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Biomethanisation of sewage sludge: Sonication pretreatment and monitoring of microbial communities

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ABSTRACT

The improvement of mesophilic biomethanisation of recalcitrant sewage sludge derived from urban wastewater treatment through the application of a sonication pretreatment was evaluated in parallel in two pilot-scale anaerobic digesters (two biological replicates: reactors RA and RB). The valorisation process was monitored through a novel and holistic approach that related the biomethanisation yield, and its main batch operational parameters, with the abundance of archaeal and bacterial communities in the anaerobic inocula. Sonication allowed achieving a methane yield coefficient derived from sewage sludge of 240 ± 20 $mL_{STP}CH_4/g$ VS (volatile solids) at the load range of 0.8–4.0 g VS/L in both reactors. The process was more stable in reactor B, with a wider range of loads being allowed (up to 5.29 g VS/L). Monitoring the presence of Archaea in the mixing liquor revealed a variation in their abundance throughout the process which was directly related to the availability of organic matter and pH. Advanced metagenomic analysis showed the phylogenetic and functional diversity of the complex microbiome involved. While Bacteria were widely distributed in 35 phyla, Archaea fitted in only two. *Euryarchaeota* was the majoritarian archaeal phylum (99.5 %) and its more abundant families are linked to methanogenic metabolism. Functional analysis revealed several relevant metabolic pathways that followed similar trends in both reactors. "Methane metabolism" clearly diminished at the end of the process in concordance with the exhaust of methane generation, while "ABC transporters" or "two-component systems", involved in bacterial survival to changing environments, followed the

ABBREVIATIONS: AD, anaerobic digestion; Alk, alkalinity; COD, chemical oxygen demand; FS, total fixed solids; GAL, Glucose, sodium acetate and lactic acid solution; NGS, next generation sequencing; OTUs, operational taxonomic units; PCR, polymerase chain reactions; RA, reactor A; RB, reactor B; SRT, solid retention time; STP, standard temperature and pressure conditions; TOC, total soluble organic carbon; TS, total solids; TSN, total soluble nitrogen; VA, volatile acidity; VS, volatile solids; WAS, waste activated sludge; WWTP, wastewater treatment plant.

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opposite pattern. This integrated approach could help to increase the methanogenic valorisation of sewage sludge.

1. Introduction

Urban wastewaters are frequently treated through activated sludge process. This has been shown to be a convenient, easy to perform and very efficient procedure (Cella et al., 2016; Martín et al., 2015; Sahinkaya, 2015) but activated sludge also has disadvantages. The main problem of this process is the production of a large quantity of polluting sludge (sewage sludge or waste activated sludge (WAS)), with a complex composition that includes recalcitrant organic compounds, inorganic micropollutants, phosphorous, with frequent significant concentration of heavy metals (Ni, Cd, Zn, Hg, etc.), abundant pathogenic microorganisms, etc (Guan and Tian, 2023; Pilli et al., 2011).

Consequently, WAS derived from wastewater treatment plants (WWTP) must be treated carefully to avoid compromising human health and the environment. In fact, WAS management, which usually includes composting for agricultural application or landfill disposal, is subjected to a very strict legislation that has been tightened during the last years (Council Directive 86/278/EEC, version consolidated on 2022). The latest applicable regulation in the Andalusian community (Spain) (Junta de Andalucía, BOJA 156, 2018) includes different methodologies for the treatment of sludge, other than composting, which makes it possible to obtain treated sludge by reducing its fermentation power and its potential to cause discomfort and damage to health and the environment. The ultimate objective pursued by these regulations is the sanitisation of the sludge derived from WWTP.

When evaluating the sustainability of the activated sludge process, organic carbon and nutrient efficient removal are key factors to be considered, as well as handling of WAS (Motlagh and Goel, 2014). To manage the large quantities of WAS produced, anaerobic digestion (AD) appears as a promising technology with many associated advantages, such as: mass and pathogen reduction, odour removal and energy recovery (Di Capua et al., 2020). Specifically, AD produces renewable biogas, a mixture of methane and carbon dioxide (with traces of other gases), which after purification can be transformed into highly demanded energy, *e.g.*, heat or electricity. Considering that the activated sludge process requires marked consumption of electrical energy for aeration of the bioreactor, and the increasing costs of electricity, AD emerges as a suitable procedure for renewable energy generation, also improving the carbon footprint. Gikas (2017) estimated energy requirements of 0.057 $kWh/m^3_{inlet\ wastewater}$ for complete wastewater treatment, and 0.087 kWh/m^3 when UV disinfection was implemented. However, the methane yield derived from AD of WAS is usually low, owed to the poor and slow volatile solids (VS) removal of the sludge, which leads to long solid retention times (SRT) that determine the size of the digesters and, thus, investment and operational costs (Bandelin et al., 2020; Pilli et al., 2011). WAS presents dual characteristics of "resource" and "pollution". While it enriches nutrients from wastewater, it also contains a portion of toxic substances. The VS removal of WAS is usually hindered by the presence of significant concentrations of heavy metals, nitrogen and emerging pollutants. Moreover, when the biodegradable organic matter content of sludge is low, it affects the efficiency of resource utilization and energy recovery. Specifically, wastewater treatment plants where prolonged aeration activated sludge is implemented generate highly stable WAS, which reduces subsequent VS removal (Guan and Tian, 2023). Those drawbacks might be palliated by applying different types of pretreatments to WAS before being subjected to AD, such as: sonication (Delmas et al., 2015; Martín et al., 2015), combined alkaline and ultrasonic pretreatment (Tian et al., 2015), combined acid and ultrasonic pretreatment (Sahinkaya, 2015), thermal pretreatment, microwave (Appels et al., 2013), freezing and thawing (Machnicka and Grübel, 2023), etc. These pretreatments can enhance the AD process by increasing the solubilization of WAS, leading to different effects depending on which one is applied (Cella et al., 2016). Frequently, the pretreatments pursue to improve the hydrolytic step of the AD, increasing the hydrolysis rate and the VS removal of WAS (Gonzalez et al., 2018). Pilli et al. (2011) reported that ultrasonication is a very effective mechanical pretreatment method to enhance the biodegradability of the sludge, and it would be very useful to all wastewater treatment plants in treating and disposing sewage sludge. Ultrasonication enhances the sludge digestibility by disrupting the physical, chemical and biological properties of the sludge. The full-scale implementation of ultrasonication have demonstrated that there might be 50 % increase in the biogas generation. In addition, the evaluation of energy balance showed that the average ratio of the net energy gain to electric consumed by the ultrasound device is 2.5. Further studies reported interesting results derived from the combination of sonication and AD of WAS, achieving VS removal values as high as 81 %, and a methane production rate of 1270 $L_{STP}/m^3 \cdot d$ (Martín et al., 2015). As reported by Bandelin et al. (2020), a significant reduction in viscosity of up to 60 % was observed after sonication of digested sludge with low energy inputs. The study, therefore, demonstrates that the choice of the most suitable sonication system essentially depends on the properties of the sludge to be sonicated. Consequently, further efforts are still required to improve the feasibility of the combined treatment.

AD is a complex microbial process, in which Bacteria and Archaea work in a coordinated and interdependent way to transform organic matter, in absence of oxygen, into biogas and a stabilised digestate. The anaerobic process takes place in three main steps: hydrolysis-acidogenesis, acetogenesis and methanogenesis. Deep knowledge on the microbial populations involved in AD might allow the optimization of the process with a view to promoting scaling-up, as well as the prediction of possible failures in the reactor (Rincón et al., 2013). In this context, the microbial communities involved in AD processes, when different substrates are treated, have been studied by different authors (Pampillón-González et al., 2017; Rincón et al., 2008; Wang et al., 2018). Broadly speaking, the microorganisms involved include hydrolytic bacteria that belong to different phyla, such as: *Firmicutes, Chloroflexi, Bacteroidetes, Proteobacteria*, and *Spirochaetes* (Nguyen et al., 2019). Acidogenic bacteria can be found in the phyla *Firmicutes, Actinobacteria, Proteobacteria* and *Bacteroidetes*. Acetogens, as *Syntrophomonas wolinii* and *Syntrophomonas wolfei,* among others, are usually linked to AD (Liu et al.,

2021), together with homoacetogenic species, as those of the genera *Acetogenium, Acetobacterium, Acetoanaerobium, Butyribacterium, Eubacterium, Clostridium*, and *Pelobacter* (Archer and Kirsop, 1990; Liu et al., 2021). Methanogens are obligate anaerobes and thus extremely sensitive to oxygen. In AD, they usually belong to the archaeal orders *Methanococcales*, *Methanomicrobiales* and *Methanosarcinales* (Nguyen et al., 2019; Wang et al., 2018).

Nevertheless, monitoring microbial communities in AD is substantially evolving from traditional visual techniques, *e.g.*, green fluorescent protein (Ozbakir et al., 2020), flavin-mononucleotide based fluorescent proteins (Drepper et al., 2007), SNAP-tag and Halo-tag (Chia et al., 2019), etc., to molecular techniques based on rDNA gene identification (from polymerase chain reactions (PCR) to next generation sequencing (NGS)). These molecular techniques are currently the most used to monitor the microbial communities present in the digesters. 16 S rDNA sequencing has been a relevant advance for the identification of prokaryotes with unusual phenotypes, slow growing or uncultivable profiles. Nevertheless, sometimes simple taxonomic identifications do not provide enough information of how the microbial community works, and there is an increasing tendency to use metagenomic functionality approaches to understand these systems (Ordaz-Diaz and Bailon-Salas, 2020). In this sense, "*understanding how individual species within communities interact with others and their environment is important for improving performance and potential applications of an inherently green technology*" (Innard and Chong, 2022). However, when compared with bacterial rDNA genes, the primers available for the amplification of archaeal 16 S rDNA genes are not efficient enough to amplify certain linages, which limits convenient evaluation of archaeal diversity (Bahram et al., 2019; Eloe-Fadrosh et al., 2016). For that reason, Bahram et al. (2019) designed and validated several novel primer pairs based on up-to-date reference genes, thus achieving a sequencing coverage which surpassed commonly used primers. These new degenerate primers targeted 16 S subregions from 250 to 1500 base pairs, uncovering a broad phylogenetic diversity of Archaea in which metabarcoding bypassed specific archaeal lineages.

In this work, microbial populations (Bacteria and Archaea) participating in the AD process of sonicated WAS were studied. Two independent anaerobic digesters, working in parallel (biological replicates) at decreasing inoculum/substrate ratios (I/S), were evaluated. The evolution of the microbial populations with the successive increasing organic loads was assessed in both reactors using advanced techniques for microbial communities' identification and primers of the latest generation. Furthermore, the phylogenetic and functional diversity of the microbiome in WAS and their evolution along the biomethanisation process were inferred using Tax4Fun and FAPROTAX that predict the functional capabilities of complex microbial communities. In this novel study, methane yield and batch operational parameters of AD were also related to the changes in the microbial populations (Bacteria/Archaea ratio) detected through metagenomics analysis, focusing on the relationship between the microbial communities and the AD performance.

2. Materials & methods

2.1. Sewage sludge

The sludge used as substrate in this research study derived from the aerobic treatment carried out in the urban WWTP of Puente Genil (Córdoba, Spain). Specifically, sludge derived from the primary and secondary settling tanks, whose streams were unified and centrifuged after being subjected to coagulation-flocculation with polyelectrolyte. Table 1 shows the main physical-chemical characteristics of WAS, expressed in dry basis (initial moisture 86.8 ± 0.7 %).

2.2. Experimental set-up

2.2.1. Sonication

"WAS" was subjected to sonication using a 6.0-L ultrasonic bath (Selecta P. 3000513), at 150 W, atmospheric pressure and 298 K, while the experimental pretreatment time was fixed at 45 min, as reported by Martín et al. (2015) and in accordance with the results reported by Feng et al. (2009). The pretreatment was carried out in closed 0.25-L NORMAX bottles inoculated with 30 g of wet substrate each. Manual agitation of the bottles was periodically carried out to avoid temperature gradients inside them. Subsequently, the bottles were first left on a workbench to cool at room temperature and then stored at 4ºC to avoid the release of volatile compounds before being fed into the anaerobic digesters. The sonication process led to significant changes in the variables related to the solubility of carbonaceous and nitrogenous matter. The main results (in dry basis) were as follow: total soluble organic carbon (TOC), 33 ± 2 g

Table 1
Physical-chemical characterisation of WAS (dry basis).

	Mean value ± Standard deviation
pH	7.70 ± 0.05
Alkalinity (g $CaCO_3$/kg)	110 ± 5
Volatile acidity (g CH_3COOH/kg)	23 ± 2
COD (g/kg)	1195 ± 20
VS (g/kg)	690 ± 15
FS (g/kg)	310 ± 5
N-NH_4^+ (g/kg)	62 ± 4
P_{total} (g/kg)	35 ± 2
TOC (g/kg)	18 ± 1
TSN (g/kg)	11 ± 1

TOC/kg; total soluble nitrogen (TSN), 17 ± 1 g TSN/kg; TOC:TSN ratio ~ 2; and volatile acidity (VA), 33 ± 2 g acetic acid/kg. No significant changes were observed in humidity, pH, alkalinity (Alk), total solids (TS), or volatile solids (VS). Specifically, the pre-treatment procedure allowed the breakage of VS in a closed system, with the consequent maintenance of their concentration and that of TS, as they did not leave the NORMAX bottles (Zielewicz, 2016). Furthermore, due to the presence of a high concentration of alkalinity in the sludge and the consequent predominance of a buffered medium, the generation of acidic compounds throughout the sonication pretreatment did not lead to significant variation of pH and/or alkalinity.

2.2.2. Biomethanisation

Two mesophilic 3.5-L Pyrex reactors were used to carry out the biomethanisation assays of the sonicated WAS, called reactor A (RA) and reactor B (RB). The digesters operated under complete mixing conditions and were fed in batch mode. The same procedures were carried out in both reactors, which were considered as two biological replicates where the same pretreated "WAS" was fed. Four connections at the top of the systems allowed feeding the reactors with WAS, as well as the ventilation of the biogas, injection of gaseous nitrogen to maintain the anaerobic conditions when samples were withdrawn, and effluent removal. A magnetic stirrer was used to homogenise the mixing liquor in each reactor, while a thermostatic jacket containing water allowed maintaining their temperature within the mesophilic range. Furthermore, 2-L Boyle-Mariotte reservoirs containing water were used to measure the volume of methane produced in each reactor. Carbon dioxide was previously absorbed in a 6 N concentrated sodium hydroxide solution located in closed bubblers between the digesters and the water reservoirs. Finally, a test tube was used to measure the volume of water displaced by methane at each reservoir. The effect of water steam pressure was considered in the measured volume, which was expressed at 0ºC and 1 atm (standard temperature and pressure (STP) conditions).

Granular sludge obtained from a full-scale digester used to treat brewery wastewater (Heineken S.A., Jaen, Spain) was used as inoculum (pH, 7.84 ± 0.08; VS, 65.30 ± 50 mg/L; methane production rate, 154 ± 5 mL_{STP} CH_4/g COD·h). Each reactor was loaded with the granular sludge as inoculum at a concentration of 7 g VS/L. Two solutions containing micronutrients and trace elements essential for microbial growth and metabolism were also added when the inoculum was loaded (Serrano et al., 2014). The initial bio-stimulation of the inoculum was carried out by adding a highly biodegradable solution (named GAL) composed of lactic acid, sodium acetate and glucose at concentrations of 21 mL/L, 25 g/L and 50 g/L, respectively.

The organic load added with GAL to the anaerobic digesters was gradually increased from 0.50 g COD/L to 2.00 g COD/L (10-days period). Subsequently, the acclimatisation of the inoculum to "WAS" was carried out by mixing waste and GAL. Specifically, the concentration of WAS in the mixture was increased from 0.2 g VS/L to 0.8 g VS/L in four steps. The time required to maximise waste biodegradation and biogas generation during the bio-stimulation (with GAL) and acclimatisation (GAL + WAS) was found to be lower than 68 hours in all cases. Finally, the reactors were fed with WAS as the sole substrate. An initial biomethanisation test with untreated "WAS" was carried out to compare the results obtained with those derived from the treatment of sonicated waste. Specifically, the reactors were loaded with four loads of untreated WAS at 1.0 g VS/L. Subsequently, increasing loads with pre-treated WAS were fed within the range of 0.8–5.3 g VS/L (equivalent to I/S ratios of 8.6–1.3 g VS/g VS). Samples were withdrawn and analysed before adding each load, while the volume of methane gas was monitored periodically throughout the biomethanisation process. Samples were subjected to centrifugation (2000 rpm) in order to recirculate the solid fraction of the digestate to the anaerobic reactors. Each experiment lasted the time required to exhaust methane generation and maximise the removal of biodegradable WAS (<110 hours). The whole process (bio-stimulation + acclimatisation + WAS biomethanisation) lasted for 117 days.

Fig. 1 shows the range of organic loads carried out in RA and RB with sonicated WAS throughout the experimental time, along with the corresponding values of the I/S ratio. As can be seen, the addition of loads was gradually increased following the same pattern in both reactors.

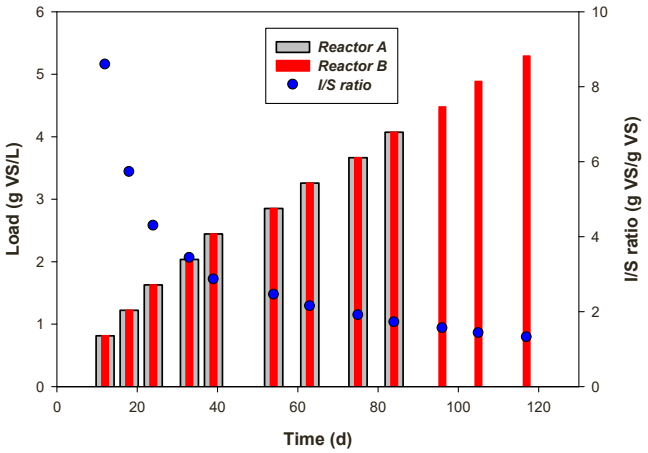

Fig. 1. Range of organic loads added to RA and RB and variation of the inoculum/substrate ratios (I/S) throughout the experimental time.

2.3. Chemical analyses

The following parameters were determined in WAS: total chemical oxygen demand (COD, g O_2/kg), total solids (TS, g/kg), total fixed solids (FS, g/kg), total volatile solids (VS, g/kg), total phosphorus (P_{total}, mg/kg), and ammoniacal nitrogen (N-NH_4^+, mg/kg). Volatile acidity (VA, g acetic acid/kg), pH, and alkalinity (Alk, g $CaCO_3$/kg) were also analysed to characterise the soluble fraction of the substrate. A Shimadzu TOC-V CSH/CSN total soluble organic carbon analyser was used to quantify the total soluble organic carbon (TOC, g/kg) and total soluble nitrogen (TSN, g/kg). In addition, the effluents of the reactors were characterised by monitoring the following variables: pH, COD (mg O_2/L), TS (mg/L), FS (mg/L), VS (mg/L), VA (mg acetic acid/L), and Alk (mg $CaCO_3$/L). All these analyses were carried out in accordance with the Standard Methods of the APHA (2023).

2.4. Metagenomic analysis

2.4.1. DNA isolation, cleaning and amplification

Microbial communities were analysed by molecular genomic techniques. Aliquot samples of 50 μL from the reactors were homogenised by pipetting and then nucleic acids were extracted following the genomic DNA purification protocol of the NucleoSpin Food DNA extraction kit (Macherey-Nagel, Düren, Germany). Different loads were selected in each reactor to monitor the evolution of the bacterial and archaeal microorganisms. In RA, 0.814, 1.629, 2.443 and 3.665 g VS/L were selected, while in RB, 0.814, 1.629, 2.850, 4.072 and 5.293 g VS/L were chosen. The inoculum and controls from the un-sonicated samples for both reactors were also included.

The presence of PCR inhibitors was checked by performing PCR tests using 16 S standard bacterial primers. No amplification was detected in any of the initial extracted samples. To clean the DNAs, each sample volume was increased to 400 μL using TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0), and then 40 μL NaCl 5 M and 400 μL phenol-chloroform-isoamyl alcohol (24:24:1) were added. Tubes were vortexed for 1 min and incubated on ice for 5 min. Phases were separated by centrifugation and the upper inorganic phases extracted to new tubes. The phenolisation procedure was repeated and a subsequent treatment with chloroform was used to eliminate all phenol residues. Finally, the inorganic gDNA extracts were precipitated with ethanol and resuspended in 20 μL of sterile mQ H_2O. Again, new PCR tests were performed but none of them amplified. Thus, a second purification step was carried out. Twenty μL of the isolates were run in a 0.8 % low melting agarose gel. Once the samples entered the gel, the fragments containing their gDNA were cut out, placed in Eppendorf tubes, and heated at 68°C for 20 minutes to melt the agarose. Each sample volume was again increased to 400 μL using TE buffer, phenolised and precipitated. The final pellet was resuspended in 20 μL of sterile mQ H_2O. PCR reactions with 16 S standard primers were performed again and all the samples amplified, indicating that there were no PCR inhibitors in the last cleaned samples.

2.4.2. Sequencing and bioinformatic analysis of 16 S amplicons

16 S rDNA was amplified from each sample using the primers 340 F/806rB (Bahram et al., 2018) and sequenced using the Ion S5 System (Thermo Fisher Scientific). This procedure, and further bioinformatic analyses were carried out at the Central Services for Analysis (SCAI) at the University of Córdoba (Genomic and Bioinformatic Units, respectively). The bioinformatics analyses are summarised as follows: Raw reads were processed to retain only high-quality sequences (Cutadapt v1.9, BBDuk v35.43). Briefly, sequencing adapters were first clipped, low-quality bases were trimmed (Phred score threshold > 20) and all reads with a length less than 100 nucleotides were filtered out. Reads quality assessment was carried out using FastQC software (v0.11.8) to evaluate the effect of every step of this process.

All further analyses were conducted using the filtered datasets and the QIIME open-reference OTU picking pipeline (Bolyen et al., 2019; Caporaso et al., 2010b). Sequence identifiers were adapted to the Illumina formatting, including the corresponding sample name within the sequence ID. The usearch61 method was employed to detect and filter out chimeric sequences; all non-chimeric sequences were then concatenated to be processed by the OTU picking procedure (Bolyen et al., 2019). Thus, those sequences passing the prefiltering step (> 60 % identity to the reference database Silva 132 (Quast et al., 2013), were searched following QIIME guidelines). The UCLUST algorithm was used to cluster OTUs at 99 % similarity (Edgar, 2010). PyNAST was used to filter out poor aligned OTUs (Caporaso et al., 2010a). Taxonomy was assigned to representative OTU sequences by UCLUST against SILVA database 132.

2.4.3. Functional analysis of the microbiome

The functional and metabolic capabilities of the microbial communities identified in Section 2.4.2 were predicted using the Tax4Fun open-source R package algorithm (Aßhauer et al., 2015) (http://tax4fun.gobics.de/). Tax4Fun uses an association matrix built from a BLASTN analysis in which 16 S rRNA gene sequences of all organisms in KEGG (Release 64.0) are searched against the SILVA database (SILVA_123_SSURef_Nr99, Release 115). Thus, the resulting OTUs after preprocessing and clustering our 16 S rDNA sequencing reads were assigned to reference sequences in the SILVA database.

The evolution of the different functionalities along the process in the reactors was analysed using the Genesis algorithm (Sturn et al., 2002). To do so, the abundances of the different metabolic pathways were normalised, and the distance measured employed was Pearson's correlation. A hierarchical clustering analysis was performed using complete linkage as the agglomeration rule.

A more detailed functional assignment of the microbiome was estimated using the FAPROTAX software (Phyton), as previously described (Gutiérrez et al., 2024).

2.5. Statistical analysis

Sigma-Plot (version 11.0) and Excel (version 2002) softwares were used to create graphs and perform the statistical analysis (mean value and standard deviation) of the experimental results.

3. Results & discussion

3.1. Stability of biomethanisation of WAS

WAS is a polluting and hazardous waste generated in WWTPs, which leads to well-known severe management problems. The optimal conditions described by Martín et al. (2015) (see Materials & Methods, Section 2.2.1.) were applied to carry out a sonication pretreatment of WAS, at laboratory scale, to reduce its recalcitrant nature to biodegradation under anaerobic conditions by increasing its solubilisation and the AD yield. A wide range of biomethanisation experiments were carried out in two reactors (biological replicates), in order to evaluate the suitability of sonication for improved WAS management, with a focus on the analysis of the microbiome in connection with the organic loads added.

The physical-chemical analyses confirmed that AD of pre-treated "WAS" was stable under the study conditions in a wide range of loads added to the reactors. However, although the experimental procedure followed in both reactors was the same, RA become inhibited after 84 d of experimentation (at organic load of 4.07 g VS/L), while RB withstood a wider range of loads (up to 117 days of experimentation; 5.29 g VS/L) (Fig. 1). This fact confirms that methanogenic microorganisms responsible for biogas production in both reactors could have been affected in different ways. The alteration of different physical-chemical and/or biochemical processes might influence the last stage of the degradation process of biodegradable organic matter.

Fig. 2 shows the variation in pH (left) and VA/Alk ratio (right) values with the different organic loads added to both reactors. No

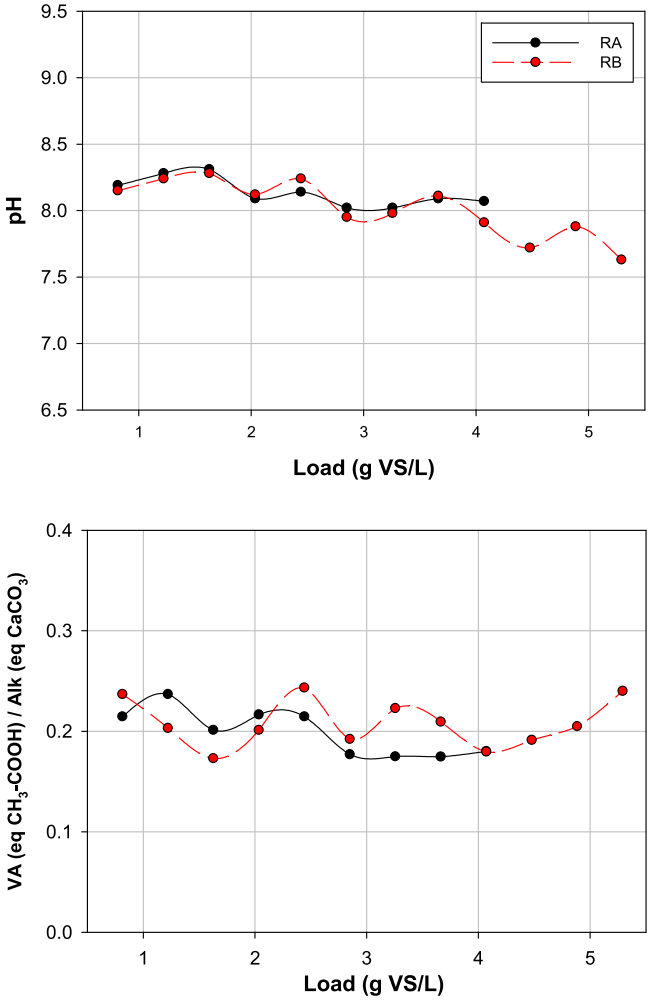

Fig. 2. Variation in pH values (left) and VA/Alk ratio (right) in the mixing liquor of the reactors with the organic load added to RA and RB.

marked differences were observed in the behaviour of RA and RB in terms of the variation of such physical-chemical variables, with the main difference being the range of organic loads added before inhibition occurred. In terms of pH, the reactors operated under stable conditions, considering that the most adequate range of pH in AD has been reported to be close to neutrality. However, it is worth noting that the optimal pH value in the mixing liquor of anaerobic digesters could be different for the diverse types of microorganisms involved in the process. Specifically, the optimal pH ranges between 7.2 and 8.4 for fermentative microorganisms, 7.0–7.2 for acetogenic bacteria and around 7.5 for methanogenic ones (Martí, 2006). In this study, no control was exercised over that variable. To monitor the stability of the AD process, it is necessary to evaluate effective early-warning indicators. The evaluation of the effectiveness of common variables, including biogas production, VA, Alk and/or the concentration of different forms of nitrogen showed that individual indicators might not provide a sufficiently early response to anticipate the digesters collapse. On the contrary, the combination of indicators such as the ratios CH_4/CO_2, CH_4/pH and CH_4/H_2S might be more sensitive to disturbance. Among the combined indicators, the VA/Alk ratio stands out as a simple auxiliary indicator with rapid detection, even at full scale (Zou et al., 2022). The VA/Alk ratio (eq CH_3COOH/eq $CaCO_3$) can be also used to measure process stability; specifically, when this ratio is lower than 0.3–0.4, the process is considered to operate favourably without the risk of acidification (Siciliano et al., 2019). The experimental values of the VA/Alk ratio were always found to be lower than 0.24, thus indicating that the process worked favourably under the study conditions in both reactors. Nearly optimal values were observed with the initial loads of raw WAS (1.0 g VS/L), while the mean values of such ratio during all the process were found to be 0.07 ± 0.02 (eq CH_3COOH/eq $CaCO_3$) (pH: 7.91 ± 0.03). Regarding Alk, the only notable difference between the reactors was that its concentration in RB was maintained and even slightly increased throughout the experimentation, while Alk levels decreased in RA at the load of 4.0 g VS/L. The final Alk of RA was 5400 mg $CaCO_3$/L, while it reached 7600 mg $CaCO_3$/L in RB. Furthermore, an increase over 80 % in the concentration of TOC, associated with the increment of approximately 40 % of VA, are in line with the results obtained in the AD of sonicated WAS.

In addition, it is worth noting that the pH value of sonicated "WAS" was slightly higher than the unpretreated WAS. Furthermore, the pretreatment increased the TSN concentration by 50 %. Nevertheless, the increase in the concentration of TOC was higher and thus the TOC/TSN ratio in pretreated WAS changed from 1.69 ± 0.03–2.10 ± 0.04. On the other hand, the relationship among TSN, temperature and pH has been widely described as an important variable affecting AD (Mutegoa et al., 2020). There is an equilibrium between free ammonia (NH_3) and ammonium species (NH_4^+), with free ammonia being toxic due to its ability to cross the cell membrane of Bacteria and Archaea and affect their internal pH. Methanogenic bacteria (*Euryarchaeota*) is the most sensitive group to high ammonia concentrations (>1800 mg/L) and the first one to be inhibited (Gallert et al., 1998; Krakat et al., 2017). In this study, while methane production was maintained, the average concentration of N-NH_4^+ in RA was found to be 550 mg/L, increasing to 1760 mg/L at the load of 4.0 g VS/L. The concentration of N-NH_4^+ observed in RB were within the same order of magnitude. However, tolerance to free ammonia and N-NH_4^+ is highly variable depending on the author (Bujoczek et al., 2000; Hansen et al., 1998; Siles et al., 2010). This fact demonstrate once again that the stability of the process cannot be followed by a single indicator (Zou et al., 2022).

3.2. Biomethanisation process yield

Fig. 3 shows the methane production ($mL_{STP}CH_4$/L) derived from AD of sonicated WAS, as a function of the organic load added to the reactors. As can be concluded from the slope of the linear fitting line, the mean methane yield was found to be 240 ± 20 $mL_{STP}CH_4$/g VS, within the loads of 0.8–4.0 g VS/L (where non-inhibitory effects were observed) in both reactors. It is important to note the decrease in the yield at the highest load added in RA (4.07 g VS/L) which could probably be due to the inhibition of the methanogenic activity. In contrast, a wider range of loads (up to 5.29 g VS/L) was allowed in RB without inhibition symptoms. The increase in methane production with the last load added to RB might be a consequence of the increment in biodegradable organic matter available

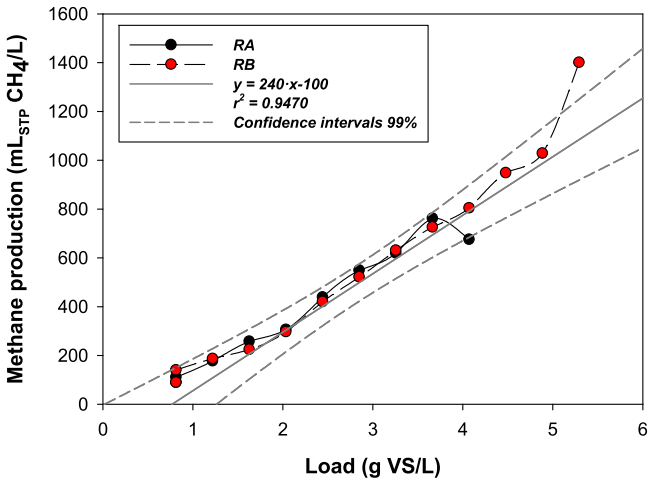

Fig. 3. Methane production yield from sonicated WAS at different organic loads added to RA and RB.

because of cellular lysis taking place under stressing conditions in the mixing liquor of the anaerobic digester (Liu et al., 2024). However, the application of pretreatments to hydrolyse complex substrates might lead to increased degradation of nitrogenous organic compounds. N-NH_4^+ from protein degradation could penetrate cell membranes and inhibit the bioactivity of microorganisms, thus affecting the performance of methanogenesis (Gallert et al., 1998; Krakat et al., 2017). Therefore, the efficiency of the process not only depends on the presence of free ammonia, but also on the different soluble and bioavailable nitrogenous compounds after the pretreatment of WAS (Liu et al., 2024).

Regarding the methane production yield derived from AD of raw WAS, it was found to be 89 and 90 $mL_{STP}CH_4/g$ VS in RA and RB, respectively, because of its well-known low VS removal. However, the sonication pretreatment improved experimental methane yield by around 167 % (from 90 to 240 $mL_{STP}CH_4/g$ VS), with the consequent environmental, economic and social benefits. Sonication allowed organic matter to be solubilised (TOC increased by 80 %), especially in the form of VA (increased by 40 %), which in general is easily degraded by the microbial communities involved in AD and leads to a consequent increase in the methane production yield. Specifically, the VS removal of sonicated "WAS" was found to be as high as 81 % (in VS) in both reactors, with the non-biodegraded fraction remaining in the digestate under the study conditions. As the concentration of nitrogen and phosphorus in "WAS" was high, with a significant fraction becoming soluble after the pretreatment, such nutrients accumulated in the digestate with recalcitrant organic matter. This fact might be advantageous if such effluent was valorised as agricultural amendment (Leite et al., 2023).

3.3. Global microbiome analysis

The microbiome of the initial inoculum and of the mixing liquor of both anaerobic reactors was analysed by 16 S rDNA sequencing. DNA was isolated, cleaned and amplified at different times after the initial loading using archaeal specific primers, as described in the Material and Methods section. All the amplified fragments were sequenced. The samples from the anaerobic reactors were firstly analysed. Processed reads were filtered to avoid spurious reads or artifacts within the sequence collections to finally obtain 1,200,709 sequences, representing 735,253 unique reads. One of the main problems for analysing these sequences is the absence of complete databases, since most microorganisms in complex/stressed environments remain unknown. For that reason, a bioinformatic procedure was developed to enrich the identification of the sequences. Briefly, the operational taxonomic units (OTUs) were firstly searched against RNA Silva database, the best available dataset for ribosomal DNA, and roughly only 10 % were identified (72,454 sequences) yielding 2579 identifications. A percentage of 25 % from the non-Silva-matching 662,799 remaining sequences were used to generate an in-house database. Thus, 36,431 new clusters were generated as reference from the 165,626 subsampled sequences (139,359 dereplicated). This second picking procedure yielded 32,362 new potential OTUs. The other 101,779 not-matching sequences were subjected to a *de-novo* (centroid) clustering, to identify 78,025 further putative OTUs. To filter out less supported or spurious OTUs, a minimum coverage filter of 2 was applied, retaining 35,522 OTUs, of which 28,965 OTUs were finally selected, once PyNAST aligning filtering was applied. Finally, the inoculum was analysed following the same process. The abundance of the selected OTUs in the different samples analysed is shown in Supplementary Table A. Using those databases, the 16 S sequencing analysis identified more than 850,000 OTUs from RA and RB at the different reactor loads. Krona diagrams representing the relative proportion of microorganisms, both Archaea and Bacteria, throughout the operation of the two reactors are shown in Supplementary Figure 1.

Unlike the samples from the reactors, the initial inoculum contains almost exclusively Archaea with more than 95 % of all counts (Supplementary Table A and Supplementary Figure 1). The vast majority of the Archaea belongs to the *Euryarchaeota* phylum, and most of its families are related to methanogenesis (*e.g.*, *Methanosaetaceae*, *Methanoregulaceae*, *Methanobacteriaceae*), which constitute an adequate inoculum for the subsequent operation of the reactors. Fig. 4 shows the total reads related to Archaea in the mixing liquor of both anaerobic digesters. As can be seen, the value was always higher in RB, although in both cases a marked decrease was observed

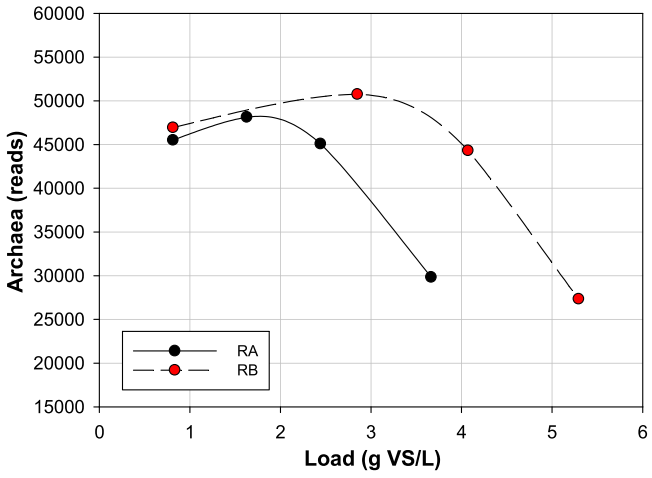

Fig. 4. Variation in the number of reads associated to the presence of Archaea in the mixing liquor of RA and RB, with the organic load added in both reactors.

for loads above 3.00 g VS/L, with the decline starting at a higher load in RB than in RA. It is worth noting that the number of reads for Archaea after biomethanisation of untreated "WAS" was found to be 18,593 and 42,210 for RA and RB, respectively (Supplementary Table A). Consequently, sonication pretreatment allowed promoting the growth of such type of important methane-producing microorganisms. Furthermore, the presence of Archaea in both reactors was successfully related to the variation of some important physical-chemical variables, such as the concentration of soluble organic matter and pH (r^2: 0.9765) (Fig. 5). As shown, the maximal presence of Archaea was found to be achieved at soluble COD values around 1100 mg/L and pH within the range of 7.91–8.15. This trend could explain the fact that methane production ceased at loads higher than 4.0 g VS/L in RA, even though pH values do not deviate from the optimal range.

Specifically, the identified Archaea fitted into two major phyla, *Euryarchaeota* and *Crenarchaeota*, the former being by far the most prominent with 418,456 total reads, as more than 99.5 % belonged to that phylum (Supplementary Table A). During the process, no major differences at the phylum level could be globally observed between the two reactors under study.

Although the primers used were designed for archaeal identification, approximately half (436,283 reads) of the 16 S sequences, of the total number of microorganisms identified (855,639 reads), were assigned to the Bacteria kingdom (Supplementary Table A). The identified Bacteria belonged to 35 different phyla, being *Choroflexi* the most abundant with 257,571 total reads, followed by *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Tenericutes* and *Synergistetes* with approximately 77-, 38-, 18-, 16- and 11-thousand reads (Supplementary Table A). When compared, close to 1.8 times more Bacteria were found in RB (279,469 reads) than in RA (156,814 reads). In general terms, the higher total reads associated to the presence of Bacteria in RB were related to the load added to the reactor and the variation in pH (Fig. 6). As can be seen, an increasing trend in Bacteria occurred with decreasing pH values at the highest organic loads (r^2: 0.8909). This fact might be a consequence of accumulation of non-degraded bacteria contained in sonicated WAS. Bacteria reads were the result of the sum of the bacteria contained in the initial inoculum and the substrate added throughout the experimental process. But the increase between the non-degraded bacteria and the added substrate was observed to be proportional.

The family level was the lowest taxonomical level analysed in this work, as inferior levels gave many uncertainties. Almost 250 families of Bacteria were identified but the *Anaerolineaceae* family (of the major bacterial phylum *Chloroflexi*) clearly predominated as it accounted for approximately half of the reads (234,791 reads; Supplementary Table A). Members of this family have been previously described as very abundant in the WAS from anaerobic digester tanks (McIlroy et al., 2017). Other relatively abundant bacterial families were found to be *Caldilineaceae*, *Gracilibacteraceae*, *Synergistaceae*, *Rikenellaceae*, *Syntrophobacteraceae*, *Christensenellaceae* and *Rhodobacteraceae*, that ranged in a decreasing order from 4.6 % to 1.0 % of all bacterial reads. Thirteen archaeal families were identified, but only 5 of them corresponded to more than 1 % of all archaeal reads: the acetoclastic *Methanosaetaceae*, the hydrogenothrophics *Methanoregulaceae* and *Methanobacteriaceae*, *Methanomassiliicoccaceae* (that cannot be classed into the typical methanogenic groups (Kröninger et al., 2017)), and the hydrogenothrophic *Methanomicrobiaceae*, with approximately 37, 34, 24, 2 and 1 %, respectively (Supplementary Table A). All these relatively abundant Archaea have been linked to methanogenic metabolism (Wen et al., 2017). *Methanosaetaceae* family members contain cytochromes and methanophenazine, and show a broad substrate spectrum (Thauer et al., 2008).

Both reactors presented similar familiar taxonomic patterns although with slight differences. Comparing the relative abundance of the different archaeal families, the main difference between reactors was that the most common family in RA was *Methanoregulaceae*, while in RB it was found to be *Methanosaetaceae*, but with very similar percentages (ranging between 24.8 % and 29.6 %). Regarding

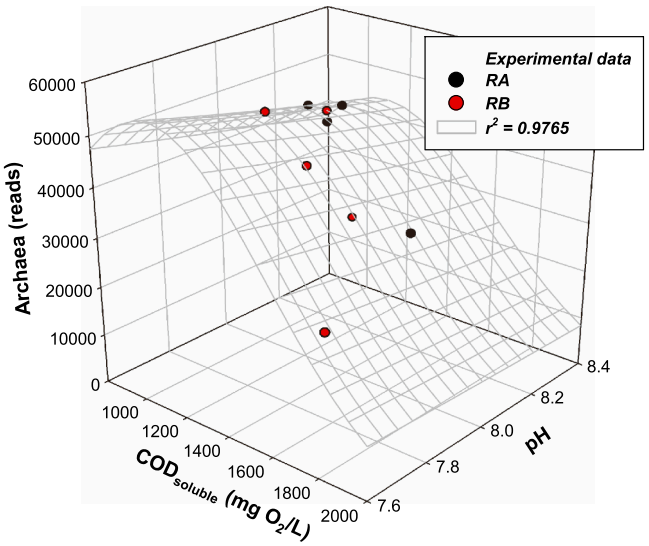

Fig. 5. Variation in the number of reads related to Archaea as a function of the concentration of soluble COD and pH in the mixing liquor of the reactors. Normality test (Shapiro-Wilk) passed (P = 0.4482; W statistic = 0.9172; significance level < 0.0001; constant variance test passed P = 0.1209).

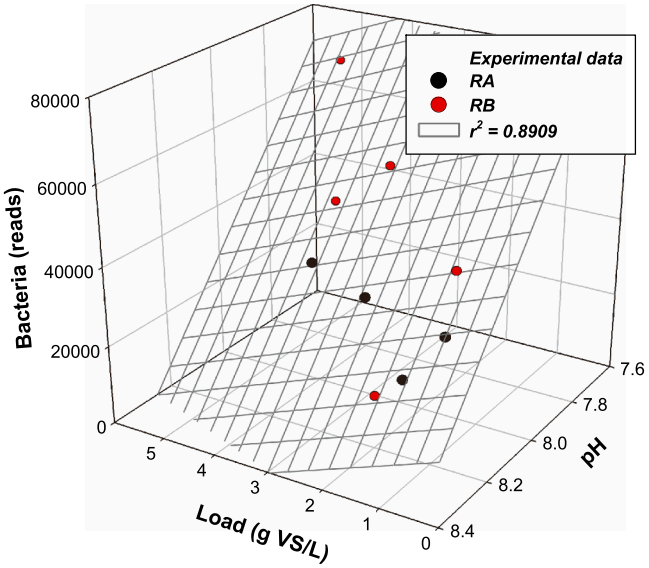

Fig. 6. Variation in the total reads associated to Bacteria as a function of the organic load added and pH in the mixing liquor of RA and RB. Normality test (Shapiro-Wilk) passed (P = 0.2726; W statistic = 0.9034; significance level < 0.0001; constant variance test passed P = 0.3558).

the most common bacterial families, several were more common in RA than in RB: *Thermoanaerobaculaceae* (10.3-fold), *Dysgonomonadaceae* (2.8-fold), *W27* (10.4-fold), *Gracilibacteraceae* (2.6-fold), *Peptococcaceae* (3.6-fold) and *Syntrophobacteraceae* (2.7-fold), while in RB the most represented families were: *Rikenellaceae* (2.8-fold), *Christensenellaceae* (4.7-fold) and *Pedosphaeraceae* (4.8-fold). As previously described in AD, the bacterial subcommunities identified here are more highly diverse than the archaeal ones. However, the latter are more correlated with the reactor performance (Yu et al., 2014; Ziganshin et al., 2011).

3.4. Functional analysis of the microbiome

In this study, the metabolic potential of the microbiome was predicted using the Tax4Fun algorithm. The OTUs were computed using KEGG Ortholog reference profiles, generating a total of 6433 functional capabilities. Those functions were grouped into 284 metabolic capabilities using KEGG Pathway reference profiles.

The top metabolic pathways identified include, in a decreasing order: "methane metabolism", "ABC transporters" responsible for translocation of many substrates, "two-component system" involved in the respond to different environmental changes, and also several pathways related to "amino acids metabolism", "porphyrin and chlorophyll metabolism", "nucleotide metabolism", "oxidative

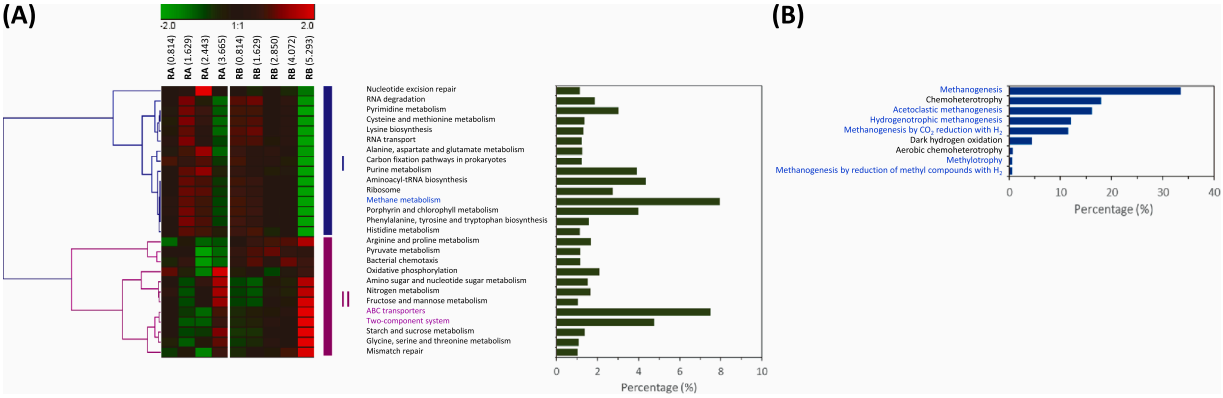

Fig. 7. Functional analysis of the microbiome during the reactor operation. **(A)** Metabolic pathways obtained with the Tax4Fun algorithm with a representation above 1 % are presented. The hierarchical clustering analysis of the differentially represented pathways is shown on the left, where routes are grouped in two main clusters (I and II). Samples are indicated on the top of the image, where RA means reactor A and RB reactor B, both followed by the organic loads (g VS/L) added (in brackets). Green rectangles indicate functions with lower representation relative to other conditions, while red rectangles represent higher levels. The colour intensity is proportional to the fold-change as represented by the scale. The percentage represented by the different metabolic pathways is shown in the graph on the right. **(B)** Metabolic processes in the microbiome predicted by the FAPROTAX analysis with a representation above 0.5 %.

phosphorylation", "nitrogen metabolism", "starch and sucrose metabolism", "carbon fixation pathways", "pyruvate metabolism", "bacterial chemotaxis", "DNA repair", etc. (Fig. 7A, right). Besides the methane metabolism, the multiple pathways identified suggested a very versatile metabolism and an intense bacterial replacement indicated by the abundance of amino acid and nucleotide metabolism pathways.

The Genesis software was then used to perform a hierarchical clustering analysis to visualise the evolution of the different metabolic pathways along the process in both bioreactors. Fig. 7A (left) shows the analysis of those routes that represent more than 1 % of the global functional analysis. Both reactors presented similar tendencies along the process, although not identical, and the metabolic pathways could be clearly grouped in two different clusters. Cluster I included pathways which sharply decreased at the end of the process. "Methane metabolism" was clearly included in this group in concordance with the exhaust of methane generation (described in Section 3.2. Biomethanisation process yield) (Xing et al., 2023). Metabolic pathways included in cluster II mostly show an opposite trend and clearly increase at the end of the whole process, *e.g.*, "ABC transporters" or "two-component systems", both involved in bacterial mechanisms of adaptation and colonisation to changing environments (Fig. 7A, left). Thus, the different mechanisms of active import and export, and the diverse and broad range of substrate exchanged mediated by ABC transporters, were essential in cell viability, virulence, and pathogenicity. They were involved in many diverse and relevant processes, *i.e.*: environmental and osmotic sensing, growth under stress conditions, etc. Since the action of transporters allowed counteracting any undesirable change occurring in the cell, they were essential for bacterial survival (Davidson et al., 2008; Herruzo-Ruiz et al., 2021; Rees et al., 2009). Similarly, vital for Bacteria was the two-component regulatory system, which was composed of at least two proteins, the sensor kinase and the response regulator. While the former sensed external stimuli, the latter responded by altering the expression profile of bacterial genes for survival and adaptation to a wide range of environments, stressors, and growth conditions (Hirakawa et al., 2020; Skerker et al., 2005).

Biological methane production was exclusively carried out by methanogenic Archaea (Kröninger et al., 2017). These microorganisms are usually classified into three major groups depending on their substrate spectrum. Hydrogenotrophic methanogenic Archaea use H_2 and CO_2 or formate as substrates; this pathway is usually considered ancestral as it is commonly found in most methanogenic orders, except *Methanomassilliicoccales*. The two other methanogenic pathways use acetate (aceticlastic) and methylated compounds (methylotrophic), and while the first one is restricted to the order *Methanosarcinales*, the second pathway can be additionally found in *Methanomassilliicoccales* and *Methanobacteriales* (Enzmann et al., 2018; Kröninger et al., 2017). All the mentioned orders were quite represented in WAS, suggesting a multiple methanogenic metabolism (Supplementary Table A): Acetoclastic methanogenesis and hydrogenotrophic methanogenesis. Thus, the families *Methanobacteriaceae* (affiliated with the order *Methanobacteriales*), *Methanomassiliicoccaceae* (order *Methanomassiliicoccales*), *Methanomicrobiaceae* and *Methanoregulaceae* (order *Methanomicrobiales*), and *Methanosaetaceae* (order *Methanosarcinales*) were present and evolved throughout the biomethanisation process of the sonicated WAS developed in the two reactors.

Functional analysis using FAPROTAX predicted that the largest percentage of processes in the microbiome were related with methanogenesis (33.5 % in total). Included in this general process, the three main subprocesses mentioned previously could be separated, all of them following a similar pattern over time (Fig. 7B). Thus, the most abundant corresponded to acetoclastic methanogenesis (16.2 %), followed closely by hydrogenotrophic methanogenesis (also known as CO_2/H_2 reduction, 12.1 %) and, finally, a much lower percentage of methylotrophy (0.6 %).

4. Conclusions

In this work, biomethanisation of "WAS" was effectively improved by applying a sonication pretreatment, especially in terms of renewable methane yield (increased by 167 % in comparison with untreated waste). AD was also found to be markedly stable within the range of organic loads evaluated, with optimal values of pH and VA/Alk ratio throughout the valorisation process, although inhibition was observed at the highest loads.

Archaeal and bacterial community structure were monitored by metagenomics of the hypervariable regions of the 16 S rDNA gene and correlated with the physicochemical characteristics of the system. The global microbial profile was successfully related to process performance and its variation throughout the experimentation in each reactor. Most of the Archaea belonged to the majoritarian *Euryarchaeota* phylum and they were related to methanogenic metabolism. Their abundance decreased at the end of the process, which probably leads to the observed depletion in the methane production. Metabolic functionalities used by microorganisms to survive under adverse environmental conditions were increased in the exhausted digestors.

Implementation and integration of physicochemical and microbiological analytical approaches could show the real picture of the processes developing during anaerobic digestion in WWTP, and, thus, become a valuable source of information to increase their efficiency.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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CRediT authorship contribution statement

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2024.103750.

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