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Valorization of sewage sludge for volatile fatty acids production and role of microbiome on acidogenic fermentation

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Abstract

This work explored the production of volatile fatty acids (VFA) through the anaerobic digestion of sewage sludge (SS). The first experiment took place at batch scale to evaluate the combined effect of using a thermal pre-treatment (120 ºC, 15 min) and different Substrate/Inoculum ratios (S/I) (1, 2, 4 and 6 gVS of substrate/gVS inoculum) on the acidogenic potential of the SS. The results showed that the thermal pre-treatment influenced positively the degree of acidification of the SS at low S/I ratios, reaching maximum of 45%. Afterwards, a continuous lab-scale experiment, was set-up to study two ranges of organic loading rates (OLR): 1300-1600 mg COD L^{-1} d^{-1} and 2400-3500 mg COD L^{-1} d^{-1}. The highest degree of acidification (22%) was achieved at the lowest OLR. Analysis of the microbial community in the reactor revealed that OTUs most

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abundant present genes related with aminoacids and carbohydrates fermentation being crucial for VFA production.

Keywords

Acidogenic fermentation; Acidogenic potential; Microbial community; Sewage sludge; Volatile fatty acids

1. Introduction

Nowadays the main drawback in Wastewater Treatment Plants (WWTP) is the amount of excess sewage sludge (SS) being generated. The management of the SS represents more than 60% of total operating costs in WWTP (Yang et al., 2012) and its disposal generates several environmental problems due to the high content of organic matter and the presence of heavy metals, pathogens and xenobiotic compounds among others (Hendrickx, 2009). A proper management of SS can prevent its accumulation and allows the valorization of this waste to produce biotechnological added-value products through anaerobic digestion (AD). AD happens naturally in the environment in the absence of oxygen when the microbial community decomposes organic materials into methane and carbon dioxide (Molino et al., 2013). It is a complex process that consists of four sequential and biochemical steps; hydrolysis, acidogenesis, acetogenesis and methanogenesis. Recently, the Volatile Fatty Acids (VFA) generated during the acidogenic step has gained attention due to their wide range of possible applications in biotechnology such as energy generation by microbial fuel cells, their use as carbon source for biological removal of nutrients in WWTP and for the production of Polyhydroxyalkanoates (PHA) with mixture cultures (Lee et al., 2014). PHA are biodegradable biopolymers with many possible applications in biomedicine, pharmacy or food industry (Reddy et al., 2003). These compounds can be produced by bacteria as energy source and as carbon storage that can be used when the amount available

nutrients becomes limited (Sudesh et al., 2000). Nowadays, the production of these biopolymers is limited both by the high cost of the substrates and the need to utilize pure cultures. It has been estimated that the use of the VFA produced from AD could decrease the cost of PHA production down to 50% of the current price (Tamis et al., 2013). The composition and proportion of VFA in the effluent is an important aspect determining the type of PHA produced and their characteristics (Serafim et al., 2004). The VFA feeding streams with even number of carbon atoms (acetic or butyric acids) results in the production of poly-3-hydroxybutyrate (PHB) (Lemos et al., 2006). Conversely, the predominance of VFA with odd number of carbon atoms (propionic and valeric acids) generates a co-polymer with higher 3-hydroxyvalerate (3HV) content, which is more suitable for industrial applications due to its thermal and mechanical properties.

SS is a good candidate for PHA production, particularly by taking into account the huge amount produced and the high concentrations of proteins and carbohydrates (Ucisik and Henze, 2008) which can be converted into VFA by anaerobic fermentation. However, according to Eastman and Ferguson (1981) it has to be considered that hydrolysis of particulate organic matter to soluble compounds is the rate limiting step during the AD of sewage sludge. For these reasons, different strategies were proposed to increase and speed-up the yield of hydrolysis, such as the use of thermal, chemical, biological or mechanical pre-treatments (Gagliano et al., 2015; Kavitha et al., 2014; Park et al., 2010; Ye et al., 2016).

The main target of studies reported in the literature is biogas production through the AD of the SS. On the contrary, in the present study the main goal is to elucidate the acidogenic potential of SS and the VFA profile suitable for PHA production. The effect of a thermal pre-treatment in combination with the Substrate/Inoculum ratio (S/I) was

investigated first in batch experiment. Afterwards, the feasibility of the process in a semi-continuous mode without thermal pre-treatment of the SS was performed in lab-scale reactor.

Whilst the diversity of microbes involved in anaerobic digestion of SS to produce methane and their response to different management practices and environmental conditions has been well documented (Guo et al., 2015), there are very few studies describing the microbial community involved in the acidogenic fermentation process. In this study the microbial populations were studied when the performance of the acidogenic fermentation in the anaerobic reactor was stable, in order to deepen the investigation and to control the acidogenic process. Additionally, identity and metabolic potential of the microbial community carrying out the process were determined by means of Illumina 16S rRNA amplicon sequencing.

2. Material and Methods

2.1 Feed sources and inoculum

The inoculum used in this work was collected from an anaerobic digester in a brewery wastewater treatment plant (WWTP) from A Coruña (Spain). The concentrations of total solids (TS) and volatile solids (VS) in the sludge were 38 ± 1.5 and 32 ± 1.8 g L^{-1}, respectively. The raw material used as substrate was sewage sludge from a WWTP from Carral, A Coruña (Spain). It was stored in a cold chamber until being used. The main characteristics of the sewage sludge are summarized in Table 1.

2.2 Batch fermentation experiments

A batch experiment was carried out in triplicate, using bottles with a working volume of 120 mL. Anaerobic sludge was added to each bottle to reach a biomass concentration of 2 g VS L^{-1}. Fresh sewage sludge was added in all the experiments in order to achieve a specific S/I ratio. $NaHCO_3$ was added to provide a mineral medium and alkalinity of 2

g L^{-1} as $CaCO_3$ in order to avoid a sudden drop of pH. Na_2S was added as reducing agent at a concentration of 1mM. 2-Bromoethanesulphonic acid sodium salt, (BES, 20 mmol L^{-1}) was also added as inhibitor of the methanogenesis. Finally, distilled water was added to make up the volume to 100 mL. The pH was not adjusted, and the headspace of the bottles was purged with nitrogen gas for several minutes to ensure the anaerobic conditions and then they were sealed. The bottles were placed in an orbital shaker at 150 rpm, in a room at 30 ± 1 °C (mesophilic operation). The experiments were run until the VFA production reached steady state.

The influence of a thermal pre-treatment was studied in combination with different S/I ratios (1, 2, 4 and 6 VS substrate/VS inoculum). A blank test was also carried out using the same concentration of inoculum but without adding sewage sludge.

2.3 Laboratory scale reactor

A glass reactor with a working volume of 2 L was maintained at 37 °C using a thermostatic bath. To keep the mixture well-stirred, the reactor was continuously stirred by means of an electric shaker at 150 rpm. The reactor was also inoculated with the same type of anaerobic sludge used in the batch assays, adding 400 mL with a concentration of 53.8 g TS L^{-1} and 47.8 g VS L^{-1}. In order to start-up the reactor with a biomass concentration of 4.98 g VS L^{-1} of feedstock, a volume of 1600 mL sewage sludge diluted with distilled water was added. The pH in the reactor remained constant at 5.6 by feeding the feedstock (SS) and it was controlled during the process. The reactor was fed 200 mL feedstock (sewage sludge diluted) daily and 200 mL of effluent were discharged, to keep HRT and SRT of 10 days. An initial Organic Loading Rate (OLR) of 900 mg COD L^{-1} d^{-1} was applied to acclimate the inoculum to the substrate for one week. Afterwards, two ranges of OLR were tested: 1300-1600 mg COD L^{-1} d^{-1}

and 2400-3500 mg COD L^{-1} d^{-1}. The HRT of 10 days was maintained during the whole operation period.

2.4 Analytical methods

Total and volatile solids (TS and VS), ammonia and phosphates content were determined according to Standard Methods (APHA/AWWA/WEF, 2012). The total and soluble chemical oxygen demand (tCOD and sCOD) were assessed by digestion of the sample with dichromate at 150 ºC. The VFA (acetic, lactic, propionic, i-butyric, butyric, n-valeric and i-valeric acids) were determined by high performance liquid chromatography (HPLC) following the method used by Bermúdez-Penabad et al. (2017). The analysis of elemental carbon (C) and nitrogen (N) were done with an elemental analyzer (Carlo Erba CHNS-O 1108).

The thermal pre-treatment of the sewage sludge was carried out in a 75 L volume autoclave (Selecta P. Autester Mod. 437-G) working at 120°C and 2 atm. The autoclave was equipped with a time programming system that allowed the pre-treatment time to be controlled. Once the pre-treatment was carried out, the samples were cooled at room temperature to avoid the loss of volatile compounds. All the samples were analyzed in triplicate.

2.5 Analysis of the microbial community

2.5.1 Sampling and DNA extraction

Ten ml of sample were collected from the reactor at the end of the experiment. DNA samples were isolated and purified using the phenol-chloroform method as previously described (Alonso-Gutiérrez et al., 2009). Quantity and purity of genomic DNA was determined using NanoDrop spectrometer (Thermo Scientific).

2.5.2 Analysis of the 16S rDNA amplicons

16S rDNA amplicons of the hypervariable region V4 were analyzed using high-throughput Illumina sequencing at the Ramaciotti Centre for Functional Genomics (UNSW, Sydney). Bacteria and archaea were analyzed together using primers "515F/806R" for PCR amplification (http://www.earthmicrobiome.org/). Raw reads have been submitted to the sequence read archive database (SRA) of NCBI under the BioProject PRJNA550518 with accession number SAMN12130848. Amplicons were sequenced using Illumina MiSeq instrument obtaining paired-end sequences 250+ 250 bp long. Sequences were filtered using Trimmomatic software (Bolger et al., 2014). Since the forward and reverse reads were almost completely overlapped, they were combined using BBMerge software (https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/bbmerge-guide/). After filtering and paired-end merging, the number of sequences per each experimental replicate ranged from 73.605 to 88.295. Sequences were analyzed using QIIME software (Caporaso et al., 2010) and the RDP naïve Bayesian Classifier software (Cole et al., 2003). Operational taxonomic units (OTU) were picked against the Greengenes database at a 97% similarity threshold.
Additional investigation was performed using CLC Microbial Genomics Module (QIAGEN Bioinformatics) to generate the abundance profile of each OTU. Additional analyses were performed using CLC Workbench software (V.8.02) with Microbial genomics module plug in (QIAGEN Bioinformatics, Germany) as previously reported Treu et al. (2018). Similarly, to what reported above, in CLC analysis the OTU were picked against the Greengenes database with a 97% similarity to calculate the α-diversity index.
Predictive functional analysis was performed according to the procedure previously described (Francisci et al., 2015). A database with COG and KEGG annotations was generated, using the IMG 4 Data Management system (http://img.jgi.doe.gov),

including 453 archaea and 15,484 bacteria available at the IMG. Species belonging to the same genus were grouped using the annotation downloaded from the same website. Using as input a list of genera, the script used recovers for each genus all the microbial species and for each species it calculates the average number of genes belonging to each COG and KEGG category (functional prediction). This process allows a comparison of COG and KEGG gene content among different genera using as input results obtained from 16S rRNA analysis.

2.6. Calculations of the parameters to evaluate the acidogenic potential of SS

2.6.1 Degree of Acidification

The Degree of Acidification (DA) was the parameter used to evaluate the acidogenic potential of the sludge, and it was defined as the sum of each individual VFA produced, expressed as COD equivalents, at the maximum concentration point (VFA max) minus its equivalent in the blank test (VFA blank) divided by the initial total COD added for each assay (tCOD initial).

$$DA(\%) = \frac{\sum VFA_{max} - \sum VFA_{blank}}{tCOD_{initial}} \qquad \text{Equation 1}$$

2.6.2 Acidification Yield

The Acidification Yield (AY) was defined as the percentage of the sum of each individual VFA produced expressed as COD equivalents, at the maximum concentration point (tVFA) divided by the initial total VS added in the reactor.

2.6.3 Odd-to-Even Ratio of VFA (Odd-to-Even)

In this study, the Odd to Even Ratio (Odd-to-Even) was considered an interesting parameter for the evaluation of the quality of the acidified effluent obtained. Odd to Even Ratio was defined as the sum of odd-equivalent carboxylic acids formed (propionic (HPr) and n-valeric (HVa) acids) divided by the sum of even-equivalent carboxylic acids formed (acetic (HAc), iso-butyric (i-HBu), n-butyric (HBu), iso-valeric

(i-HVa), and n-caproic (n-HCa)acids (Silva et al., 2013) (Equation 2). The designation of odd-equivalent carboxylic acids refers to the metabolites resulting in the PHA production. Acids containing an even number of carbon atoms result in the synthesis of hydroxybutyrate (HB), whereas the acids containing an odd number of carbons led to the production of hydroxyvalerate (HV) (Pardelha et al., 2012). The isomer (i-HVa), has an odd number of carbon atoms, but it is degraded to acetic acid and for this reason is considered as an even-equivalent acid (Wang et al., 1999).

$$Odd - to\ even\ = \frac{[HPr] + [HVa]}{[HAc] + [i-HBu] + [HBu] + [i-HVa] + [n-HCa]}$$

Equation 2

3. Results and discussion

3.1.1 Effect of pretreatment on the SS

The application of a thermal pre-treatment to the SS resulted in an increase of the soluble COD from 17.02 g L–1 to 19.68 g L–1 (Table 1). Similarly, the concentrations of N-NH_4^+ and P-PO_4^{-3} increased to 45% and 19%, respectively. The percentage of VS with respect to the TS and the pH did not vary; meanwhile, the tCOD decreased from 79.49 to 76.44 g L–1. These results are in accordance with previous studies that demonstrated that the application of a thermal pre-treatment increases the sCOD proportion respect to the tCOD and it does not affect significatively the percentage of VS respect to the TS (Gagliano et al., 2015). The decrease of the tCOD with the use of a thermal pre-treatment is related with the loss of volatile soluble compounds. Some authors have previously reported the loss of the tCOD during an application of a thermal pre-treatment in substrates such as food wastes or sewage sludge (Serrano et al., 2015; Tampio et al., 2014).

3.1.2 Effect of the thermal pre-treatment at different S/I ratios on the acidogenic fermentation of the SS

The influence of thermal pre-treatment (120ºC, 15 min) and the several substrate/inoculum ratios (1, 2, 4 and 6 gVS of substrate/gVS inoculum) on the acidogenic fermentation of SS was studied in batch experiments.

Figures 1(a) and (b) depict the acidogenic fermentation performance during the batch assays for each S/I ratio tested, with and without thermal pre-treatment. It can be observed that the fermentation started immediately after the inoculation in all the conditions tested. In the assay with on S/I ratio of "1", without thermal pre-treatment, a maximum VFA as COD concentration between 1800–2200 mg L^{-1} was achieved, while in the same assay with a thermal treatment a concentration between 2200–2600 mg L^{-1} was achieved. In the assays with on S/I ratio of "2" maximum VFA as COD concentrations of 3300–3800 mg L^{-1} and 4100–4800 mg L^{-1} were achieved without and with thermal pre-treatment, respectively. In the assays with the S/I ratio of "4", the VFA as COD concentrations obtained were 6300–6600 and 6900–7300 mg. L^{-1}, without and with pre-treatment, respectively. Finally, for the S/I ratio of "6" the maximum VFA as COD concentration achieved were very similar in both cases, in around 9500 mg. L^{-1}. At all the S/I ratios tested, once the maximum VFA concentration was reached its value remained constant or, otherwise, decreased slightly at the end of the experiment.

During the batch assays, the VFA composition was analyzed. In all the assays, six VFA, including acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric acids were detected. It can be observed in Fig. 2 (a) and (b) that acetic acid was the dominant product for all the conditions tested, followed by propionic, butyric and n-valeric acids. Similar patterns of predominant VFA were observed in previous studies using different types of sludges (Dong et al., 2016; Zhang et al., 2009). The butyric acid concentration

increased with the S/I ratio with and without thermal pre-treatment assays, meanwhile the proportion of acetic acid on the VFA profile decreased. Other authors, working with other organic wastes, have reported that increasing the organic load leads to a higher accumulation of VFA and subsequently, a decrease of pH favoring the long chain fatty acids production (Gameiro et al., 2016). On the other hand, the thermal pre-treatment leads to a different VFA profile, presenting higher proportions of propionic, n-butyric, iso-butyric, iso-valeric and n-valeric acids at all the S/I ratios tested in this study.

The maximum degree of acidification (DA) reached in the different assays are summarized in Table 2. The DA increased with the thermal pre-treatment for S/I ratios of "1" and "2", reaching maximum DA of 36% and 45%, without and with thermal pre-treatment, respectively. With the S/I ratio of "4", the DA obtained was 35% without thermal pre-treatment and 41% with thermal pre-treatment. In the case of the S/I ratio of "6" a maximum DA of 36 and 37 % were reached without and with thermal pre-treatment, respectively. The results obtained show that the use of a thermal pre-treatment favors the acidification which is more significant at low S/I ratios, related to the cell rupture which allows the production of higher soluble organic matter content . Besides, the low biomass concentration could allow better mixing conditions in the bottles. At higher S/I ratios, the effect of a thermal pre-treatment (S/I "4" and "6") on the DA was less significant. This behavior was also previously observed in studies with other types of solid wastes where the highest DA was achieved at low S/I ratios (Gameiro et al., 2016). The release of heavy metals and ammonia in the liquid phase after a thermal pre-treatment could have inhibited the anaerobic digestion process of SS (Chen et al., 2007).

The biodegradation of VS respect to the TS content at different S/I ratios can be observed in Table 2. At the S/I ratios studied without thermal pre-treatment the

reduction of the percentage of VS was slightly higher at low S/I ratios. At S/I ratio of "1" it was obtained the highest destruction of VS but did not influence the maximum DA achieved.

At all S/I ratios studied with thermal pre-treatment the highest destruction of VS respect to the TS was also achieved at the lowest S/I ratio which also presented the highest DA. However, at the S/I ratios of "4" and "6" the destruction of VS did not vary significantly, and the DA followed the same trend.

The Odd to Even ratios obtained were 0.21, 0.34, 0.37 and 0.37 for S/I of "1", "2", "4" and "6" respectively, without thermal pre-treatment. The Odd to Even ratio increased with the S/I ratio due to the increment of propionic acid proportion respect to acetic acid. In addition, it can be observed that the fermentation of SS resulted in a significant increase of soluble phosphorus (PO_4^{-3}) and ammonium (NH_4^+) concentrations. The concentrations of nitrogen and phosphorus released increased at higher S/I ratios. Both concentrations, of nitrogen and phosphorus, were higher in the assays with a thermal pre-treatment (Table 2).

Considering the use of VFA for the future production of PHA, the VFA profile obtained is an important parameter, besides the total amount of VFA produced. The most suitable conditions of the acidogenic fermentation of SS for VFA production are the use of a thermal pre-treatment in combination with 1 and 2 gVS substrate/gVS inoculum S/I ratios in batch experiments. However, considering the high DA obtained at high S/I ratios, even without applying thermal pre-treatment, it was decided to evaluate the acidogenic fermentation of SS in lab-scale reactor without any thermal pre-treatment to reduce the economic costs for a continuous process.

3.2 Lab-scale reactor

Based on the results obtained in batch experiments, a semi-continuous reactor was operated to determine the effect of two organic loading rates (OLR) on the VFA profile and on the DA. Besides, the microbial community present in the last operation stage of the reactor was analyzed to elucidate the microorganisms involved in the acidogenic fermentation of the SS.

3.2.1 Lab-scale reactor experiment

At the beginning of the experiment, an OLR of 900 mg COD L^{-1} d^{-1} and an HRT of 10 days were applied for one week. These data are not shown, considering it was the start- up of the reactor and the period of acclimation of the inoculum to the substrate. The first range of OLR applied was 1300–1600 mg COD L^{-1} d^{-1} obtaining VFA concentration in the range of 250–350 mg COD L^{-1} d^{-1} in the effluent of the reactor (Fig 3 a). The VFA profile was dominated by acetic, propionic, butyric and iso-valeric acids. Small amounts of iso-butyric and valeric acids were also observed in Figure. 3 (b). As observed in Table 3 can be observed that the maximum DA reached was 22 %. The second range of OLR tested was 2400-3500 mg COD L^{-1} d^{-1} and the VFA concentration achieved was in the range of 500-650 mg COD L^{-1} d^{-1} (Fig. 3a) in the effluent of the reactor. The VFA profile was dominated by acetic, propionic, butyric and iso-valeric acids (Fig. 3b). Considering the relative percentages of each acid respect to the total VFA concentration reached it can be observed that the butyric and n-valeric acids proportion increased with the OLR, as also previously reported in the batch assays. It can be observed in Table 3 that the maximum DA reached was 18.5 %. Therefore, it can be concluded that at higher OLR the DA decreases, which could be related to the overload of the substrate as well as to the accumulation of heavy metals and other inhibitory compounds of the DA.

Figure 3c shows the concentrations of ammonium and phosphorus achieved during the experiment. In the first range of OLR, the maximum concentrations of ammonium and phosphorus achieved were 662 mg L^{-1} and 62 mg L^{-1}, respectively. In the second range of OLR, the maximum concentrations of ammonium and phosphorus achieved were 1100 mg L^{-1} and 80 mg L^{-1} , respectively.

In this study, the Acidification Yield (AY) reached in the semi-continuous reactor without continuous control of pH was higher than that reported in other studies. There are some published studies about the acidogenic fermentation of SS carried out at alkaline pH obtaining similar levels of VFA production (Table 4).

3.2.2 Bacterial community characterization

The microbial community present in the last stage of the reactor operation was investigated to identify the microorganisms potentially involved in the acidogenic fermentation of the sewage sludge. Additionally, a predictive analysis was performed to check the metabolic pathways possibly involved in the process. For the microbial analysis 60,000 reads per sample were used and, as evidenced by previous findings, (Campanaro et al., 2018b) it was high enough to explain the complexity of the AD microbiome.

The phyla with a relative abundance higher than 0.1% and representing more than 97% of the total number of reads analyzed are represented in Fig 4. Results show that *Proteobacteria (*37%), *Bacteroidetes (*33%*)* and *Firmicutes (*25%) are the most abundant phyla in the acidogenic fermentation of SS. *Actinobacteria* is the fourth phylum in terms of abundance and it represented only 2% of the total number of reads. These results are in accordance with those Campanaro et al. (2018a) who confirmed that *Proteobacteria, Bacteroidetes* and *Firmicutes* are the dominant phyla involved in the fermentation of the organic matter. Due to the relevance of the most abundant phyla,

their composition at lower taxonomic level was investigated and is depicted in Figure. 4. Additionally, their putative role is discussed more in detail below.

The phylum *Proteobacteria* is represented by three different taxonomic classes with similar abundance (*Alphaproteobacteria (37%), Betaproteobacteria (36%),* and *Gammaproteobacteria (24%)*), while *Deltaproteobacteria* represents only 2% of the phylum. Each one of the three most abundant classes are mainly represented by one single order (*Rhizobiales, Burkholderiales and Pseudomonadales,* respectively) (Fig. 4). The presence of *Alphaproteobacteria* has been previously associated with the degradation of carbohydrates (Chen et al., 2017), while *Betaproteobacteria* is the dominant class in protein-rich fermented secondary sludge reactors (Wu et al., 2015). Since in the present study the proportion of *Alphaproteobacteria* and *Betaproteobacteria* was very similar, it can be concluded that the type of sewage sludge strongly influences the balance among members of the *Proteobacteria* phylum. The phylum *Bacteroidetes* is dominated by two main orders, *Bacteroidales* (86%) and *Flavobacteriales* (13%) (Fig. 4). Species belonging to the phylum *Bacteroidetes* are well-known for their involvement in protein degradation (Kindaichi et al., 2004). The majority of proteolytic microorganisms are able to metabolize amino acids to produce NH_3 and VFA such as acetate, propionate and succinate (Rivière et al., 2009). Particularly, the order *Flavobacteriales* was found in anaerobic digesters treating wastewater with high nitrogen content, and this is due to their ability to grow at high ammonia concentrations (Guo et al., 2015). Regarding the phylum *Firmicutes* (Fig.4), it was mainly represented by the order *Clostridiales* (96% of the phylum). This taxon is mainly involved in the hydrolysis of complex organic matter, thanks to its ability to produce proteases, cellulases, lipases, and other extracellular enzymes (Levén et al., 2007) that provide the ability to efficiently use the substrates available in the anaerobic

digestion of the sewage sludge. These taxonomic results evidenced that the main phyla present in the reactor are related to the degradation of complex organic material like proteins and carbohydrates which are subsequently fermented to produce acetic, propionic, butyric and iso-valeric acids as main products.

The taxonomic assignment of the OTUs having relative abundance higher than 1% is reported in Figure 5a. The most abundant OTU (*Bacteroidales sp.*) can be assigned only at order level and it accounts for 25-30% of the reads depending on the sample. Similarity search of the OTU *Bacteroidales sp.* evidenced that it is distantly related (85% similarity) with *Prevotellamassilia timonensis*, which is a hemicellulose-decomposing bacteria present in the roots of different vegetables (Ueki et al., 2007). The second most abundant OTU was *Comamonas sp.* (10-11%). In decreasing order of abundance, it can be noted the presence of OTUs assigned to the genera *Psychrobacter (2.5-5%), Calmidomonas* (3-5%), *Methylocapsa* (3-4%), *Rubrivivax* (2-3.5%), *Acidaminococcus* (3-4%) and *Prevotella* (2%-2.5%).

A predictive functional analysis was applied in order to investigate the properties of the most abundant taxa previously assigned at genus level. This analysis was performed considering both COG classes and KEGG metabolic pathways (see section 2.5.2). As expected, results obtained using COG is representative of functions associated with the conversion of the sewage sludge into volatile fatty acids (VFA) during anaerobic digestion (Fig. 5b). For example, all the genera identified encode proteins involved in amino acids and carbohydrates transport and metabolism. Besides, to a lesser extent, other proteins are also involved in lipid transport and metabolism. Since COG categories represent very general functional processes, KEGG pathways and modules were analyzed in order to have a more detailed prediction of the pathways used for

crucial AD functions such as “carbohydrates fermentation”, “proteins degradation” and “VFA production”.

KEGG analysis (Fig 5c) confirmed the ability of the most abundant genera to ferment carbohydrates as well as proteins, a functional property revealed by the presence of genes related with glycolytic pathway and proteases. The presence of genes related with VFA production was also reported in all the genera except *Rhodobacter*. Due to the high concentration of proteins in the SS, it is expected that amino acid utilization pathways are widespread in the most abundant taxa. There are two main pathways for amino acids fermentation: Stickland reaction, which involves the coupled oxidation and reduction of amino acids to organic acids, and degradation of amino acids in cooperation with hydrogen-utilizing bacteria. Stickland pathway has been reported only in *Clostridium* species which are present in the reactor. On the other hand, KEGG analysis revealed that the most abundant OTUs assigned at genus level encodes enzymes involved in the Wood-Ljungdahl pathway of hydrogen-utilizing bacteria. It can be concluded, that both pathways for amino acids fermentation are involved in the fermentation of proteins during the fermentation of SS. *Comamonas* is the second most abundant genus and it is known from the literature that some species are able to perform denitrification under anaerobic conditions. This process was also previously reported during the AD of mixed sludge (Supaphol et al., 2011) suggesting that nitrate removal is crucial to keep low the nitrogen level. KEGG analysis confirmed the presence of the denitrification pathway in *Comamonas sp,* as well as in other genera present in the microbial community like *Pseudomonas sp, Rubrivax sp and Rhodobacter sp*. (Fig. 5c).

4. Conclusions

This study reports the effect of the use of a thermal pre-treatment at different S/I ratios on the acidogenic fermentation of SS. The positive effect of thermal pretreatment on the

acidification at low S/I ratios was demonstrated, meanwhile the effect on high S/I ratios was not significant. The influence of the OLR on the acidogenic fermentation of SS was demonstrated in lab-scale reactor obtaining a maximum of 22% DA at an OLR of 1300-1600 mg COD L^{-1} d^{-1}. The study of the microbial community reveals that the most abundant OTUs are involved in proteins and carbohydrates utilization.

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"E-supplementary data of this work can be found in online version of the paper"

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Table Captions

Table 1. Chemical characterization of SS with and without thermal pre-treatment.

Table 2. Mass balance, maximum degree of acidification (DA), odd to even ratio, final concentrations of ammonium and phosphorus achieved and VS/TS percentages for each condition tested.

Table 3. Effect of the OLR on the DA, on the ammonium and phosphorus concentration and on the Odd to Even Ratio.

Table 4. Comparison of the VFA production reported in this study with results selected from the literature.

Figure captions

Fig. 1. Acidogenic fermentation of SS at S/I ratios of 1, 2, 4 and 6 g VS substrate/g VS inoculum (S/I 1, S/I 2, S/I 4 and S/I 6) (a) without thermal pre-treatment and (b) with thermal pre-treatment. Error bars represent standard deviations.

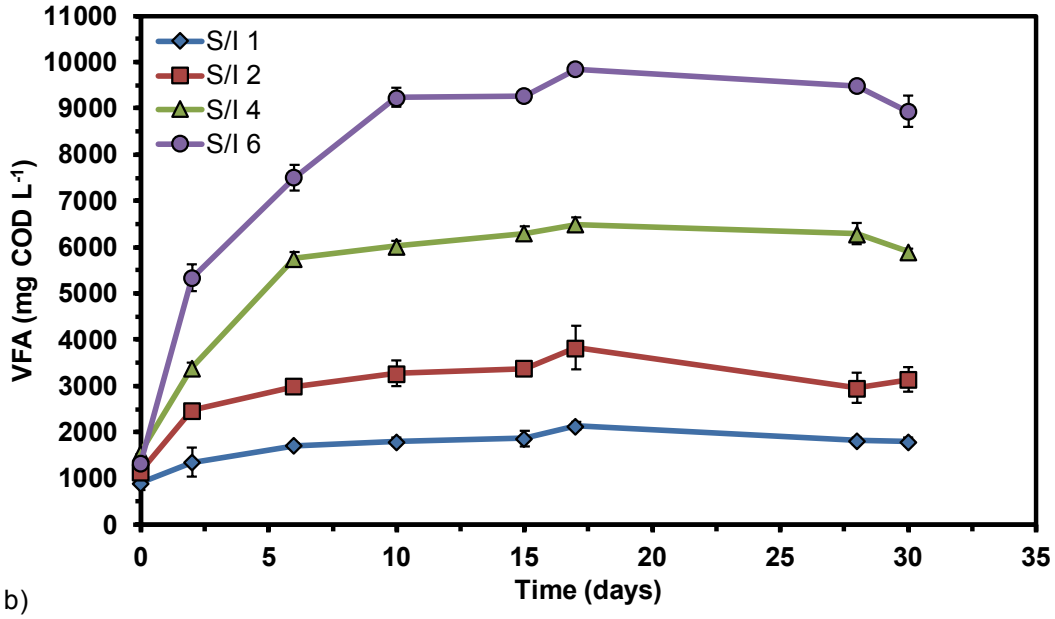
Fig. 2. Sewage Sludge VFA profile obtained at day of maximum DA at each S/I ratio (S/I 1, S/I 2, S/I 4 and S/I 6) (a) without thermal pre-treatment and (b) with thermal pre-treatment.

Fig. 3. Effect of the organic loading rate on the (a) concentration of VFA in the effluent as mg COD per day, (b) VFA profile and (c) NH_4^+ and PO_4^{-3} in an acidogenic reactor operating in a semi-continuous mode.

Fig. 4. The relative abundance of the phyla *Proteobacteria*, *Bacteroidetes* and *Firmicutes* at class and order level.

Fig. 5. (a) Relative abundance of OTUs at the steady state of the reactor. Taxonomic assignment of OTUs is reported in the right part of the figure; “others” refer to the OTUs having abundance lower than 1%. (b) Comparison between the numbers of genes belonging to COG categories.

a)



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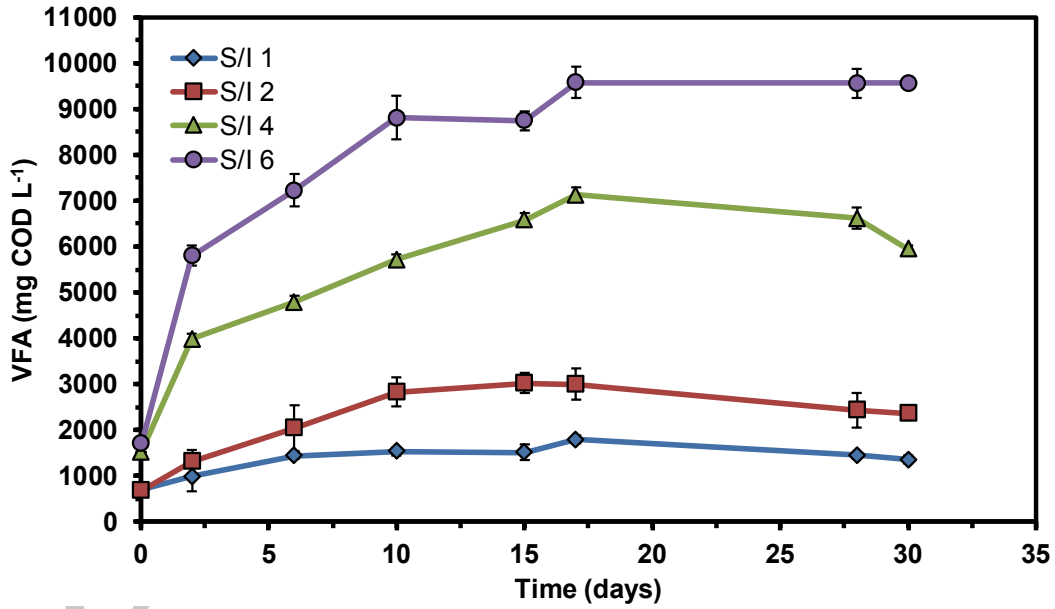


Fig. 1

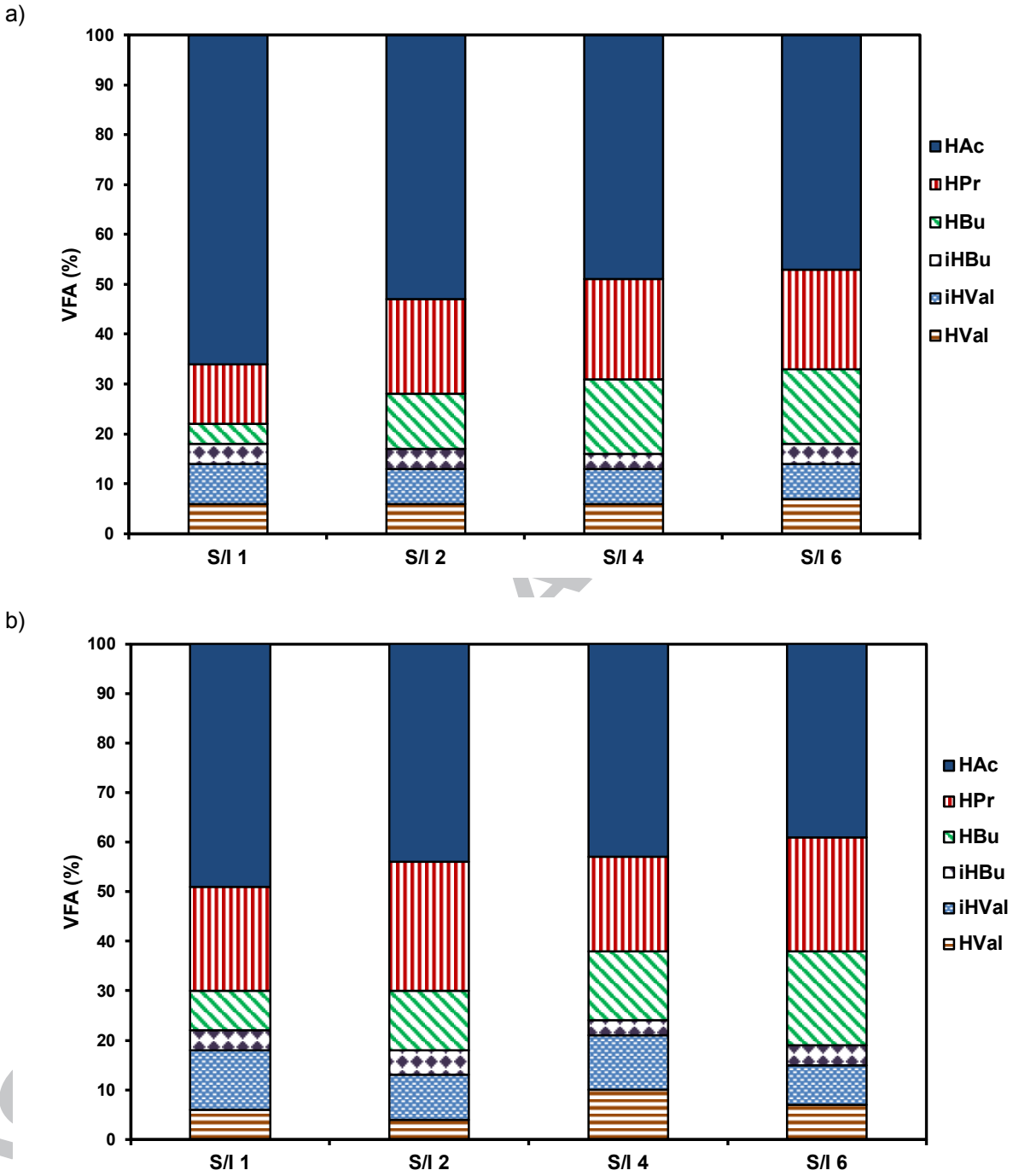
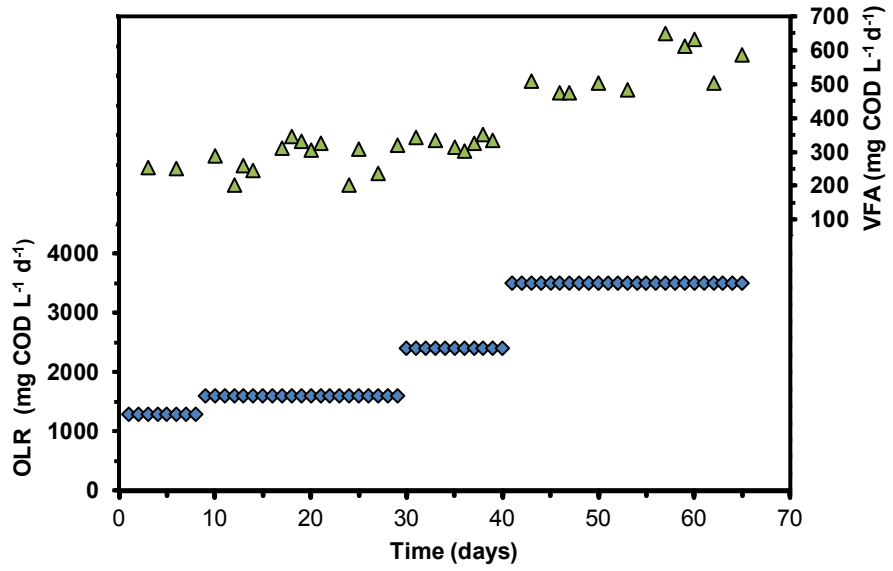


Fig. 2

a)



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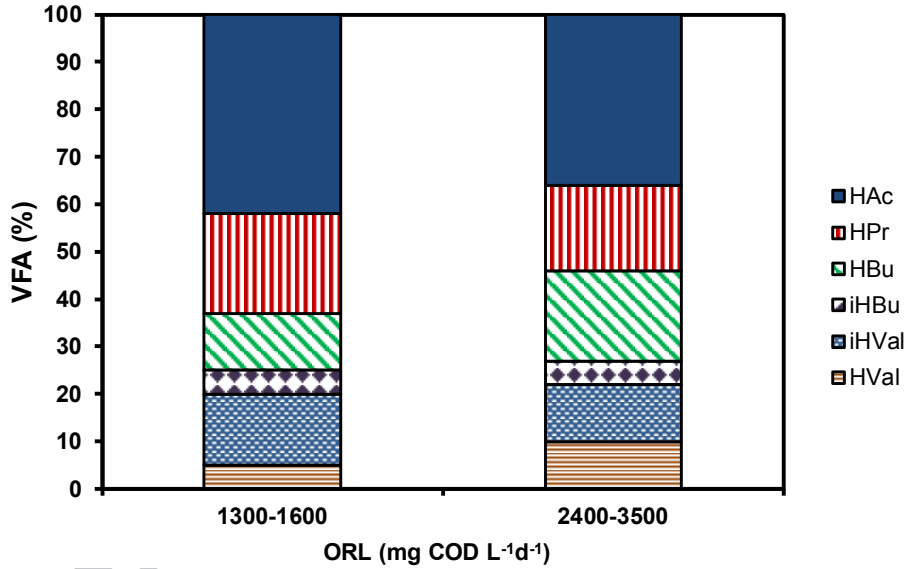

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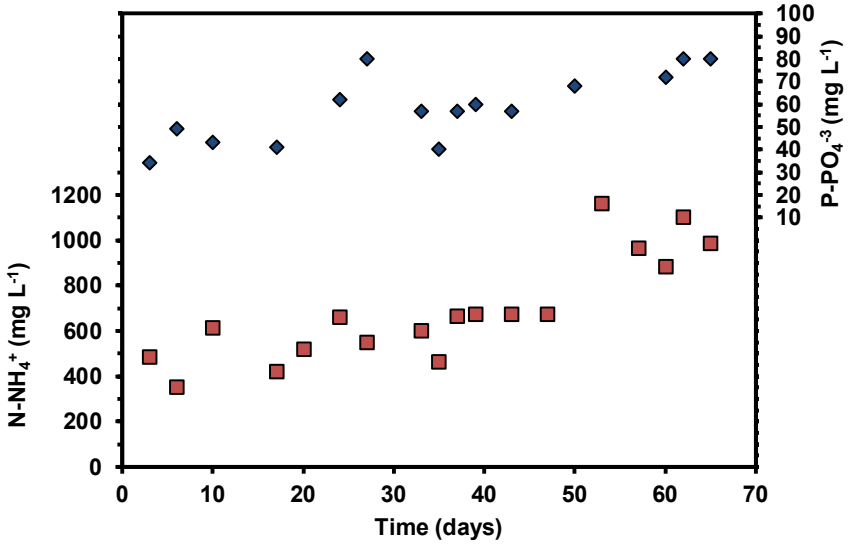

Fig. 3

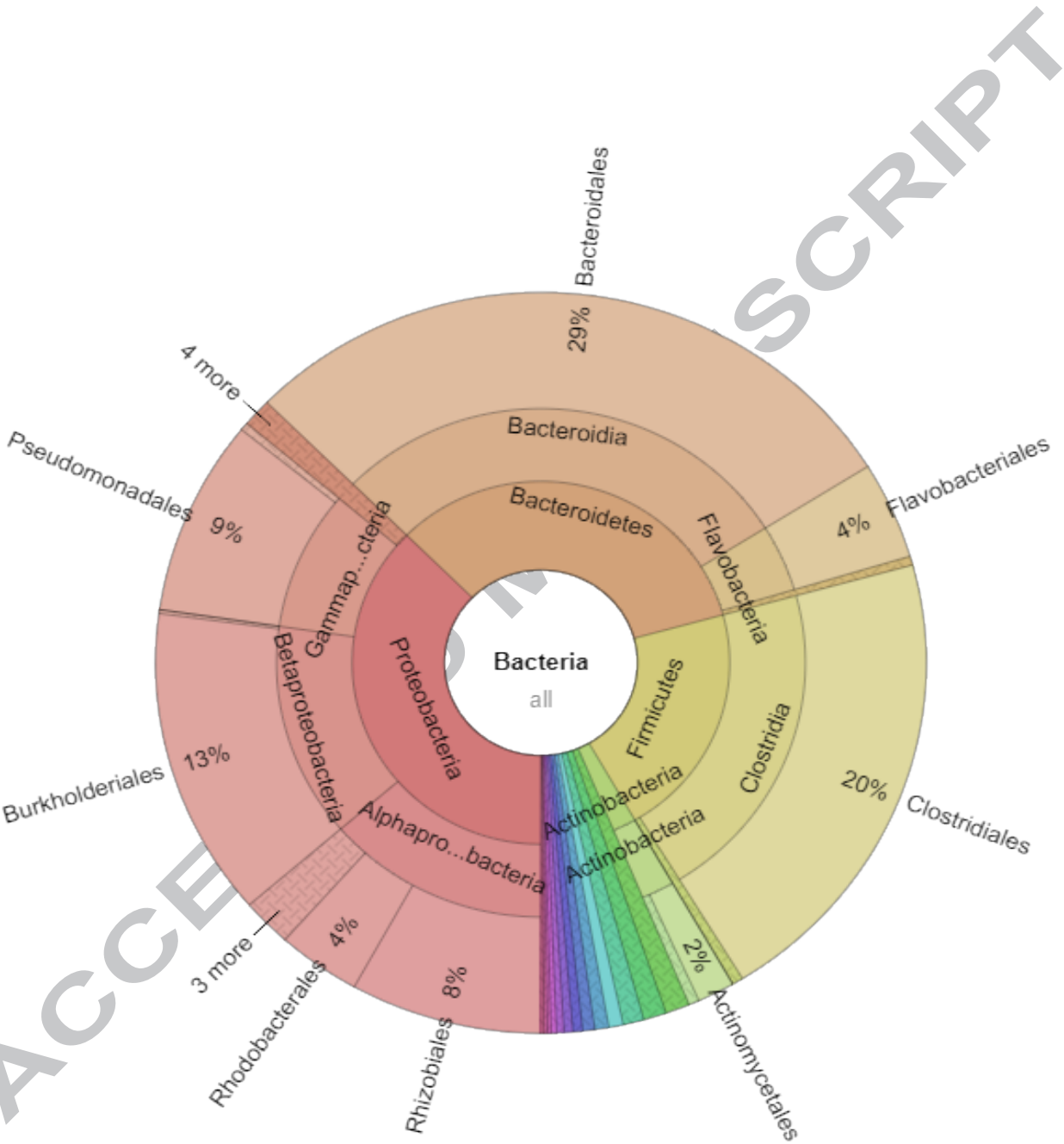

Fig.4

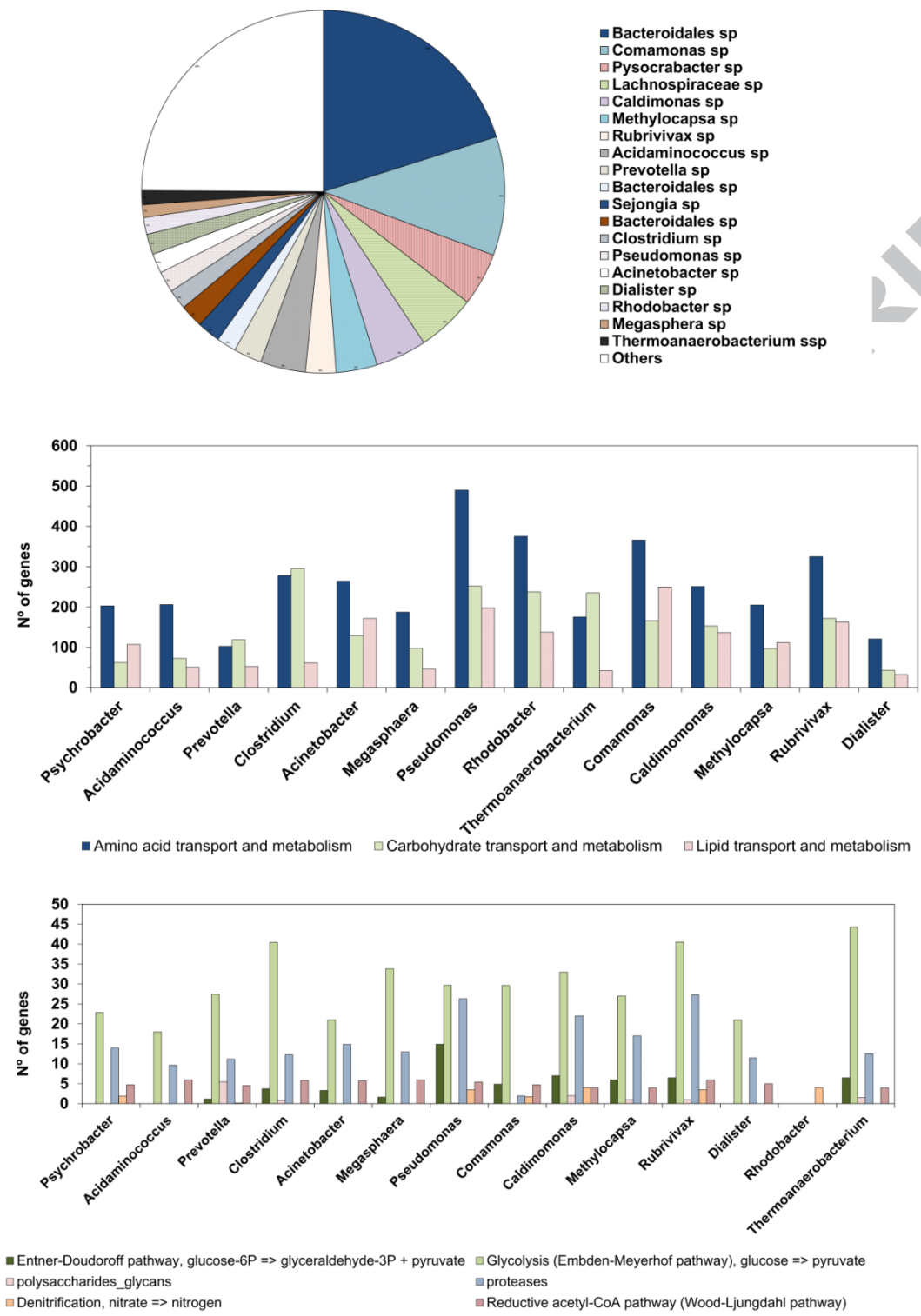

Fig. 5

Table 1. Chemical characterization of SS with and without thermal pre-treatment.

S/I ratio	TS (g L^{-1})	VS (g L^{-1})	VS/TS (%)	tCOD (g L^{-1})	sCOD (g L^{-1})	N-NH$_4^+$ (mg L^{-1})	P-PO$_4^{-3}$ (mg L^{-1})	pH
SS without pretreatment	48.80 ± 2.15	38.42 ± 1.74	79	79.49 ± 2.51	17.02 ± 0.18	2184 ± 84.6	58.7 ± 1.9	5.6
1	4183 ± 251	2353 ± 94	56	36 ± 3	0.21	189 ± 11	10.8 ± 1.7	
SS with pretreatment	53.02 ± 4.58	41.93 ± 3.58	79	76.44 ± 0.36	19.68 ± 0.54	3184 ± 47.3	49.4 ± 2.5	5.6
2	8426 ± 251	3824 ± 421		36 ± 5	0.34		370 ± 32	17 ± 1.2
4	16735 ± 251	6620 ± 346		35 ± 3	0.37		437 ± 37	35.7 ± 4.4
6	25102 ± 251	9846 ± 185		36 ± 1	0.37		934 ± 24	38.8 ± 1.0
1	4100 ± 360	2599 ± 241		45 ± 6	0.40		276 ± 19	12.7 ± 0.4
2	8103 ± 360	4405 ± 361		45 ± 5	0.46		642 ± 45	20.1 ± 1.7
4	16093 ± 360	7324 ± 75		41 ± 1	0.42		795 ± 69	44.8 ± 4.4
6	24139 ± 360	9575 ± 372		37 ± 1	0.43		1106 ± 6	40.9 ± 0.3
-	-	766 ± 26		-	-		50 ± 6	1.9 ± 0.21

Table 2. Mass balance, maximum degree of acidification (DA), odd to even ratio, final concentrations of ammonium and phosphorus achieved and VS/TS percentages for each condition tested.

Table 3. Effect of the OLR on the DA, on the ammonium and phosphorus concentration and on the Odd to Even Ratio.

Type of Sludge	OLR (mgCOD $L^{-1} d^{-1}$)	DA (%)	HRT (d)	pH	N-NH_4 (mg L^{-1})	Temperature (°C)	P-PO_4 (mg L^{-1})	Acidification yield (mg COD_VFA/ VSS added)	VFA profile	Odd to Even Ratio	Ref.
PS	**1300–1600** n.a	22 5		9	662 35		62 0.202		HAc >HPr>HBu	0.34	(Ahn and Speece, 2006)
PS	**2400-3500** n.a	18.5 5		9	1100	55	80 0.261		HAc> HPr>HBu	0.38	(Ahn and Speece, 2006)

WAS	6000	5	u.c	37	0.062	HAc> HPr>HBu	(Ucisik and Henze, 2008)
PS	10100	5	u.c	37	0.197	HPr> HAc>HBu	(Ucisik and Henze, 2008)
WAS thermal pretrated	3750	6	9	35	0.348	HAc> HPr>HVa>HBu	(Dong et al., 2016)
Mix Sludge	6600	6	9	55	0.423	HAc> HPr>iHVa>HBu	(Chen et al., 2017)
Sewage Sludge	1600	10	u.c	37	0.405	HAc> HPr=HBu >iHVa	This study

Table 4. Comparison of the VFA production reported in this study with results selected from the relevant literature.

PS: Primary Sludge WAS: Waste Activated Sludge u.c: uncontrolled

Highlights:

Acidogenic potential of the sewage sludge was evaluated in batch and continuous mode.

Thermal pre-treatment positively influenced the acidogenic yield at low S/I ratios.

Reactor process performance was studied evaluating two organic loading rates.

Acetic acid was the dominant product in all the conditions tested.

Exists a correlation between the microbial community and the VFA profile.