lignin. In addition, species of *Bacilli* are known to secrete catabolic enzymes, such as proteases (37). Therefore, the presence of these classes in the S3 sample is convenient for the subsequent composting process.

Similarly to *Firmicutes* phylum, the distribution of the classes within phylum *Bacteroidetes* was not affected by the centrifugation process and *Bacteroidia* was again the predominant class.

Separation process II: Decanting

The results obtain for the decanted supernatant (S5) are shown in Fig. 4. After the decanting process a great variety of phyla were detected, whose distribution varies significantly in comparison with the liquid before decanting (S4).

FIGURE 4

The phylum *Firmicutes* was reduced to 37% of total abundance. On the contrary, other phyla such as *Bacteroidetes*, *Proteobacteria*, *Spirochaetes* or *Tenericutes* increase their total abundance reaching values of 10%, 37%, 8% and 6% respectively.

To understand these change, it is important to take into consideration that the decanting process takes place into an open tank during 45 h as previously commented, in S4, *Clostridia* was the class most abundant and these bacteria are strict anaerobes, thus it is expectable that, during decanting step, its concentration decrease, being replaced for other aerobic or facultative microorganisms.

The composition of relative abundance for the classes belonging to *Bacteroidetes* and *Firmicutes* phyla were almost not affected by the decanting being again *Bacteroidia* and *Clostridia* classes the most representative respectively. These classes contain well-known fermentative bacteria which are closely involved in degradation of organic materials and volatile fatty acids. Their presence is potentially associated with hydrolysis in wastewater treatment plant (38).

The phylum *Proteobacteria* was the most affected by decantation, with an increase of the *γ-proteobacteria* class and a drastic decrease of the *α-proteobacteria* class. The *α-proteobacteria* class is mostly anaerobic microorganisms, so the decanting process in an open tank for almost 2 days compromises their survival. On the contrary, the class of *γ-proteobacteria* includes groups of aerobic bacteria that can grow during this process, as *Pseudomonas*. The high relative abundance of *γ-proteobacteria* is important since these microorganisms together with *β-proteobacteria* class, as *Nitrosomonas* bacteria, play a crucial role in biological treatment processes as nitrification-denitrification (38), which is the final destination of the decanted product.

In conclusion, the study indicated that the methodology employed, based on PGM sequencing and the amplification of all variable regions of the 16S gene allowed us to obtain an exhaustive taxonomic classification of bacterial populations throughout the processes considered. The main phyla detected throughout the digestion, centrifugation

and decanting processes corresponded with microorganisms previously identified in anaerobic digestion of sludge. However, the relative abundance for the phyla throughout the process was very different depending on the treatment phase.

In the digestate (S2) from anaerobic digestion, *Firmicutes* was the predominant phylum, constituted mainly by bacteria of the genus *Clostridia* followed by the genus *Bacilli*. Their presence was crucial for a correct development of the anaerobic process. In related to class level, only important variation was observed within *Proteobacteria* phylum where *α-proteobacteria* was the most abundant after the process.

The relative abundance of the phyla in the clarified (S4) from centrifugation was very similar to that found in the digestate (S2) and only a light decreasing of *Actinobacteria* phylum was detected. This may be an advantage due to the recirculation of S4 sample to the initial phase of anaerobic digestion. On the contrary, *Firmicutes* showed a sharp decrease in the solid phase (S3), while the phylum *Proteobacteria* increased. This fact indicates the different predisposition of these microorganisms to be removed by centrifugation. *Proteobacteria* classes were again the most affected by the process being *γ-proteobacteria* the dominant one.

Finally, after the decanting process, in the supernatant (S5), it was observed an increase in the relative abundance of phyla that were minority in the clarified from the centrifugation step (S4), especially the phyla *Proteobacteria*, *Spirochaetes* and *Tenericutes*. On the other hand, *Firmicutes* phylum suffered a sharp descent again in its relative abundance. This was attributed to the presence of oxygen in the decanter which is an open tank, where the water remains for almost 2 days. Although the oxygen dissolve concentration in the decanter was always below 0.5 mg/L, it is possible that in the upper levels aerobic bacteria are favoured. At the class level, an increase of *γ-proteobacteria*,

396 which includes denitrifying microorganisms, was observed. The presence ammonia-
397 oxidizing bacteria as *Nitrosomonas* and denitrifiers as *Pseudomonas*, may be beneficial
398 for the subsequent biological treatment. It is necessary to consider that the small quantities
399 of biodegradable organic effluents that are occasionally spilled into the tank may
400 contribute to the differences detected in the microbiology. The variations observed on the
401 relative abundances of bacterial phyla and classes are important to understand the
402 influence of operation ways over the efficiencies of the following biological processes.

403 **ACKNOWLEDGMENTS**

404 The work upon which this paper is based on was co-financed by COGERSA, SAU
405 (Projects FUO-EM-138-15, FUO-139-16), IDEPA (Project IDE/2015/000245), Spanish
406 MINECO (Project CTM2015-63864-R) and FEDER funds from European Union.
407 Technical assistance from the Scientific-Technical Services of the University of Oviedo
408 is gratefully acknowledged.

REFERENCES

1. **Cardinali-Rezende, J., Debarry, R.B., Colturato, L.F., Carneiro, E.V., Chartone-Souza, E., and Nascimento, A.M. (2009).** Molecular identification and dynamics of microbial communities in reactor treating organic household waste. Appl. Microbiol. Biotechnol, 84 (4), 777-789.
2. **Venkiteshwaran, K., Bocher, B., Maki, J., and Zitomer, D. (2015).** Relating Anaerobic Digestion Microbial Community and Process Function. Water Research, 8, 37–44. https://doi.org/10.4137/MBI.S33593.
3. **Liu, A.C., Chou, C.Y., Chen, L.L. and Kuo, C.H. (2015).** Bacterial community dynamics in a swine wastewater anaerobic reactor revealed by 16S rDNA sequence analysis. Journal of Biotechnology, 194, 124-131.
4. **Fykse, E.M., Aarskaug, T., Marslien, E.H. and Dybwad, M. (2016).** Microbial community structure in a full-scale anaerobic treatment plant during start-up and first year of operation revealed by high-throughput 16S rRNA gene amplicon sequencing. Bioresource Technology, 222, 380-387.
5. **Zeng, Y., De Guardia, A. and Dabert P. (2016).** Improving composting as a post-treatment of anaerobic digestate. Bioresource Technology, 201, 293–303.
6. **Sancha, M., Laca, A., Laca, A., González, J.M., Mayo, B. and Diaz, M. (2014).** Approaches for Microbiological Characterization of a Landill Leachate Treatment. Journal of Residuals Science & Technology, 11 (2) 1544-8053/14/02 039-06.
7. **Vincent, A.T., Derome, N., Boyle, B., Culley, A.I. and Charette, S.J. (2017).** Next-generation sequencing (NGS) in the microbiological world: How to make the most of your money. Journal of Microbiological Methods 138, 60-71.

8. **Chen, Z., Wang, Y., Li, K., and Zhou, H. (2014).** Effects of increasing organic loading rate on performance and microbial community shift of an up-flow anaerobic sludge blanket reactor treating diluted pharmaceutical wastewater. Journal of Bioscience and Bioengineering, 118 (3), 284–288. https://doi.org/10.1016/j.jbiosc.2014.02.027
9. **Zhou, S., Nikolausz, M., Zhang, J., Riya, S., Terada, A., and Hosomi, M. (2016).** Variation of the microbial community in thermophilic anaerobic digestion of pig manure mixed with different ratios of rice straw. Journal of Bioscience and Bioengineering, 122 (3), 334–340. https://doi.org/10.1016/j.jbiosc.2016.02.012
10. **Nelson, M., Morrison, M. and Yu, Z. (2011).** A meta-analysis of the microbial diversity observed in anaerobic digesters. Bioresource Technology, 102, 3730–3739.
11. **Sundberg, C., Al-Soud, W.A., Larsson, M., Alm, E., Yekta, S.S., Svensson, B.H., Sorensen, S.J., and Karlsson, A. (2013).** 454 pyrosequencing analyses of bacterial and archaeal richness in 21 full-scale biogas digesters. FEMS Microbiol. Eco., 85 (3), 612-626.
12. **Zamanzadeh, M., Hagen, L.H., Svensson, K. and Linjordet, R. (2016).** Anaerobic digestion of food waste - Effect of recirculation and temperature on performance and microbiology. Water Research, 96, 246-254.
13. **Shu, D., He, Y., Yue, H. and Wang, Q. (2015).** Microbial structures and community functions of anaerobic sludge in six full-scale wastewater treatment plants as revealed by 454 high-throughput pyrosequencing. Bioresource Technology, 186, 163–172.

14. **Hu, M., Wang, X., Wen, X. and Xia, Y. (2012).** Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresource Technology, 117, 72–79.
15. **Zhang, T., Shao, M. and Ye, L. (2012).** 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. The ISME Journal, 6, 1137-1147.
16. **Abendroth, C., Vilanova, C., Günther, T., Luschnig, O. and Porcar, M. (2015).** Eubacteria and archaea communities in seven mesophile anaerobic digester plants in Germany. Biotechnology of Biofuels, 8 (87). DOI 10.1186/s13068-015-0271-6
17. **Yang, Z., Xu, R., Zheng, Y., Chen, T., Zhao, L. and Li, M. (2016).** Characterization of extracellular polymeric substances and microbial diversity in anaerobic co-digestion reactor treated sewage sludge with fat, oil, grease. Bioresource Technology, 212, 164–173.
18. **Narihiro, T., Nobu, M. K., Kim, N.-K., Kamagata, Y. and Liu, W.T. (2015).** The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. Environmental Microbiology, 17, 1707–1720. doi: 10.1111/1462-2920.12616
19. **Gulhane, M., Pandit, P., Khardenavis, A., Singh, D., and Purohit, H. (2017).** Study of microbial community plasticity for anaerobic digestion of vegetable waste in Anaerobic Baffled Reactor. Renewable Energy, 101(February), 59–66. https://doi.org/10.1016/j.renene.2016.08.021
20. **Riviere, D., Desvignes, V., Pelletier, E., Chaussonnerie, S., Guermazi, S., Weissenbach, J., Li, T., Camacho, P. and Sghir, A. (2009).** Towards the

480 definition of a core of microorganisms involved in anaerobic digestion of sludge.
481 ISME J, 3(6), 700-714.
482 21. **Regueiro, L., Veiga, P., Figueroa, M., Alonso-Gutierrez, J., Stams, A.J.M.,**
483 **Lema, J.M. and Carballa, M. (2012).** Relationship between microbial activity
484 and microbial community structure in six full-scale anaerobic digesters.
485 Microbiological Research, 167(10), 581-589.
486 22. **Maspolim, Y., Zhou, Y., Guo, C., Xiao, K. and Ng, W.J. (2015).** Determination
487 of the archaeal and bacterial communities in two-phase and single-stage anaerobic
488 systems by 454 pyrosequencing. Journal of Environmental Sciences, 36, 121-129.
489 23. **Dao, H., Kuroda, K., Nakahara., N., Danshita, T., hatamoto, M. and**
490 **Yamaguchi, T. (2016).** 16S rRNA gene-based comprehensive analysis of
491 microbial community compositions in a full-scale leachate treatment system.
492 Journal of Bioscience and Bioengineering, 122 (6), 708-715.
493 24. **Sun, L., Pope, P. B., Eijsink, V. G. H. and Schnürer, A. (2015).**
494 Characterization of microbial community structure during continuous anaerobic
495 digestion of straw and cow manure. Microbial Biotechnology, 8: 815–827.
496 doi:10.1111/1751-7915.12298
497 25. **Pervin, H. M., Batstone, D. J., and Bond, P. L. (2013).** Previously unclassified
498 bacteria dominate during thermophilic and mesophilic anaerobic pre-treatment of
499 primary sludge. Systematic and Applied Microbiology, 36(4), 281–290.
500 https://doi.org/10.1016/j.syapm.2013.03.003
501 26. **Ziganshin, A.M., Liebetrau, J., Pröter, J. and Kleinsteuber, S. (2013).**
502 Microbial community structure and dynamics during anaerobic digestion of

various agricultural waste materials. Applied Microbiology and Biotechnology, 97(11), 5161-5174.

27. **McDonald, J. E., Houghton, J. N. I., Rooks, D. J., Allison, H. E. and McCarthy, A. J. (2012).** The microbial ecology of anaerobic cellulose degradation in municipal waste landfill sites: evidence of a role for fibrobacters. Environmental Microbiology, 14: 1077–1087. doi: 10.1111/j.1462-2920.2011.02688.x

28. **Michalke, K., Wickenheiser, E. B., Mehring, M., and Hirner, A. V. (2000).** Production of Volatile Derivatives of Metal(loid)s by Microflora Involved in Anaerobic Digestion of Sewage Sludge. Applied and Environmental Microbiology. 66(7), 2791–2796.

29. **Guo, J., Peng, Y., Ni, B.-J., Han, X., Fan, L., and Yuan, Z. (2015).** Dissecting microbial community structure and methane-producing pathways of a full-scale anaerobic reactor digesting activated sludge from wastewater treatment by metagenomic sequencing. Microbial Cell Factories, 14(1), 33. https://doi.org/10.1186/s12934-015-0218-4

30. **Jaenicke, S., Ander, C., Bekel, T., Bisdorf, R., Dröge, M., Gartemann, K. H., and Goesmann, A. (2011).** Comparative and joint analysis of two metagenomic datasets from a biogas fermenter obtained by 454-pyrosequencing. PLoS ONE, 6(1). https://doi.org/10.1371/journal.pone.0014519

31. **Li, A., Chu, Y., Wang, X., Ren, L., Yu, J., Liu, X., and Li, S. (2013).** A pyrosequencing-based metagenomic study of methane-producing microbial community in solid-state biogas reactor. Biotechnology for Biofuels, 6(1), 3. https://doi.org/10.1186/1754-6834-6-3

32. **Di Maria, F., Barratta, M., Bianconi, F., Placidi, P. and Passeri, D. (2017).** Solid anaerobic digestion batch with liquid digestate recirculation and wet anaerobic digestion of organic waste: Comparison of system performances and identification of microbial guilds. Waste Management, 59, 172–180.

33. **Yi, J., Dong, B., Jin, J. and Dai, X. (2014).** Effect of Increasing Total Solids Contents on Anaerobic Digestion of Food Waste under Mesophilic Conditions: Performance and Microbial Characteristics Analysis. PLoS ONE, 9, (7) e102548.doi:10.1371/journal.pone.0102548

34. **Wang, Z., Zhang, X-X., Lu, X., Liu, B., Li, Y., Long, C. and Li, A. (2014).** Abundance and diversity of bacterial nitrifiers and denitrifiers and their functional genes in tannery wastewater treatment plants revealed by high throughput sequencing. PLOS ONE, DOI:10.1371/journal.pone.0113603.

35. **Gao, D., Zhang, T., Tang, C., Wu, W., Wong, C., Lee, Y., Yeh, D. and Criddle, C. (2010).** Membrane fouling in an anaerobic membrane bioreactor: Differences in relative abundance of bacterial species in the membrane foculant layer and in suspension. Journal of Membrane Science, 364, 331–338.

36. **De Gannes, V., Eudoxie, G., and Hickey, W. J. (2013).** Prokaryotic successions and diversity in composts as revealed by 454-pyrosequencing. Bioresource Technology, 133, 573–580. https://doi.org/10.1016/j.biortech.2013.01.138

37. **Partanen, P., Hultman, J., Paulin, L., Auvinen, P., and Romantschuk, M. (2010).** Bacterial diversity at different stages of the composting process. BMC Microbiology, 10(1), 94. https://doi.org/10.1186/1471-2180-10-94

38. **Guo, J., Ni, B. J., Han, X., Chen, X., Bond, P., Peng, Y., and Yuan, Z. (2017).** Unraveling microbial structure and diversity of activated sludge in a full-scale

551 simultaneous nitrogen and phosphorus removal plant using metagenomic
552 sequencing. Enzyme and Microbial Technology, 102(March), 16–25.
553 https://doi.org/10.1016/j.enzmictec.2017.03.009
554

FIGURE CAPTIONS

Figure 1. Process flow diagram of anaerobic digestion plant. Flow data have been shown in different unit of measurement depending on the operational mode: continuous process (m^3/day) and discontinuous process (m^3/week). The centrifugation process was carried out for 7 hours per day only 5 days per week.

Figure 2. Relative abundance for the phyla detected in the initial sludge (A) and digestate (B) samples (S1 and S2, respectively). In boxes are shown the relative abundance obtained for the classes within *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla.

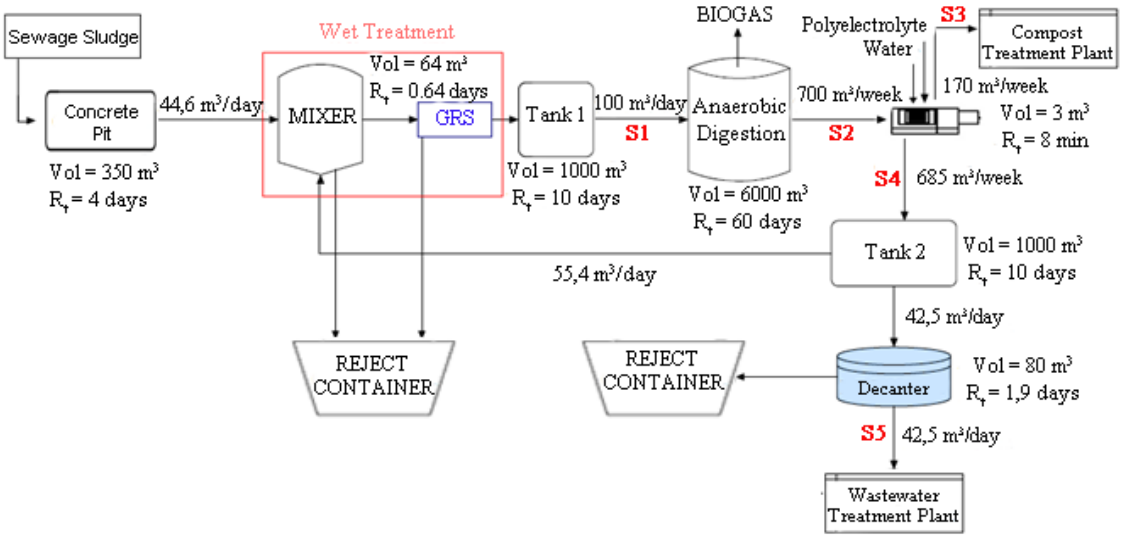
Figure 3. Relative abundance for the phyla detected in the solid phase (A) and clarified (B) samples (S3 and S4, respectively). In boxes are shown the relative abundance obtained for the classes within *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla.

Figure 4. Relative abundance for de phyla detected in the decanted product (S5). In boxes are shown the relative abundance obtained for the classes within *Firmicutes*, *Bacteroidetes* and *Proteobacteria* phyla.

TABLE CAPTIONS

Table 1. Characteristics of the samples analysed

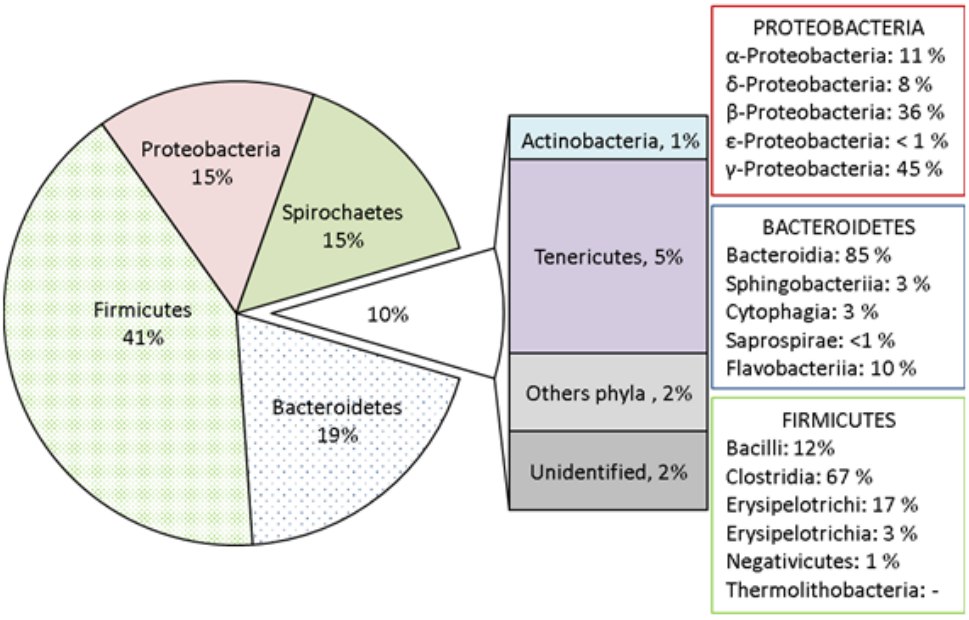
Figure 1


Sewage Sludge
Concrete Pit
Vol = 350 m³
R_t = 4 days
44,6 m³/day
Wet Treatment
MIXER
Vol = 64 m³
R_t = 0.64 days
GRS
Tank 1
Vol = 1000 m³
R_t = 10 days
100 m³/day
S1
BIOGAS
Anaerobic Digestion
Vol = 6000 m³
R_t = 60 days
Polyelectrolyte
Water
S3
Compost Treatment Plant
700 m³/week
170 m³/week
S2
Vol = 3 m³
R_t = 8 min
S4
685 m³/week
Tank 2
Vol = 1000 m³
R_t = 10 days
55,4 m³/day
42,5 m³/day
REJECT CONTAINER
REJECT CONTAINER
Decanter
Vol = 80 m³
R_t = 1,9 days
S5
42,5 m³/day
Wastewater Treatment Plant

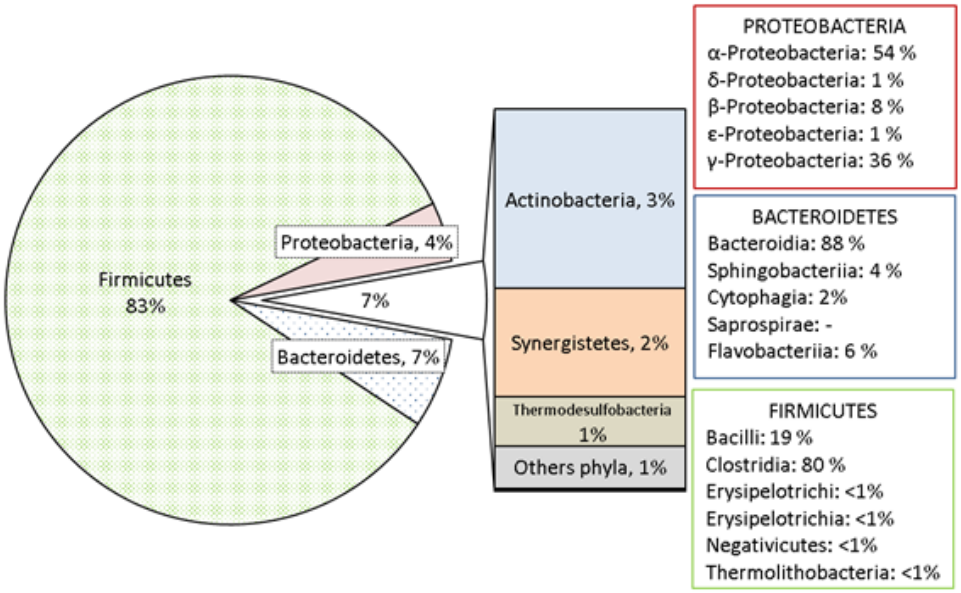
576

Figure 2

A)



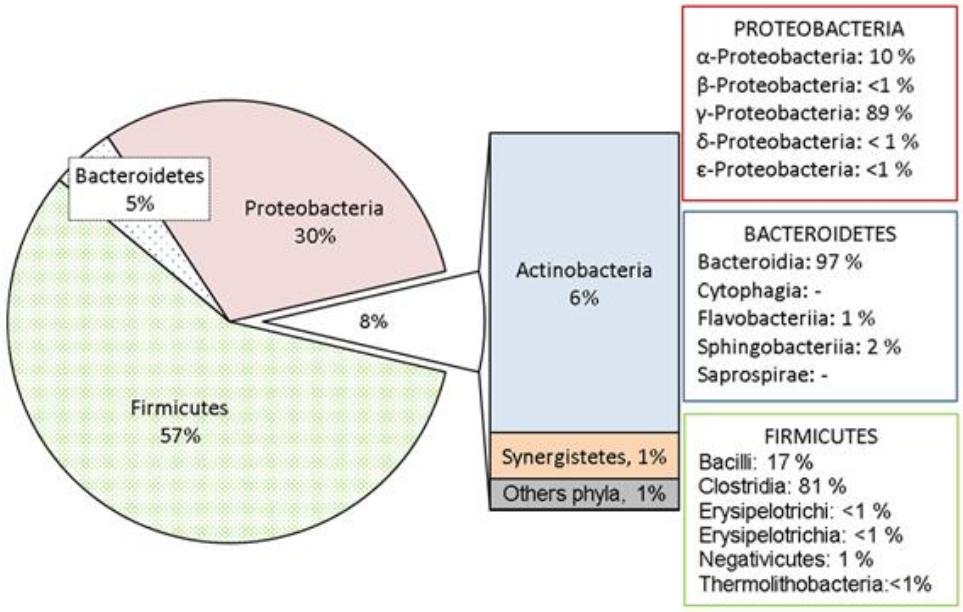
B)



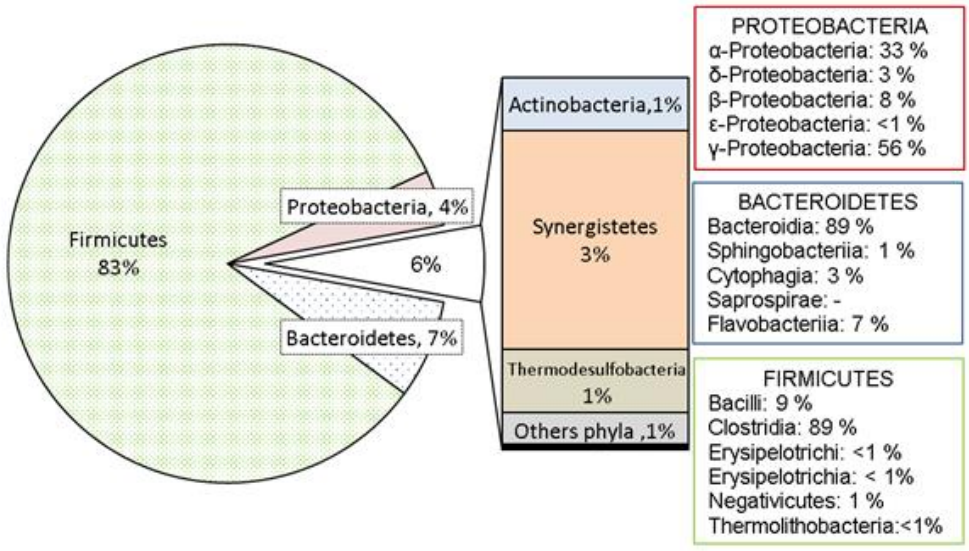
577

Figure 3

A)

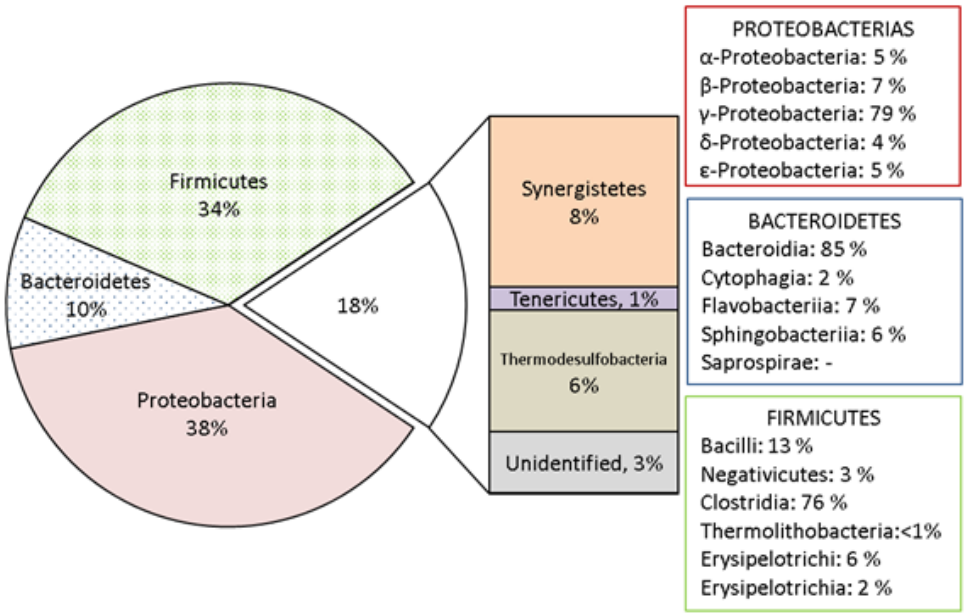


B)



578

Figure 4


Firmicutes
34%
Bacteroidetes
10%
Proteobacteria
38%
18%
Synergistetes
8%
Tenericutes, 1%
Thermodesulfobacteria
6%
Unidentified, 3%
PROTEOBACTERIAS
α-Proteobacteria: 5 %
β-Proteobacteria: 7 %
γ-Proteobacteria: 79 %
δ-Proteobacteria: 4 %
ε-Proteobacteria: 5 %
BACTEROIDETES
Bacteroidia: 85 %
Cytophagia: 2 %
Flavobacteriia: 7 %
Sphingobacteriia: 6 %
Saprospirae: -
FIRMICUTES
Bacilli: 13 %
Negativicutes: 3 %
Clostridia: 76 %
Thermolithobacteria:<1%
Erysipelotrichi: 6 %
Erysipelotrichia: 2 %

579

Table 1. Characteristics of the samples analyzed. The average values correspond to four samples taken along 2016.

	Parameters					
Samples	pH (ud.)	COD (mg/L)	NH_4 (mg/L)	BOD_5 (mg/L)	TS (mg/L)	Moisture (%)
S1	6.99 ± 0.26	N/A	N/A	N/A	81193 ± 7360	92 ± 0.69
S2	8.13 ± 0.07	56375 ± 1431	5501 ± 421	3750 ± 395	63670 ± 1422	94 ± 0.13
S3	8.21 ± 0.38	67475 ± 13530	17274 ± 1125	N/A	223125 ± 11442	79 ± 1.26
S4	8.39 ± 0.17	10405 ± 4269	4385 ± 428	1525 ± 540	10347 ± 2724	99 ± 0.33
S5	8.42 ± 0.09	10600 ± 4988	3989 ± 177	2735 ± 1572	10198 ± 3544	99 ± 0.30