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E.J. Martínez(a), J.G. Rosas(b), A. Sotres(a), A. Morán, J. Cara(a), M.E. Sánchez(a), X. Gómez(a)*

(a) Chemical and Environmental Bioprocess Engineering Group, Natural Resources Institute (IRENA), University of León, Av. de Portugal 41, 24009, Leon, Spain

(b) Department of electric engineering, School of Industrial Engineering and Informatics, University of León, Campus de Vegazana, 24009, León, Spain.

*Correspondence to: Xiomar Gomez, Chemical and Environmental Bioprocess Engineering Department, Natural Resources Institute (IRENA), University of León, Avda de Portugal nº 41, León 24071, Spain. *E-mail of the corresponding author: xagomb@unileon.es Telephone number: +34 987 29 5349

ORCID number and E-mail of the first author E.J. Martínez: 0000-0002-4426-4353, ejmartr@unileon.es

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Codigestion of sludge and citrus peel wastes: Evaluating the effect of biochar addition on microbial communities

E. Judith Martínez(a), Jose Guillermo Rosas(b), Ana Sotres(a), Antonio Moran, Jorge Cara(a), Marta Elena Sánchez(a), Xiomar Gómez(a)*

(a) Chemical and Environmental Bioprocess Engineering Group, Natural Resources Institute (IRENA), University of León, Av. de Portugal 41, 24009, Leon, Spain

(b) Department of electric engineering, School of Industrial Engineering and Informatics, University of León, Campus de Vegazana, 24009, León, Spain.

*E-mail of the corresponding author: xagomb@unileon.es Telephone number: +34 987 25349

Abstract

In this study, the effects on process performance and changes in microbial populations with the addition of biochar to the anaerobic digestion of sludge and orange peels were evaluated. Biochar had a positive influence in batch digestions, leading to a decrease in the lag phase and an increase in methane yields; this was even more evident for citrus peel wastes, which reached an increase of approximately 33% when 10 g L⁻¹ of biochar was added and 56% when 30 g L⁻¹ was added. Particle size analysis performed for the

AD Anaerobic digestion
SS Sewage sludge
CEO Citrus essential oil
$q_e$ Adsorption capacity
PCR Polymerase chain reaction
OTUs Operational Taxonomic Units
PCA Principal components analysis
DIET Direct interspecies electrons transfer
SSA Specific surface area
PSD Particle size distribution
SMP Specific Methane potential
experiments shows greater surface area available in biochar systems for biomass immobilization.

Analysis of the microbial communities by means of 16S rRNA gene pyrosequencing shows that the biochar addition favoured the electro-active microorganisms consortia creating a synthrophic metabolism between eubacterial and archaeal populations, which resulted in an improvement of the anaerobic digestion performance.

The codigestion of the mixture under a semicontinuous regimen showed an improvement in methane yields of approximately 60% and at hydraulic retention times of 30–20 days (reaching methane production values above 500 L CH4 kg VS⁻¹ at an OLR of 1.49 kg VS m⁻³ d⁻¹). The enhancement observe in biochar supplemented fermentations may be explained by the adsorption of inhibitors and the relatively high surface area favoured the adhesion and growth of microorganisms.

**Keywords:** Anaerobic digestion; biochar; high throughput sequencing; orange peel wastes; sewage sludge.

1. **Introduction**

Biowastes can be transformed into useful resources through anaerobic digestion (AD), which is an attractive technology for achieving pollution control and the recovery of energy. Nevertheless, AD is a complex process where the intrinsic characteristics of substrates, the presence of inhibitors and the reactor configuration are closely related to the stability of the process. Consequently, different approaches have been applied to attain higher benefits in terms of energy production and material recovery. These approaches include codigestion, pretreatment of substrates, improvement of the reactor
configuration and the use of additives for reducing the effect of inhibitors and increasing microbial activity.

AD is an effective technology widely applied for the treatment of sewage sludge (SS), although several disadvantages such as low methane production and biodegradability are well known [1,2]. This technology is usually optimized in many wastewater treatment plants (WWTPs) by adding a cosubstrate or applying pretreatment strategies to improve biogas yield and balance energy demands [3].

Valorization of citrus peel waste as a cosubstrate may represent an important source of income for the food industry and to provide a new insight into waste management and production of high-value products [4]. However, codigestion of this material also requires the prior removal of inhibitory compounds, which would result in increased treatment costs. Nearby, 50–60% of the total mass of the oranges consumed becomes citrus peel waste, which is mainly composed of membranes, seeds and peels [5,6]. Due to their main characteristics, the biological alternatives for their treatment such as composting or ethanol fermentation are not always successful [7]. These characteristics include a low pH of approximately 3–4, high water and organic matter contents, and the presence of limonene, the main compound of citrus essential oils (CEO).

Limonene, which is a cyclic terpene, is the main compound present in CEO, with a complex chemical structure (cyclohexane ring and ethylene group), making it resistant to hydrolysis. Several studies have reported on its negative effects, which can severely affect hydrolytic–acidogenic and methanogenic activity [6]. The concentration thresholds reported for inhibitory effects show a wide range of values (24–192 CEO mg L⁻¹ d⁻¹). This high variability may be explained by the different conditions tested in their experiments and the type of substrates used [5,6,8].
Different approaches have been used for improving the AD of citrus wastes, e.g., microbial acclimation or codigestion with other feedstocks in an attempt to reduce its toxic effect [7,9]. However, the removal of limonene by different mechanisms (e.g., steam distillation, solvent extraction or adsorbents) is more effective because this removal avoids further accumulation of limonene metabolites derived from microbial activity, which may also act as inhibitors [5,8].

The use of low cost adsorbents such as biochar may become a feasible solution to avoid inhibitory conditions. In addition, it should be borne in mind that biochar is frequently produced from agro-industrial wastes; as such, this approach for preventing inhibition in AD results in a holistic valorisation, thus allowing the integration of biological and thermal processes with the aim of producing higher added value products, achieving simultaneously environmental solutions and reducing the carbon footprint of industrial processes. The integration of AD and thermal conversion processes as it is the case of pyrolysis, results in higher energy gains, opening up new interesting pathways for the valorisation of residual biomass. The integration of these two processes allows for the use of pyrolysis by-products which can be easily integrated in the same valorisation unit leading to a significant reduction of the organic material needing final disposal [10]. Although thermal processes may be seemed as expensive technologies due to their high energy demand, pyrolysis has demonstrated capable of energy self-sufficiency implying that no extra fuel is necessary for the operation of this type of technology [11,12].

The addition of biochar to AD reduces the negative effects of toxic compounds and promotes the immobilization of microbial biomass, due to the microporous structure.
Even though the mechanism of adsorption in biological systems has not been fully studied, several authors have reported on the conjunction of adsorption and immobilization for explaining the enhancements observed [13,14].

Mumme et al. [15] reported on the positive effects of biochar in avoiding the toxicity caused by ammonia and others compounds. Similarly, Fagbohungbe et al. [8] reported on the benefits for treating citrus peel wastes. It is well known that biochar provides a relatively high surface area which favours the formation of biofilm and presents conductive characteristics which may favour biological activity [16]. However, the effect of these features on anaerobic microbial communities is still poorly understood, particularly if special attention is to be payed to biochar–microbe interactions.

The aim of this study was to assess the influence of biochar addition on the anaerobic codigestion of orange peels and sewage sludge. Bearing in mind the possible mechanisms of improvement via adsorption and/or biomass immobilization, the process was evaluated in terms of microbial populations, methane production and changes in the main parameters such as volatile fatty acids, limonene, polyphenols, ammonia and particle size.

The experiments were carried out in the Chemical and Environmental Bioprocess Engineering Group at University of Leon during the years 2016 – 2017.

2. Materials and methods

2.1 Characteristics of the substrates and biochar

Sewage sludge (SS) and digested sludge (used as inoculum) were obtained from the WWTP of the city of León, Spain. The total solids (TS) and volatile solids (VS) contents of the inoculum were 35.5 ± 0.2 and 20.9 ± 0.2 g kg⁻¹, respectively. Sewage
sludge (SS) was stored at 4 °C prior to its further use. Orange peels (Op) were manually
chopped to obtain a particle size of 2–5 mm. No pretreatment was applied prior to
digestion. The physical and chemical characteristics of the Op are shown in Table 1.

Table 1.

Biochar was produced from vineyard prunings from a vineyard in Barcelona, Spain.
The production of biochar was performed in a semi–continuous electrically heated
reactor. A full description of the process can be found elsewhere [17]. The biochar was
initially obtained using an electrical unit in order to develop an alternative management
of vineyards’ residues from a Spanish winery. The aim was obtaining data for the
design and development of an energy self-sufficient pyrolysis plant which would aid in
the reduction of greenhouse gas emissions by biochar application to vineyards. The
main characteristics of the substrates and biochar are shown in Table 1.

2.2 Adsorption experiments

Adsorption experiments were carried out to evaluate the adsorption capacity of biochar.
The experiments were run using 250 mL Erlenmeyer flasks provided by magnetic
stirrers. The flasks contained 10 g L⁻¹ of biochar and 100 mL of different solutions
containing acetic, propionic and butyric acid. Two replicates were tested for each
experiment. The solutions were prepared with a concentration range of 1 000–9 000 mg
L⁻¹ for acetic, between 400 and 3 000 mg L⁻¹ for propionic and from 400 to 4 000 mg L⁻¹
for butyric acid. The temperature was set at 25 °C, and the initial pH was 7.0. The
initial and final concentrations were measured, and the data obtained were used to
calculate the adsorption capacity (qe) of the biochar and the removal achieved for each
compound (expressed in %). The results were fitted to a pseudo-second order model,
and adsorption kinetics constants were calculated based on the work of Martínez et al.[18].

Initial concentrations against residual adsorbate concentration in the equilibrium were fitted to the isothermal models of Langmuir and Freundlich. The Langmuir sorption isotherm describes the sorption process at specific homogenous sites within the adsorbent, assuming that the maximum adsorption corresponds to a monolayer saturated with adsorbate molecules [18], Eq. (1):

\[
q_e = \frac{Q \cdot K_L \cdot C_e}{1 + K_L \cdot C_e} \tag{1}
\]

where \(Q\) is a constant related to the adsorptive capacity, \(C_e\) is the equilibrium concentration and \(K_L\) is a parameter of the adsorption energy.

The Freundlich isotherm is an empirical model and suggests that sorption energy exponentially decreases upon occupation of the sorption site by an adsorbent, Eq. (2):

\[
q_e = K_F \cdot C_e^{1/n} \tag{2}
\]

where \(K_F\) is a parameter related to the adsorption capacity and \(n\) refers to the process intensity.

2.3 Experimental set-up of anaerobic digestion

2.3.1 Batch test

Batch digestion of the individual substrates, Op or SS, and of a mixture of the two at a VS ratio of 1:1 (Op:SS) was tested (setting three replicates) using Erlenmeyer flasks of 250 mL. The flasks were filled with inoculum and substrate also at a VS ratio of 1:1 (inoculum-substrate, I:S). Sodium bicarbonate was added as a buffer against pH.
changes, at a concentration of 10 g L⁻¹. Reactors containing inoculum were used as blanks to subtract the background gas production. Temperature was controlled by a water bath at 37 ± 1 °C, and agitation was provided by magnetic stirrers. Gas volumes were measured using bottle gasometers and corrected to standard temperature and pressure (STP, 0 °C and 760 mmHg). Digestion systems were denoted as Op when digesting orange peels, SS for sewage sludge and Op+SS for the mixture. Reactors containing char were denoted Op+SS 10 Biochar and Op+SS 30 Biochar based on the biochar content tested. Systems without substrate but containing the same amount of char were also tested in order to observe if char itself promotes biogas production.

Methane production was fitted to the modified Gompertz equation [19]:

\[ P(t) = P_1 + P_{\text{max}} \cdot \exp[- \exp(R_{\text{max}} \cdot e / P_{\text{max}})(\lambda - t) + 1] \]

where \( P(t) \) is the cumulative methane yield (L kg VS⁻¹), \( P_{\text{max}} \) is the maximum methane yield (L kg VS⁻¹), \( R_{\text{max}} \) is the maximum methane production rate (L kg VS⁻¹ d⁻¹), \( \lambda \) is the lag–phase time (d) and \( e \) is Euler’s number (approx. 2.718).

Data analysis was performed using OriginPro software. A modification to the model was proposed when an extended lag phase was observed [19]. This modification considers the addition of a new parameter corresponding to the initial methane production obtained from the experiment (\( P_1 \)).

2.3.2 Semicontinuous digestion

Semicontinuous digestion was performed in completely stirred reactors (working volume of 3 L) equipped with mechanical stirrers and outer-jackets to circulate heating water at a temperature of 37 ± 1 °C. Feeding was manually performed once a day every day. The start-up of the reactors was done using SS as substrate at a hydraulic retention
time (HRT) of 30 days for 15 days to allow for a progressive adaptation. Afterwards, they were fed with the mixture of Op and SS. The organic loading rate (OLR) was increased by reducing the HRT from 30 to 20 to 10 days subsequently. Biochar was added to the reactor with the beginning of each HRT to reach a content of 10 g L\(^{-1}\). The biochar concentration in this reactor was calculated based on the following expression:

\[
C_f = C_i e^{-\frac{1}{HRT}t}
\]

where \(C_f\) is the biochar concentration (g L\(^{-1}\)) at the time \(t\) and \(C_i\) is the initial biochar concentration (g L\(^{-1}\)).

The reactor denoted RC_Op+SS stands for the control system treating the mixture of Op and SS. The reactor denoted RB_Op+SS represents the system treating the mixture with the addition of biochar.

2.4 Analytical techniques

For liquid samples, ammonia, chemical oxygen demand (COD), TS, VS, pH, and alkalinity were measured in accordance with APHA Standard Methods [20]. Total organic carbon (TOC) and total nitrogen (TN) were measured for 25 ml of the supernatant of centrifuged liquid sample (5000 rpm – 4193 x g, time of 5 min) using the Analytik Jena Multi N/C_3100 system by thermocatalytic oxidation, Total carbon (TC) and inorganic carbon (TIC) are determined separately. The difference results in TOC, TOC = TC - TIC. The analysis of metals for the dried solid sample (0.3 g) was carried out using a PerkinElmer Optima 2000 DV inductively coupled plasma (ICP) atomic emission spectrometer as described in Fierro et al [21].
Biochar analysis was carried out using a LECO CHN-600 apparatus for measuring C, H, and N in accordance with ASTM Standard D-5373. Ash content was determined using a LECO MAC-300 thermogravimetric analyser (TGA). The Brunauer-Emmett-Teller (BET) Surface Area method was performed with a Micromeritics model ASAP 2420 by adsorption isotherms of N₂ at 196 °C. Analysis of organic matter for substrates (sewage sludge and orange peels) were measured by the use of the Walkley–Black method [22]. TOC of substrates was calculated from the organic matter value, using a correlation factor of 1.72.

D-Limonene was determined by bromate titration [23]. Total polyphenols (TP) were measured by colourimetry at 760 nm on a Beckman DU640 spectrophotometer using the Folin–Ciocalteau reagent in a mixture containing 650 µL of de-ionised water, 50 µL of sample, 600 µL 7.5% Na₂CO₃ and 200 µL Folin–Ciocalteau reagent. Gallic acid was used as standard for the calibration curve.

VFAs were measured by gas chromatography using a Varian CP3800 GC and a flame ionisation detector equipped with a Nukol capillary column from Supelco. Biogas composition was analysed using the same chromatograph when it was equipped with a thermal conductivity detector. A packed column (HayeSep Q 80/100; 4 m) followed by a molecular-sieve column (1 m) was used. The carrier gas was helium. Hydrogen sulfide (H₂S) was detected using a pulse flame photometric detector (PFPD) with an FA-II capillary column and helium as carrier gas.

Particle size analysis was performed using a Beckmann Coulter LS 13 320 laser diffraction particle size analyser. The scatter generated was estimated based on the Fraunhofer optical model. Samples were obtained from the SS batch digestion system.
Liquid samples were diluted in tap water prior to the analysis. Ten measurements were performed for each sample.

**2.5 Microbial community analyses**

Samples for microbiological analysis were obtained from batch digestion tests and were denoted following the nomenclature described previously for these tests: Op, Op 10 Biochar and Op 30 Biochar for the orange peel digestions, and SS, SS 10 Biochar and SS 30 Biochar for the sewage sludge digestions. The sample denoted SS Feed contained the sewage sludge that was used as substrate in the digestion systems.

Genomic DNA was extracted at the end of the batch experiments with the PowerSoil® DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA), following the manufacturer’s instructions. All PCR reactions were carried out in a Mastercycler (Eppendorf, Hamburg, Germany), and PCR samples were checked for size of the product on a 1% agarose gel.

The entire DNA extract was used for High Throughput Sequencing of 16S rRNA gene based massive libraries for eubacterial and archaeal communities. The primer set used was 27Fmod (5’-AGRGTGTTGATCMTGGCTCAG-3’) /519R modBio (5’-

GTNTTACNGCGGCKGCTG-3’) for the eubacterial population analysis and Arch 349F (5´- GYGCASCAGKCGMGAAW-3´) / Arch 806R (5´GGACTACVVGGGTATCTAAT-3´) [24] for the archaeal population analysis. The obtained DNA reads were compiled in FASTq files for further bioinformatics processing following the procedure described by Sotres et al. (2016). Operational Taxonomic Units (OTUs) were then taxonomically classified using the Ribosomal Database Project (RDP) (https://rdp.cme.msu.edu/). The raw pyrosequencing data obtained from this analysis were deposited in the Sequence Read Archive (SRA) of the
Microbial richness estimators ($S_{obs}$ OTUs and Chao1) and diversity indices ($H'$, inv Simpson and sampling coverage) were calculated using MOTHUR software, version 1.35.1, for each sample, after normalising the number of reads of all samples to those of the sample with the lowest number of reads. Dynamics and similarity of the microbial community structures were evaluated by principal components analysis (PCA) and Venn diagrams based on the total of all OTUs obtained by high throughput sequencing. The Venn diagram analysis was performed using VENNY software (http://bioinfogp.cnb.csic.es/tools/venny/). Rstudio was used for performing PCA on the OTU abundance matrix of both eubacterial and archaeal OTU populations, and it was also used to produce heat maps.

3. Results and discussion

3.1 Adsorption assays

The adsorption experiments with organic acids (acetic, propionic and butyric) were performed to obtain an approximation of the adsorption capacity of the biochar used ($q_e$). The experimental value of $q_e$ was 10.70 mg g$^{-1}$ for acetic acid, 5.56 mg g$^{-1}$ for propionic acid and 3.15 mg g$^{-1}$ for butyric acid, when the lower concentrations of the organic acids were used (1000 mg L$^{-1}$ of acetic, 400 mg L$^{-1}$ of propionic and 400 mg L$^{-1}$ of butyric acid). In all of the cases, the adsorption capacity $q_e$ increased with an increase in the initial concentration. When the difference in concentrations was higher, the driving force governing the process was also higher, favouring the mass transfer (data are shown in supplementary material Fig. SM1 and Table SM1). The results were fitted to the pseudo-second order model, and adsorption kinetics constants were calculated.
based on this model. The calculated values of $q_e$ were similar to the experimental data obtained, indicating that the sorption systems belong to the pseudo-second order kinetics model with regression coefficients higher than 0.95.

Different initial concentrations of VFAs (acetic, propionic and butyric) against residual adsorbate concentration in the equilibrium were fitted to the isothermal models of Langmuir and Freundlich. The values of the characteristic parameters obtained from the fitting of both models (supplementary material Table SM2) indicated a better fit to the Freundlich model for any of the VFA used (determination coefficients higher than 0.99). The $n$ value (which is an indicator of the adsorption intensity) obtained for this model was >1 for acetic acid, reflecting the favourable adsorption of this VFA. It has to be noted that Freundlich parameters are only descriptive and unlike the Langmuir model, it does not predict the formation of a boundary monolayer for adsorption, showing a greater incorporation of the adsorbate.

The adsorption in water or in organic mixtures such as anaerobic digestion liquor is a complex phenomenon due the differences in the adsorbent surfaces and specific interactions of polar molecules with oxygen-containing surface groups [25]. Cuetos et al [26] observed a mild retention of acids onto the activated carbon surface, which may alleviated an inhibitory stage for microorganisms in the digestion of residual blood. The adsorption capacity of biochar when used in digestion liquors may be affected since the presence of a great variety of species may interfere and compete for adsorption sites.

### 3.2 Batch digestion

The cumulative methane production curves obtained from the different batch assays are shown in Fig. 1. The fitted curves of the Gompertz model are also represented with
model parameters that are summarised in Table 2. No additional biogas production was 
observed during the evaluation of the systems loaded only with char (data not shown).

Fig. 1 HERE

Table 2. HERE

The methane production curves presented a sigmoid profile showing an extended lag 
phase when Op are digested, even with the addition of 10 g L$^{-1}$ of biochar. This 
behaviour is due to the need of acclimation of microorganism to the complex substrate 
and high initial concentration of limonene (~600 mg L$^{-1}$).

The addition of biochar resulted in a reduction of the lag phase and a significant 
increase in the methane production (see Table 2). Similarly, the assessment of biochar 
addition on digestion of citrus peel wastes by Fagbohungbe et al. [8] revealed the 
reduction of the lag phase from 13.4 to 6.8 days with wood biochar and a slight increase 
of methane production with the addition of coconut shell biochar. Different methane 
yields for orange peels and related biowastes have been reported in the literature, under 
similar conditions of temperature but higher I:S ratio. Ruiz and Flotats [7] obtained an 
average yield of 356 L CH$_4$ kg VS$^{-1}$, while Martin et al. (2010) reported 230 L CH$_4$ kg 
VS$^{-1}$ but in this case extracting limonene prior to digestion. Higher yield values have 
been reported at thermophilic temperatures, around 400–600 L CH$_4$ kg VS$^{-1}$ [27] In the 
present study, the poor yield and extended lag phase reported for Op can be attributed to 
the different operational parameters and higher limonene content.

The methane yields obtained from SS with and without the addition of biochar are 
presented in Fig.1b. The process shows a small lag phase compared with that of the Op
system. The addition of biochar increased methane yield by approximately 33% when 10 g L\(^{-1}\) of biochar was added and by 56% when 30 g L\(^{-1}\) of biochar was added.

The results from the codigestion of Op and SS are shown in Fig. 1c. The methane yield obtained from the Op+SS reactor was notably higher than that of the Op system (~89%) and comparable to that of the SS reactor. This behaviour was explained by a dilution effect caused by the way the feed was prepared and the type of the experimental set-up. Reactors were initially loaded at a I:S ratio of 1:1, which was also the same for the Op+SS mixture; therefore, the global amount of Op in the codigestion reactor was lower than that of the Op digestion. The concentration of limonene when digesting the mixture may have not reached inhibitory levels (336 against mg 620 L\(^{-1}\) in the Op reactor).

The benefits of biochar addition are evident from data reported in Table 2, when treating the mixture Op+SS. The addition of biochar to the batch systems presents an enhancement of the process, by increasing the gas production rate, improving yields or decreasing the lag phase. These effects may be associated with the physical mechanisms of adsorption of inhibitory compounds. In fact, the great improvement observed when treating Op is related to the lower values of limonene and polyphenols measured (see Fig. 2a–b). However, with regard to VFA, there is not a clear trend; the Op reactor reached lower values during the first 10 days, with the exception of the particular low concentration observed for butyric when biochar was added at its higher level. This may be explained by the presence of a great diversity of compounds in the digestion liquor which cause interference in the adsorption performance of biochar. Therefore, a lower adsorption capacity of VFA was obtained in digestion tests, which was associated with the presence of other counter-ions and compounds that may compete with organic acids for adsorption sites.
Fig. 2c also shows a rapid increase in VFA concentration regardless of whether or not biochar had been added. Studies by Wang et al. [28] about the effects of VFA on methane yield reported that acetic acid concentrations of 2 400 mg L$^{-1}$ and butyric acid concentrations of 1 800 mg L$^{-1}$ may not cause a significant inhibition of the activity of methanogens, while a propionic acid content of 900 mg L$^{-1}$ could result in significant inhibition. The digestion of Op resulted in a rapid degradation of the substrate leading to VFA build-up, mainly of acetic acid (reaching $\sim$12 000 mg L$^{-1}$) and butyric acid (reaching $\sim$5 000 mg L$^{-1}$), to levels above inhibitory thresholds. Nonetheless, the concentration at which VFAs can cause inhibition depends on the previous conditions, hence the impossibility of defining VFA levels to indicate the state considered as ‘normal’ for the anaerobic process [29]. However, in the case of propionic acid, the values for this system were initially much lower than those from reactors with biochar addition. At the end, these later systems consumed this acid while the Op reactor maintained an increasing trend reaching a final concentration of 2 100 mg L$^{-1}$, a much higher value than that of biochar systems.

After 30 days of operation, the acetic and butyric acid concentrations decreased significantly with biochar addition, with especially low values attained for butyric acid. The final values of VFA for biochar containing reactors were 1 790 mg L$^{-1}$ for Op 10 Biochar and 1 459 mg L$^{-1}$ for Op 30 Biochar, indicating that although a better assimilation of the organic material can be associated with the presence of biochar, methanogenic microorganisms were not capable of consuming all VFAs derived from the hydrolysis–acidification stages.
It may be reasonable to assume that the presence of limonene and polyphenols were causing interference in the adsorption of VFA. Further work is necessary to elucidate the effect of biochar on the adsorption of these compounds and their interaction with VFA. The concentration of limonene and polyphenols was lower for biochar supplemented systems at the end of the digestion tests (Fig. 2a – 2b), indicating that there is an adsorption effect taking place for these two compounds and explaining the different results in VFA adsorption tests of single acids and digestion tests.

The evolution of VFA reported in Fig. 2c indicates that adsorption may not be playing a crucial role on the enhancement obtained in methane yields when biochar was added, since initial values of VFA were similar to those of the Op reactor. A mechanism that has been suggested when using conductive carbon materials is the direct interspecies electrons transfer (DIET), a syntrophic metabolism where free electrons flow from one cell to another without being shuttled by reduced molecules such as molecular hydrogen or formate [30]. This mechanism has been suggested as responsible for obtaining better degradation rates of simple substrates and higher biogas yields in anaerobic systems when conductive carbon materials are added [26].

An explanation behind the improvement of digestion may be gained from the behaviour of the SS digestion set-up when studying the particle size distribution. In this case, the presence of inhibitory substances lacks relevance, so the effect of adsorption of toxic compounds is not considered. Modifications in the physical structures of aggregates formed due to the addition of biochar may have caused an improvement in microorganism consortia. Samples from the SS reactors were obtained at the end of the digestion.
The particle size analysis performed for the SS digestion test shows the impact of biochar addition in the main parameters measured. Table 3 summarizes the results obtained at the end of the experiments. The value for specific surface area (SSA) was 33% higher for SS 10 Biochar and 47% for SS 30 Biochar samples when compared with the SS digested sample.

**Table 3.**

The particle size distribution (PSD) of biochar was centred between $1-1000 \, \mu m$ of particle size. The distribution shows three main peaks, one located between $40-400 \, \mu m$, one corresponding to small particles between $1-40 \, \mu m$ and the other between $400-1000 \, \mu m$. This sample had a mode of $185.4 \, \mu m$ particle size and an SSA of $2890 \, cm^2 \, g^{-1}$ (see supplementary material Fig. SM2a).

The PSD shows a similar bimodal distribution for all SS samples after digestion. The main peak is centred in the smaller particle range, with samples SS and SS 10 Biochar presenting also a minor peak centred between $100-300 \, \mu m$ (see supplementary material Fig. SM2b). The major differences observed were associated with the values of SSA. The greater surface area available in biochar systems may have favoured biomass immobilization and assisted the microbial consortia in the access to substrate, improving the assimilation of acid intermediaries and, as a consequence, methane yields.

Similar experiments carried out by several authors using carbon additives in anaerobic digestion also report on an improvement thanks to the influence of the supporting material causing the selection of some microorganisms over others [13,14].
Watanabe et al. [31] demonstrated the improvement caused by the addition of cedar charcoal when SS and crude glycerol were codigested. The better performance was explained by the attachment on charcoal particles of microorganisms capable of producing methane from glycerol.

### 3.3 Microbial community structure

The reads and the coverage values obtained for eubacterial and for archaeal communities are shown in Tables SM3 and SM4 (see supplementary material). The number of quality reads per sample ranged from 80,093 to 103,331 for eubacteria and from 18,604 to 102,847 for archaea. The differences in species richness indicators ($S_{obs}$, OTUs and $Chao1$ estimator) and diversity indices ($H'$ and $1/Simpson$) are described in Tables SM3 and SM4 (see supplementary material).

The PCA results for the eubacterial community can be seen in Fig. SM3a. The microbial community on the Op and SS digestion was modified based on that obtained from samples of the Inoculum and SSFeed. The two anaerobic systems clustered together, being separated from each other. In the Venn diagram, substantial differences in the total of OTUs in the Op digestion system when biochar was added (Op 10 Biochar, Op 30 Biochar) are represented (see supplementary material Fig. SM3b). It is highlighted that not a single OTU was shared between the Op sample and the samples obtained when biochar was added (29.5% of the total OTUs were shared between Op 10 Biochar and Op 30 Biochar). On the other hand, there are not such dramatic differences with the SS digestion set-up, since 35.3% of the total OTUs were shared between the SS sample and those with biochar added (SS 10 Biochar, SS 30 Biochar).

The PCA results for the archaeal community (see supplementary material Fig. SM4a) were similar to those for the eubacterial community. Nevertheless, the Venn diagram
showed that biochar addition did not induce a dramatic shift on the total of observed OTUs for the archaeal community with the Op digestion, since 245 OTUs, equivalent to 52.1% of the total OTUs were shared between Op, Op 10 Biochar and Op 30 Biochar samples (see supplementary material Fig. SM4b). Similarly, 47.7% of the total OTUs were common in the sludge set-up (SS, SS 10 Biochar and SS 30 Biochar samples). As a result, biochar addition led the sharpest change in the eubacterial community of the Op digestion.

The effect of biochar addition was studied by phylogenetic identification in the eubacterial and archaeal communities. Heat maps at the family level (Fig. 3) for the eubacterial and archaeal communities showed two clusters clearly differentiated for Op and SS samples. Although no significant difference in family composition for the archaeal community were observed (Fig. 3a), shifts in the relative abundance of some families were noteworthy. When Op and Op 30 Biochar samples were compared, an increase was observed in the relative abundance of *Methanomicrobiaceae* from 1.2% to 9.1%, *Methanosarcinaceae* from 3% to 5.4% and *Thermoplasmataceae* from 3.2% to 14.7%. On the other hand, slight differences were observed in the case of the SS digestion experiment, increasing the abundance of three families by less than 2% when 30 g L$^{-1}$ of biochar was added. *Methanobacteriaceae* and *Methanoregulaceae* and two families *Methamassiliicoccaceae* and *Cenarchaeaceae* were identified only after biochar addition.

*Methanosetaeaceae* was the dominant family found in samples obtained from Op and SS experimental set-up, accounting for 41–49% (Op experiment) and 59–60.5% (SS experimental set-up), respectively.
experiment) of the total archaeal community. *Methanosaetaceae* was initially predominant in the SS<sub>Feed</sub> (45%) and in the inoculum sample (43.1%) (data not shown).

Eubacterial communities at the family level were more sensitive than archaeal communities to the biochar addition, as shown in Fig. 3b. In the Op system, *Peptococcaceae* turned out to be the most affected family, being completely inhibited after biochar addition. Some families were completely inhibited after biochar addition in both Op and SS systems, such as *Symbiobacteriaceae* and *Peptococcaceae* (within the phylum *Firmicutes*), and *Pseudomonadaceae* and *Rhodobacteraceae* (within the phylum *Proteobacteria*).

The most abundant group in all samples was the family *Anaerolineaceae* (within the phylum *Chloroflexi*), which performs syntrophically in cooperation with *Methanosaetaceae*, also abundant in all samples as described above (Fig. 3a). Both groups have been described to be the predominant microorganisms and to be involved in the process of methanogenic degradation of alkanes [32]. The next most abundant group, also present in all samples, was the family *Clostridiaceae*, which belongs to the phylum *Firmicutes*.

Microbial community structure was also studied at the genus level (Fig. 4 and 5). The results obtained for Op digestion revealed that 9 genera increased their relative abundance after biochar addition, namely, *Bellilinea*, *Trepomena*, *Cythophaga*, *Dechloromonas*, *Clostridium*, *Petrimonas*, *Proteiniphilum*, *Bacteroides* and *Eubacterium*. In addition, 5 genera were identified only in Op 10 Biochar and Op 30 Biochar: *Spaerochaeta*, *Spirochaeta*, *Thermolithobacter*, *Petrotoga* and *Acidovorax*.

Although minor, some changes were also detected for the archaeal genera, with the relative abundance of *Thermogymnomonas* and three hydrogenotrophic methanogens,
Methanofollis, Methanoculleus and Methanolinea, being increased. In addition, Methanobacterium decreased in abundance after biochar addition.

**Fig. 4 HERE**

**Fig. 5 HERE**

In the SS digestion, changes were also detected in the relative abundance of some genera. For the eubacterial community, the biochar addition had a positive effect on *Clostridium, Curvibacter, Petrimonas, Eubacterium*, and *Syntrophomonas* genera. This last genus, *Syntrophomonas*, is an anaerobic, syntrophic and fatty acid oxidizing bacteria, previously described in anaerobic digestion works using biochar [14], and additionally these bacteria participate in a methanogenic syntrophy with H₂ using archaea such as *Methanospirillum* [33], also present in this study. *Geobacter* was identified when biochar was added to the anaerobic digestion SS 30 Biochar but not at the lower biochar level.

In the archaeal structure, only *Methanobacterium* and *Methanolinea*, both hydrogenotrophic methanogens, slightly increased their relative abundance.

Another two important genera, *Clostridium* and *Geobacter*, were also detected in batch experiments such for Op and SS digestion. *Clostridium* increased their abundance in both anaerobic systems after the addition of Biochar, being the most abundant genus in system SS 30 Biochar, accounting for 25% of the total population, and being the second most abundant in Op 30 Biochar. *Clostridium* is known to be a homoacetogenic bacteria and active fermenter, and a correlation between this genus and high methane production has been previously described in the literature, which may signify a syntrophic association with methanogens [34]. The well-known exoelectrogenic *Geobacter* was one of the bacterial models used to study the conductive properties of biochar, and the
impact of these bacteria on direct electron transfer (DIET) [35], mainly with
*Methanosarcina* and *Methanosaeta*, was also evident in our experiments.

Even though differences in the eubacterial community populations were observed
between Op and SS anaerobic digestion, some bacteria were favoured under the biochar
influence in both systems. *Treponema* is a *Spirochaeta* also described together with
*Geobacter* in conductive biofilms [36], which also increased their abundance with
biochar addition, probably explaining its presence at the higher level tested (30 g L\(^{-1}\))
but not at the lower level. *Petrimonas* (in the family *Porphyromonadaceae*) have a
fermentative type metabolism, with the final fermentation products of glucose being
acetate, H\(_2\) and CO\(_2\). The genus *Dechloromonas* (belongs to the family
*Rhodocyclaceae*), are described as H\(_2\) producing bacteria. Hence, it is likely that the
addition of biochar aids in the formation of co-cultures that produce H\(_2\) or formate,
providing electrons for CO\(_2\) reduction (to produce methane) by H\(_2\) utilizing
methanogens, as *Methanolinea, Methanobacterium, Methanosarcina,*
*Methanomassiliicoccus,* and *Methanofollis*, which were favoured by the addition of
biochar, while the acetoclastic *Methanosaeta*, the most abundant group in both systems,
decreased their relative abundance.

### 3.4 Semicontinuous digestion

The results of semicontinuous digestion are presented in Fig. 6(a–e) and Table 4. After
an adaptation period with sludge feeding, the reactors RC_Op+SS (control) and
RB_Op+SS (biochar addition) were fed in a semicontinuous mode with the mixture and
evaluated with a decreasing HRT. The biochar addition and concentration in the reactor
is reflected in Fig. 6b.

**Table 4. HERE**
A fluctuating process was observed for the codigestion system due to the presence of limonene in the feed, which severely affected the microbial activity (Fig. 6c). Biochar significantly improved the specific methane production (SMP) at the different HRTs studied (30−20 days) (P < 0.05, one-way ANOVA, Tukey post hoc test) and also attained a slight reduction in H$_2$S concentration in biogas along with lower levels of VFA content (Table 4).

Ammonium content in both reactors was similar (P > 0.05, one-way ANOVA) indicating that the addition of biochar had no effect on the evolution of this compound. The sludge sample had an initial ammonium content of 678 ± 89 mg L$^{-1}$. The digestion of sludge leads to the degradation of proteins releasing in consequence nitrogen in the form of ammonium. Reactors presented ammonium values in the range of 1300 to 1800 mg L$^{-1}$. Since the organic co-substrate added does not present a high content of nitrogen, its addition to the digester will not lead to ammonium inhibitory problems. In the case of a hypothetical implementation of this type of co-digestion in a WWTP accumulation of this compound in the water line due to the recycling of digestate supernatant from sludge decanters should therefore not to be expected. In terms of ammonium load, the use of this type of cosubstrate will not derive in the emergence of additional environmental problems regarding sludge disposal.

Results were in accordance with the benefits associated with the adsorption of inhibitory compounds like limonene and with the immobilisation of biomass. The dramatic variability in methane production observed in this reactor during the initial HRTs and the similar methane production achieved at an HRT of 10 days with that of RC_Op+SS (Fig. 6a) can be attributed to the adsorption-desorption phenomena and saturation of the added biochar, which resulted in the release of limonene into the bulk
solution, thereby affecting the microbial activity. No significant changes were observed for the other parameters measured when evaluating the effect of biochar addition (Fig. 6d–e). Nevertheless, under mesophilic conditions, the addition of biochar to attain a content in the range of 3–20 g L\(^{-1}\) significantly improved the Op+SS system, reaching methane production values above 500 L CH\(_4\) kg VS\(^{-1}\) at an OLR of 1.49 kg VS m\(^{-3}\) d\(^{-1}\), similar to the results from batch tests.

The SMP obtained for both systems significantly decreased with the increase in OLR, from 1.49 to 4.48 kg VS m\(^{-3}\) d\(^{-1}\). The performance of both reactors was quite similar at the end of the 20 d HRT, and this was also true with the further decrease to 10 d. The reloading of char on day 105 (its contents reached 15 g L\(^{-1}\)) was not sufficient and gas performance was initially affected. This behaviour was also followed by a decrement in other parameters (VFA build-up, limonene and polyphenols increments). Analogous experiments by different authors have suggested an inhibition of the process by high organic loading. The results of Serrano et al. [9] with SS and Op codigestion (70:30 wet weight respectively) showed a lower methane yield, 165 L CH\(_4\) kg\(^{-1}\) VS, with an OLR being increased from 0.4 to 1.6 kg VS m\(^{-3}\) d\(^{-1}\). The studies carried out by Martin et al. [5], also an Op digestion, presented a decrement in methane production with the increase in OLR for values above 3.5 g COD m\(^{-3}\) d\(^{-1}\).

4. Conclusions

The addition of biochar had a positive influence on the anaerobic digestion evaluated. In batch systems, a decrease in the lag phase and an increase in methane yields were observed. The benefits were more noticeable in systems with higher content of biochar...
(30 g L\(^{-1}\)) and were more significant for the Op system which presented a greater improvement in methane yield. The digestion can be improved due to the conductive properties of biochar, which may aid in H\(_2\)/formate transfer between syntrophic microorganisms rather than the formation of aggregates directly connected between the microbes.

The microbial community composition shows differences in both SS and Op systems. However, pyrosequencing analysis showed that biochar addition led to similar populations shifts in both anaerobic digestion reactors, where biochar favoured the electro-active microorganisms consortia creating a syntrophic metabolism through conductive carbon materials. The most highlighted changes could be the enrichment of well know homoacetogenic bacteria such as *Clostridium* and *Eubacterium, Geobacter, Syntrophomonas* and *Anaerolineaceae*, which perform syntrophically with H\(_2\) using archaea and also with *Methanosaeta*. Therefore, the addition of biochar allowed the formation of co-cultures that improved the production of methane and as a consequence, the performance of anaerobic digestion. An enhancement on the average methane yield was obtained for the codigestion of Op and SS under semicontinuous regimen. Higher amounts of biochar would be necessary to maintain the stability of the process, especially during substrate-induced inhibition. Biochar addition avoids system decay due to its adsorption capacity for inhibitors.

5. **Acknowledgements**

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projects cofinanced by FEDER funds. A. Sotres thanks the regional ‘Junta de Castilla y León’ for the postdoctoral contract associated with project ref: LE060U16.

6. References


the concentration of essential oil on orange peel waste biomethanization:


Table 1. Substrates and Biochar characterisation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Op</th>
<th>SS</th>
<th>Parameters</th>
<th>Biochar</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>5.15±0.05</td>
<td>5.66±0.05</td>
<td>Moisture (wt.%)</td>
<td>1.59±0.60</td>
</tr>
<tr>
<td>(\text{COD}_{\text{soluble}}) (mg L(^{-1}))</td>
<td>23000±460</td>
<td>3869±193</td>
<td>Volatile matter(^a) (wt.%)</td>
<td>21.0±2.0</td>
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<tr>
<td>TOC soluble (%)</td>
<td>43.72±0.5</td>
<td>34.69±0.5</td>
<td>Ash(^a) (wt.%)</td>
<td>47.4±1.8</td>
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<tr>
<td>VFA (mg L(^{-1}))</td>
<td>70±4</td>
<td>2602±169</td>
<td>Fixed carbon(^{a,c}) (wt.%)</td>
<td>31.6±3.8</td>
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<tr>
<td>TS (g kg(^{-1}))</td>
<td>311±37</td>
<td>28.7±0.6</td>
<td>C(^b) (wt.%)</td>
<td>74.5±5.3</td>
</tr>
<tr>
<td>VS (g kg(^{-1}))</td>
<td>302±35</td>
<td>23.28±0.5</td>
<td>N(^b) (wt.%)</td>
<td>0.7±0.2</td>
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<tr>
<td>Ammonia (mg L(^{-1}))</td>
<td>n.m</td>
<td>687±89</td>
<td>H(^b) (wt.%)</td>
<td>1.51±0.24</td>
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<tr>
<td>Organic matter (%)(^a)</td>
<td>75.2</td>
<td>59.68</td>
<td>S(^b) (wt.%)</td>
<td>0.05±0.04</td>
</tr>
<tr>
<td>N Kjeldahl (%)(^a)</td>
<td>0.82</td>
<td>7.34</td>
<td>O(^{b,c}) (wt.%)</td>
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<tr>
<td>C/N</td>
<td>53.3</td>
<td>4.72</td>
<td>O/C</td>
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<tr>
<td>Cu (mg kg(^{-1}))</td>
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<td>3.32±0.02</td>
<td>H/C</td>
<td>0.24±0.01</td>
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<td>Zn (mg kg(^{-1}))</td>
<td>5.34±0.10</td>
<td>19.61±0.25</td>
<td>HHV (MJ kg(^{-1}))</td>
<td>12.89±1.84</td>
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<tr>
<td>P (mg kg(^{-1}))</td>
<td>601±6</td>
<td>681.06±13.29</td>
<td>(\text{SBET isotherm. N}_2) (m(^2) g(^{-1}))</td>
<td>240±4.8</td>
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</tbody>
</table>

Chemical oxygen demand (COD), total organic carbon (TOC), volatile fatty acids (VFA), higher heating value (HHV), surface area (\(\text{SBET isotherm. N}_2\)), n.m.: not measured.

\(^a\) in a dry matter basis; \(^b\) in a dry ash free basis; \(^c\) Calculated by difference.
Table 2. Kinetic Gompertz parameters of batch digestion

<table>
<thead>
<tr>
<th></th>
<th>P1 (LCH₄ kg SV⁻¹)</th>
<th>Specific methane potential (LCH₄ kg SV⁻¹)</th>
<th>P_max (LCH₄ kg SV⁻¹)</th>
<th>R_max (LCH₄ kg SV⁻¹d⁻¹)</th>
<th>λ (d)</th>
<th>R²</th>
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<td>Orange peels batch digestion</td>
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<td></td>
<td></td>
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<tr>
<td>Op 2</td>
<td>14.71±3.9</td>
<td>103±5</td>
<td>90.94±2.36</td>
<td>10.89±0.2</td>
<td>1</td>
<td>7</td>
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<tr>
<td>Op 10 Biochar 3</td>
<td>18.63±2.6</td>
<td>209±10</td>
<td>196.87±2.95</td>
<td>14.27±0.2</td>
<td>8</td>
<td>0.990</td>
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<tr>
<td>Op 30 Biochar 9</td>
<td>29.04±4.2</td>
<td>298±15</td>
<td>280.99±4.21</td>
<td>14.15±0.2</td>
<td>8</td>
<td>0.991</td>
</tr>
<tr>
<td>Sewage sludge batch digestion</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>SS 7</td>
<td>7.75±2.76</td>
<td>273±14</td>
<td>271.15±4.06</td>
<td>18.73±0.3</td>
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<td>0.997</td>
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<td>SS 10 Biochar 6</td>
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<td>364±18</td>
<td>367.95±5.51</td>
<td>23.13±0.4</td>
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<td>0.999</td>
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<td>SS 30 Biochar 5</td>
<td>16.22±3.0</td>
<td>425±21</td>
<td>412.96±6.19</td>
<td>33.39±0.6</td>
<td>6</td>
<td>0.998</td>
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<tr>
<td>Orange peels and sewage sludge batch codigestion</td>
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<td>Op+SS 5</td>
<td>12.81±2.1</td>
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<td>298.73±4.48</td>
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<td>Op+SS 10 Biochar 5</td>
<td>0</td>
<td>500±28</td>
<td>501.92±25.40</td>
<td>66.34±1.1</td>
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<td>Op+SS 30 Biochar 0</td>
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<td>704±36</td>
<td>704.10±32.10</td>
<td>75.53±3.2</td>
<td>3.25</td>
<td>0.995</td>
</tr>
</tbody>
</table>

P₁ is the initial methane production obtained, P_max is the maximum methane yield, R_max is the maximum methane production rate and λ is the lag–phase time.
Table 3. Main parameters of particle size analysis applied after sludge digestion

<table>
<thead>
<tr>
<th>Samples</th>
<th>Mean µm</th>
<th>Median µm</th>
<th>Mode µm</th>
<th>SSA cm² mL⁻¹</th>
<th>d10 µm</th>
<th>d50 µm</th>
<th>d90 µm</th>
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<td>19.2</td>
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<td>19.6</td>
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<td>19.6</td>
<td>57.1</td>
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<td>SS 30 Biochar</td>
<td>21.2</td>
<td>16.3</td>
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<td>8634</td>
<td>3.37</td>
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<td>43.3</td>
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Table 4. Main parameters of semicontinuous digestion of orange peels and sewage sludge

<table>
<thead>
<tr>
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<th>RC_Op+SS</th>
<th>RB_Op+SS</th>
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<tr>
<td><strong>HRT (days)</strong></td>
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<td>25</td>
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<tr>
<td><strong>Evaluation period (days)</strong></td>
<td>60</td>
<td>10</td>
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<tr>
<td><strong>Specific methane production (L CH₄ kg VS⁻¹)</strong></td>
<td>318±73</td>
<td>288±39</td>
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<tr>
<td><strong>Methane content (%)</strong></td>
<td>56.0±0.5</td>
<td>56.0±0.5</td>
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<tr>
<td><strong>H₂S content (ppm)</strong></td>
<td>100±2</td>
<td>85±1</td>
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<tr>
<td><strong>Ammonia (g L⁻¹)</strong></td>
<td>1.63±0.19</td>
<td>1.75±0.87</td>
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<tr>
<td><strong>pH</strong></td>
<td>7.64±0.10</td>
<td>7.59±0.10</td>
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<tr>
<td><strong>Total organic carbon soluble (g L⁻¹)</strong></td>
<td>0.24±0.12</td>
<td>0.49±0.20</td>
</tr>
<tr>
<td><strong>Total solid (g L⁻¹)</strong></td>
<td>23.2±2.4</td>
<td>21.0±3.1</td>
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<tr>
<td><strong>Volatile solid (g L⁻¹)</strong></td>
<td>14.4±0.1</td>
<td>14.4±0.1</td>
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<tr>
<td><strong>Volatile Solid feed (g)</strong></td>
<td>4.5±0.2</td>
<td>5.4±0.3</td>
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<tr>
<td><strong>Volatile solid removal (%)</strong></td>
<td>67±2</td>
<td>67±3</td>
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</table>
**Figure Captions**

**Fig. 1** Cumulative methane production and Gompertz adjustment curve: orange peels (Op) (a), sewage sludge (SS) (b) and codigestion systems (Op+SS) (c)

**Fig. 2** Parameters measured during batch digestion of orange peels (Op) and with biochar addition at 10 g L⁻¹ (Op 10 Biochar) and 30 g L⁻¹ (Op 30 Biochar): limonene (a), total polyphenols (b) and volatile fatty acids (acetic, propionic and butyric acid) (c)

**Fig. 3** Heat maps and hierarchical cluster at the family level, of the archaeal (a), and of the eubacterial community samples (b). The histogram shows the relative abundance of each family within a sample

**Fig. 4** Taxonomic classification at genus level for eubacterial (a) and archaeal (b) community of orange peels digestion. Groups making up less than 1% of the total number of sequences per sample were classified as “others”

**Fig. 5** Taxonomic classification at genus level for eubacterial (a) and archaeal (b) community of sewage sludge digestion. Groups making up less than 1% of the total number of sequences per sample were classified as “others”

**Fig. 6** Parameters measured during semicontinuous digestion of orange peels and sewage sludge: Specific methane production (a), biochar concentration (b), limonene (c), total polyphenols (d) and total volatile fatty acids (acetic, propionic and butyric acid) (e)

*For any of the figures not color is required in printed version*

*All graphs were created in Origin pro2015*
(a) Specific methane production (L CO₂ mg VS)

(b) Biochar concentration (mg L⁻¹)

c) Limonene (mg L⁻¹)

d) Total polyphenols (mg GAE L⁻¹)

e) Total VFA (mg L⁻¹)

---

- **RC_Op+SS**: Red Circle, Organic loading rate
- **SMP average RC_Op+SS**: Light Blue Line
- **RB_Op+SS**: Red Circle, Organic loading rate
- **SMP average RB_Op+SS**: Light Blue Line
- **Organic loading rate**: Black Line

---

**Time (days)**: 0-15 d (adaptation period)