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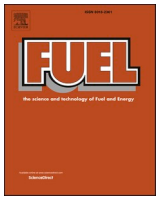

Full Length Article

Start-up of the mesophilic anaerobic co-digestion of two-phase olive-mill waste and cattle manure using volatile fatty acids as process control parameter

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ABSTRACT

In this work, the start-up and stabilization stages of mesophilic anaerobic co-digestion of 2POMW and CM in a semi-continuously fed stirred tank reactor (SSTR) were analyzed. Volatile fatty acids (VFAs) were monitored and used as the main control parameter for the start-up and stabilization stages, as well as to evaluate the potential inhibition episodes. The results showed that accumulation of propionic acid was the key factor in the inhibition of the methanogenic phase, leading to process imbalance. To avoid the problems associated with inhibition by high VFA concentrations, several reinoculations were performed using a suitable inoculum adapted to VFA degradation. The start-up phase was carried out in batch conditions for 97 days, reaching a final concentration of propionic acid of 12.77 mg/L. From that moment, the reactor was fed in a semi-continuous mode with a hydraulic retention time (HRT) of 40 days. A total period of 140 days was required to achieve a stable performance of the reactor with a methane productivity of 0.34 $L_{CH_4}/L_R d$.

1. Introduction

Anaerobic digestion is a biological process widely used in the treatment of agro-industrial waste [1,2]. Complex organic compounds are degraded by different populations of microorganisms to produce a biogas, with high methane percentage, and a stabilized effluent that can be used in agriculture [3]. However, the difficulties related to the start-up and stabilization of an anaerobic reactor depend on the characteristics of the substrates used and can affect the treatment performance. The co-digestion of different substrates may be a good alternative in waste management to solve the problem of the accumulation of toxic and inhibitory compounds, improve the nutrient balance and increase the biodegradability of organic matter, resulting in increased biogas production yields [4,5].

The olive oil industry generates large amounts of by-products and wastes in a short period of time. The characteristics of these substrates depend on the extraction system employed (mainly centrifugation in two or three phases). The olive mill wastewater (OMWW) is a by-product of the three-phase centrifugation system composed of vegetation and process waters. It is characterized by a high organic load, a high C/N ratio and an acidic pH, between 4 and 6 [6]. In addition, a solid by-product is obtained in this system (olive mill waste). The two-phase olive-mill waste (2POMW) is produced in olive mills with a two-phase centrifugation system. This by-product is characterized by high moisture content, a slightly acid character and a very high content of organic matter, consisting mainly of lignin, hemicellulose and cellulose [7]. Currently, 80% of the olive oil production in Spain is generated by using the two-phase extraction system and 800 kg of 2POMW per ton of processed olives are produced approximately by this system. According to the International Olive Council, the global olive oil production in 2020/2021 was 2.9 million tons and, hence, around 2.3 million tons of 2POMW were generated [8].

Cattle manure (CM) contains the excreted material from the animal (feces and urine), used bedding, as well as waste food, water and soil. According to the National Institute of Statistics of Spain [9], 44 millions tons of cattle manure were produced in 2021, being a potential source of contamination. The anaerobic digestion of CM has been widely studied [10,11] and its co-digestion with other substrates can result in a

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significant increase in methane yields [12].

There are several studies about anaerobic co-digestion of OMWW with various substrates: cattle manure, household waste and sewage sludge [13], poultry manure [14] and olive mill wastes [15]. However, only a single paper has been found in the literature for the co-digestion of 2POMW with other wastes. In this case, Goberna et al. evaluated the performance of a continuous anaerobic reactor for the co-digestion of CM and 2POMW in mesophilic and thermophilic temperatures [16].

The start-up and stabilization stages of an anaerobic digester usually require long periods of time due to the diversity of microorganism groups involved in the process and their complex interrelationship. The acidogenic microorganisms, in the hydrolytic and acidogenic phases, transform complex organic matter into volatile fatty acids (VFAs) and alcohols, producing CO_2 and H_2. The group of acetogenic microorganisms includes the propionate-utilizing acetogens and the butyrate-utilizing acetogens that metabolize these VFAs (butyric and propionic acids, respectively) to acetic acid, also generating CO_2 and H_2 in this process. In the methanogenic phase, methane is produced by the methanogenic microorganisms consuming acetic acid, CO_2 and H_2. The simultaneous development of all these microorganism populations in single-phase anaerobic reactors requires a very complex combination of environmental conditions to balance the different growth rates [17]. However, certain compounds, such as phenolic compounds present in OMWW, can cause inhibition of acetoclastic methanogenesis [18], slowing down the rate of the start-up process. Previous studies about the discontinuous co-digestion of 2POMW and CM showed an initial inhibition of methanogenesis due to the presence of polyphenols in the 2POMW [5].

The VFAs are produced in the hydrolytic-acidogenic phase and they are metabolized subsequently to generate methane and carbon dioxide. The starting of acetoclastic methanogenesis with VFAs degradation indicates an effective process start-up. Some authors have described a series of indirect parameters related to VFAs, such as the acidogenic substrate as carbon (ASC) and the dissolved acid carbon (DAC), that allow the analysis of the hydrolytic and acidogenic stages of anaerobic digestion [19] and more specifically in acidogenic reactors for hydrogen production [20].

Volatile fatty acids represent a critical parameter in the operation and control of anaerobic digestion [21]. Ahring et al. [22] investigated the VFAs as control parameters in the anaerobic digestion of manure (75% CM and 25% swine) at thermophilic temperatures. The levels of VFAs are good indicators of the metabolic state of the anaerobic digestion process and can help to detect possible disturbances in the system. Moreover, the accumulation of some acids, such as propionic acid, above certain limiting concentrations has a strong influence on the inhibition of the acetoclastic methanogenic stage. Asinari di San Marzano et al. [23] related the presence of propionate (between 1 and 2 g/L) with reduced methane productivities. Wang et al. [24] observed inhibition in the growth of methanogenic bacteria when the propionic acid concentration was over 900 mg/L. The imbalance in the levels of acetic acid and propionic acid can cause inhibition of methanogenesis. Moreover, Hill et al. [25] reported that acetic acid levels higher than 800 mg/L or a propionic to acetic ratio (HPr/HAc) higher than 1.4 indicate anaerobic digestion failure. Also, Lens et al. [26] observed that propionic acid degradation was influenced by the presence of butyric acid. Additionally, the presence of high concentrations of acetate together with a high hydrogen partial pressure influences the degradation of butyrate [27] and propionate [28]. The growth rates of the different populations of acetogenic microorganisms can influence the accumulation of VFAs in the reactor [29].

In this paper, the start-up stage of a semi-continuously fed stirred tank reactor (SSTR) for mesophilic treatment of a mixture of 2POMW and CM (60:40) was investigated. In this study, the analyses of individual VFAs and other indirect parameters (non-solubilized carbon (NSC), acidogenic substrate as carbon (ASC) and dissolved acid carbon (DAC)) have been used. These parameters are based on classical control parameters and provide complementary information about process evolution.

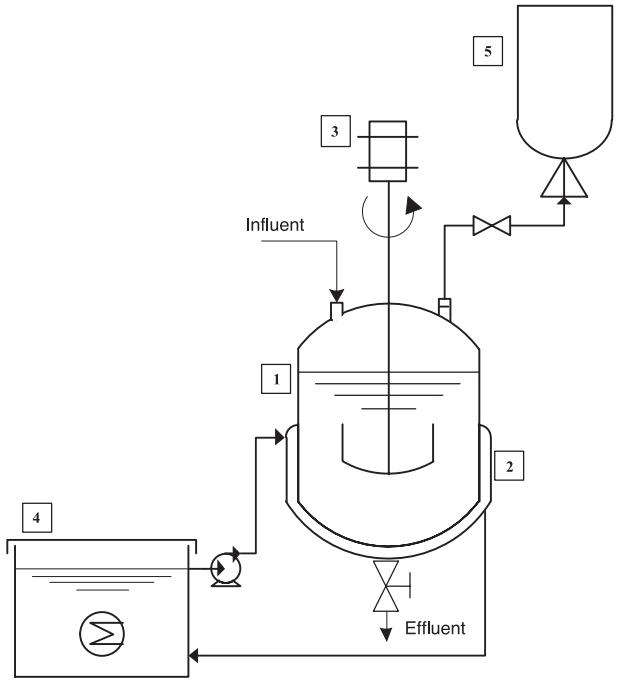

Fig. 1. Diagram of the laboratory-scale (CSTR). (1) Reactor, (2) jacket, (3) Motor agitation, (4) thermostatic water bath (7 L) and (5) Tedlar® bag (40 L).

Table 1
Characteristics of the substrates used (2POMW and CM) and the initial mixture.

Parameter	Units	2POMW	CM	Mixture
pH	–	5.35	7.52	6.57
Moisture	%	68.50	81.70	91.69
Total solids (TS)	g/kg	315.00	182.95	83.01
Volatile solids (VS)	g/kg *	282.64	143.91	56.84
Soluble chemical oxygen demand (CODs)	g O_2/kg *	142.16	43.20	28.15
Dissolved organic carbon (DOC)	g C/L	52.55	16.46	11.03
Total volatile fatty acid (TVFA)	g HAc/L	1.534	1.099	1.275
Total phenols	g/L	1.363	–	0.250
Organic matter (VS versus TS)	%	89.73	78.66	68.47
C	%	52.06	45.62	94.52
N	%	1.10	2.18	3.67
C/N	–	47.28	20.90	25.70
Total Alkalinity	g $CaCO_3$/L	2.60	5.50	4.85
Total Kjeldahl Nitrogen (TKN)	g/kg *	3.47	3.99	3.67

* Expressed in wet weight

2. Materials and Methods

2.1. Semi-continuously fed stirred tank reactor (SSTR)

The assay was conducted in a glass reactor of 5 L total volume and 4 L active volume (Fig. 1). The reactor was equipped with a discharge valve and several openings located at the top: an inlet for a stirring paddle, a biogas outlet port and a feeding inlet port. The reactor temperature was maintained at mesophilic regime (35 ± 1 °C) by circulating water through a jacket connected to a thermostatically controlled water bath (7 L of volume). A mechanical stirrer (operating continuously at 15 rpm) was connected to a stainless-steel rod with shovel-shaped end for adequate homogenization of the reactor contents. The biogas was collected in a TEDLAR® bag (40 L of volume) to accumulate the volume produced and sampling for composition analysis.

Table 2
Characteristics of the inoculums used in the reinoculations.

Parameter	DSS[(2)]	ESBC [(3)]	
	Day 0	Day 8 (stage 2)	Day 55 (stage3)
pH	7.2	7.3	7.6
TS (g/kg)	17.23	7.95	6.04
VS (g/kg) [(1)]	10.23	5.63	4.64
CODs (g O_2/kg) [(1)]	6.83	25.65	10.00
DOC (g C/L)	4.20	2.76	–
TVFA (g HAc/L)	0.020	0.128	2.460
Acetic acid (mg HAc/L)	17.98	101.44	2012.31
Propionic acid (mg HPr/L)	1.51	22.15	228.51
Butyric acid (mg n-HBu/L)	0.56	n/d	76.65

[(1)] Expressed in wet weight.
[(2)] Digested Sewage Sludge.
[(3)] Exhausted sugar beet cossettes (ESBC).

2.2. Substrates and inocula characterization

Fresh 2POMW was collected from an olive oil mill located in Olvera (Cádiz, Spain) operating with a two-phase centrifugation system. Sampling was performed in mid-season (September-November) to collect a fresh substrate. The 2POMW was sieved (<5 mm) to remove remains of seed. The CM was obtained from a semi-intensive livestock farm of dairy cattle in El Puerto de Santa Maria (Cádiz, Spain). This waste was composed of excreted material from the animals (feces and urine), used bedding (straw) and soil. CM was also sieved (<5 mm) prior to its use to remove straw and avoid obstruction of the discharge valve of the reactor. Both substrates were preserved at 4 °C to avoid variations in the composition and their characteristics are shown in Table 1.

About the inoculums, their characteristics are shown in Table 2. Firstly, a methanogenic inoculum was used at the starting-up of the assay and it was obtained from the effluent of a mesophilic sewage sludge digester of a municipal wastewater treatment plant. Later, a second inoculum was used in the adaptation phase and it was obtained from a laboratory mesophilic reactor treating exhausted sugar beet cossettes (ESBC) coming from an industrial sugar plant located in Jerez de la Frontera (Cádiz, Spain). This reactor was working at stable conditions at a HRT of 20 days with an average methane productivity of 0.64 $L CH_4/L_R \cdot d$.

2.3. Experimental procedure

For the analysis of the start-up process, two different periods were established: a first period for the adaptation of anaerobic microorganisms and a second period for stable semi-continuous operation at 40-d HRT [30]. The VFAs evolution was selected as the main parameter for process monitoring.

2.3.1. Adaptation of anaerobic microorganisms

The adaptation of anaerobic microorganisms to the mixture of co-substrates was carried out in batch mode at mesophilic temperature (35 °C ± 1). The reactor was started with a mixture 60:40 (w/w) of 2POMW and CM in order to obtain an optimal C/N ratio (20–30) for bacterial growth in anaerobic digestion as proposed by several authors [31,32]. Moreover, in accordance with previous studies [5], the tests were performed with a TS content of 10%, by dilution with distilled water. Therefore, the reactor was filled up to 80% of effective volume (3200 mL) with the co-substrates mixture and inoculated with 800 mL of the first inoculum source (DSS). During this batch phase, control of pH was maintained at 7.5 by the addition of the required volume of a Na_2CO_3 solution (2.8 M).

To solve the inhibition problems associated with high VFA levels and to accelerate the start-up of the methanogenic stage, it was decided to reinoculate the batch reactor two times (days 8 and 55 of experimentation) with a second inoculum source (ESBC) from a mesophilic effluent coming from an ESBC anaerobic reactor. The re-inoculations were carried out by direct addition of the inocula without prior removal of an equal volume of the reactor. This practice was used to avoid changing in the initial ratio of co-substrates mixture. The re-inoculations did not exceed 40% of the active volume of the reactor. Finally, before starting semi-continuous feeding, the final reactor volume was adjusted to 4 L (working volume). The inoculation details are shown in Table 3.

Table 3
Inoculum adaptation stage in batch mode.

Stage	Time interval (days)	Inoculum	Inoculation volume (mL)
1	0–8	Digested Sewage sludge	800
2	8–55	Effluent from ESBC reactor	500
3	55–98	Effluent from ESBC reactor	1000

2.3.2. Semi-continuous operation

As previously mentioned, to avoid variations during the test period in the feed composition, it was stored at 4 °C. The proportions of both co-substrates in the feed was the same as used in the batch reactor adaptation test (60:40; 2POMW:CM).

Before reactor feeding, TS concentration of the feedstock was adjusted to 10% by adding distilled water and the pH was adjusted around 8 by the addition of the required volume of a Na_2CO_3 solution (2.8 M). The reactor was batch fed daily with 100 mL of the above-described feed. Finally, the reactor was operated at 40-d HRT, giving an organic loading rate of 1.9 $gVS/L_R \cdot d$.

2.4. Analytical methods

In order to evaluate the batch adaptation phase of the inoculum and the start of methane production, the following physico-chemical parameters were determined in duplicate: Total solids (TS), volatile solids (VS), pH, total Kjeldahl nitrogen (TKN), dissolved organic carbon (DOC) and alkalinity. All these parameters were analyzed according to Standard Methods [33]. TS, VS and pH were determined directly on samples of the reactor effluent. For the rest of parameters, prior to the analytical determinations, the effluent samples were leached with distilled water (10:100 w/v) for 30 min and filtered through a glass-fiber filter of 0.47 μm pore size. The pH measurement was performed daily and the other parameters were measured three times a week. DOC was determined using a total organic carbon analyzer (Shimadzu TOC-5000) by the combustion-infrared method.

The biogas volume produced in the reactor was collected directly in a TEDLAR® bag (40 L volume) and measured using a high-precision flow gas meter (RITTER Drum-type Gas Meters, 0.1 mbar). Gas volumes were expressed at standard temperature and pressure (STP). The biogas composition (hydrogen, methane and carbon dioxide) was determined by gas chromatography (Shimadzu GC-14B) using a stainless steel column packed with Carbosive SII and a thermal conductivity detector (TCD). Helium was used as carrier gas with a flow rate of 30 mL/min and the temperature program used was: 7 min. at 55 °C; ramped at 27 °C min. until 150 °C. The detector temperature was 255 °C and the injector temperature 100 °C. The quantification of biogas was performed every two days during the semi-continuous phase of the assay.

The individual VFA concentrations (acetic, propionic, butyric, isobutyric, valeric, isovaleric, caproic, isocaproic and heptanoic acids) were determined by gas chromatography (Shimadzu GC-2010) using a flame ionization detector (FID) and a capillary column filled with Nukol™ (polyethylene glycol modified by nitroterephthalic acid). Hydrogen was used as carrier gas with a flow rate of 42.1 mL/min. In addition, synthetic air and hydrogen for the gas cromatograph flame ionization were used at 400 and 40 mL/min flow rate, respectively.

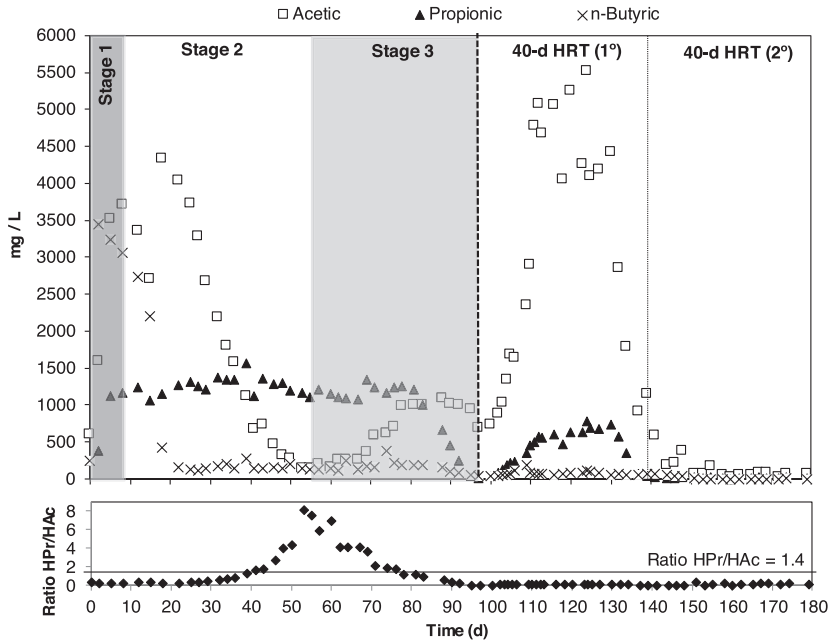

Fig. 2. Individual VFA evolution (acetic, propionic and n- butyric acids) and HPr/HAc ratio. The central dashed line differentiates the discontinuous and semi-continuous operating regime.

Nitrogen was used as makeup gas (30 mL/min). The temperatures of the injection port and detector were 200 and 250 °C, respectively. The samples were leached and filtered through a Teflon filter of 0.22 μm and were prepared by acidifying with a solution of phosphoric acid (1:2 v/v) and spiked with phenol as internal standard (500–600 mg/L). The frequency of analysis was at least three times a week and daily at critical phases of the assay. The total volatile fatty acid concentration (TVFA) was calculated as the sum of individual acid concentrations, weighted by their molecular weights, in order to be expressed as acetic acid concentration.

2.4.1. *Indirect parameters for anaerobic digestion evaluation: Non-solubilized carbon (NSC) and acidogenic substrate as carbon (ASC)*

NSC is the fraction of the organic carbon that has not been solubilized in the hydrolytic step. In this study, NSC was used to evaluate the hydrolytic activity at the start of the adaptation stage and to quantify the non-hydrolyzable organic matter. The ASC is the fraction of solubilized organic matter that has not been transformed into VFAs and thus can be used to evaluate the performance of the acidogenic phase during the assay.

NSC and ASC were calculated indirectly through other parameters such as VS, DOC and DAC (Eqs. (1), (3) and (4)). TOC was calculated through equation (2) from VS according to Fdez-Güelfo et al. [19].

$$NSC = TOC - DOC \tag{1}$$

$$TOC = VS \cdot 0.51 \tag{2}$$

$$ASC = DOC - DAC \tag{3}$$

$$DAC = \sum_{i=2}^{i=7} \cdot (A_i Hn_i 12)/MW_i \tag{4}$$

Dissolved acid carbon (DAC) represents the carbon content of the VFAs. DAC was determined using equation (4), where A_iH, represents

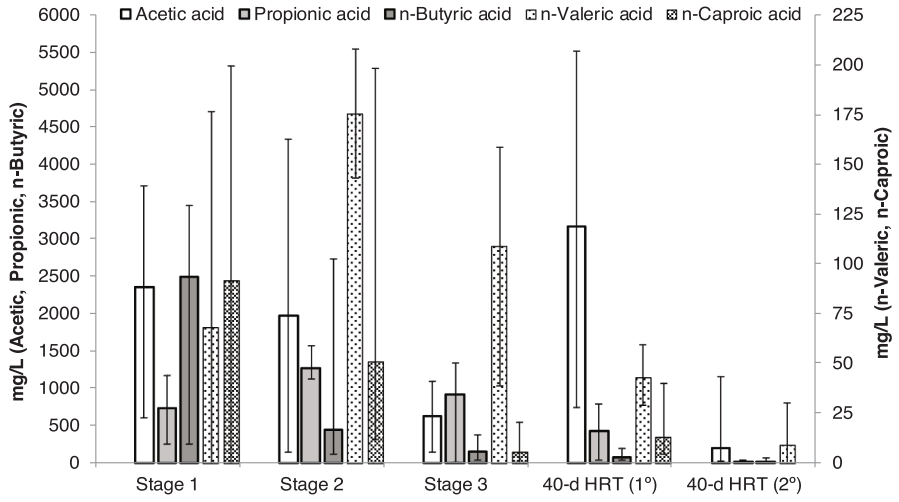

Fig. 3. Individual VFAs: average concentrations in bars; maximum and minimum values represented by the extremes of whiskers.

the concentration of each individual VFA, measured by gas chromatography; n_i is the number of carbon atoms of each A_iH; and MW_i is the molecular weight of each A_iH. NSC, TOC, DOC, VS, ASC, DAC and AiH are expressed as concentration units.

3. Results and discussion

For clarity, the discussion of the results has been organized into two major periods: an initial period of batch operation, for inoculum adaptation, and a subsequent period, operating in semi-continuous feeding mode, for process stabilization. Similarly, for a better understanding of the results in the inoculum adaptation stage, this period has been subdivided into three sub-stages corresponding to the different reinoculations developed, as it was described in *Materials and Methods* section.

3.1. Evolutions of the volatile fatty acids

The evolution of the VFA concentrations can help to establish when the inoculum is perfectly adapted so that it is appropiate to initiate the semi-continuous processing stage for the stabilization of the process. The evolution of the main VFAs and the average, maximum and minimum values of their concentrations are shown in Figs. 2 and 3, respectively. After inoculation with DSS (Stage 1), the hydrolysis and acidogenesis of the organic matter occurred during the first 8 days of the assay. In this stage, increases of acetic acid (HAc) and propionic acid (HPr) concentrations were observed, with maximum concentrations of 3713 and 1167 mg/L, respectively.

It is noticeable that, in contrast to HAc evolution, the HPr was not degraded and its concentration remains practically constant in later phases (Fig. 2). Van Lier et al. [29] observed that addition of 50 mM of acetate (3000 mgHAc/L) inhibited the propionate degradation in an UASB thermophilic reactor. This inhibition continued even when the acetate concentration was below the level of detection. In addition, Mawson et al. [34] observed that HAc concentration higher than 2000 mg/L also caused inhibition of HPr degradation. Moreover, in the present work, the n-butyric acid reached a maximum concentration of 3449 mg/L at 2 days of the assay, while the final values in this phase were around 3000 mg/L. Van Lier et al. [29] have indicated that inhibition of HPr degradation occurs in the presence of butyrate even when a culture enriched in butyrate-oxidizing microorganisms was added.

High concentrations of long-chain VFAs were also detected in this work. Thus, n-valeric and n-caproic concentrations were 176 and 199 mg/L, respectively, as a consequence of the hydrolysis process. The TVFA concentration reached 10337 mg/L (measured as HAc), indicating the inhibition of the process. This TVFA concentration is clearly higher than the 6 g/L proposed by Siegert and Banks [35] as inhibitory for the biogas production.

According to the results obtained in this work, the hydrolysis and acidogenesis of the co-substrates occurred adequately in stage 1. However, an inhibition of the acetoclastic methanogenesis was observed due to the short-chain VFAs accumulation, especially propionic and butyric acids. Likewise, values of the ratio of total VFA to alkalinity about 0.5 were observed in this period. This value is above the upper range (0.1–0.5) observed by de la Rubia Romero [36] in thermophilic anaerobic digestion of sewage sludge and by Fdez-Güelfo et al. [19] in the anaerobic digestion of the organic fraction of municipal solid waste (OFMSW) and it is indicative of instability of the process.

On the basis of the results obtained in stage 1 and to solve the inhibition problem detected, on day 8 of the assay (Stage 2), a reinoculation of the reactor was performed. In this case, effluent from a mesophilic reactor treating ESBC was used. This reactor was available in the research group and it had shown a high ability to degrade propionic and butyric acids. In this new stage (from 8 to 54 days), degradation of some of the major VFAs was observed. Acetic acid reached a maximum concentration of 4339 mg/L at day 20 (Fig. 2).

Propionic acid presented an average value of 1268 mg/L for this period, giving a HPr/HAc ratio greater than 1.4 after day 39 of the assay, which may be responsible for the inhibition of the acetoclastic methanogenesis process (Fig. 2). Some authors have observed inhibition of the methanogenic phase due to imbalance between the concentrations of acetic and propionic acids [17,37]. In this work, the HPr/HAc ratio was increased progressively throughout this period to reach a maximum value of 8.2 at day 53. On the other hand, the total VFA/alkalinity ratio was slightly below 0.1, discounting any inhibition of methanogenesis originated by the imbalance of alkalinity. Additionally, the concentration of n-valeric acid presented a maximum value of 208 mg/L around day 32, with a mean value in the period around 180 mg/L.

During stage 2, the removal yields of acetic and n-butyric acids were 97 and 95%, respectively, and the total VFA removal was 78%. However, the HPr/HAc ratio remained at inhibitory values of 7.6 due to the accumulation of propionic acid.

As a consequence of the accumulation of propionic acid observed, a new reinoculation was performed on day 55 (Stage 3) using an inoculum coming from the same reactor of ESBC. The system response was an initial decrease in the HPr/HAc ratio due to the VFA profile in the inoculum. Subsequently, the trend in the reactor was a progressive decrease in the HPr/HAc ratio to a final value of 0.02. The observed evolution of VFAs, after the reinoculation process, could be related to two main factors: an increase in acetic acid concentration as a result of the β-oxidation pathway of long-chain VFAs ($C \geq 4$) and, on the other hand, the effective degradation of propionic acid around day 81. The propionic acid concentration at the end of this stage was 12.77 mg/L and

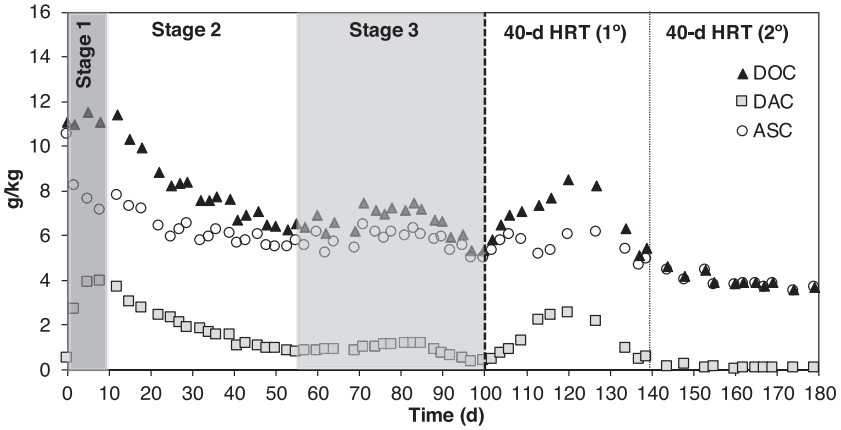

Fig. 4. Evolution of dissolved organic carbon (DOC), dissolved acid carbon (DAC) and acidogenic substrate like carbon (ASC). The central dashed line differentiates the discontinuous and semi-continuous operating regime.

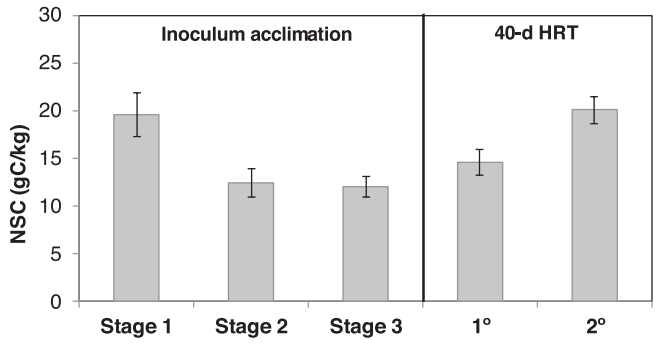

Fig. 5. Average non-solubilized carbon (NSC).

the removal efficiency for propionic acid reached 99%. Therefore, during this period, the propionic acid accumulated from the beginning of the process was removed. This fact, coupled with a low total VFA concentration (889 mg/L expressed as acetic acid), suggested the existence of an adapted microbiota and, hence, it was decided to start the semi-continuous co-digestion process with a 40-d HRT.

On day 98, the semi-continuous operation was started. At the beginning of this period, an increase of VFAs occurred again, due to the fast hydrolysis-acidogenesis of organic matter provided by the daily feed. So, acetic and propionic acids reached concentrations of 5516 and 739 mg/L, respectively (Fig. 3).

In addition, the concentrations of the long-chain volatile fatty acids ($C \geq 5$) were reduced, mainly the n-valeric acid from 208 to 60 mg/L. Additionally, the decrease of alkalinity implied an increase in total VFA/alkalinity ratio, reaching values around 0.4 on day 125 of the assay. This can affect the stability of the process, as it was described by other authors for sewage sludge digestion reactors [38].The removal of acetic, propionic and long-chain VFAs ($C \geq 5$) occurred from day 127 being coupled with a net methane production (Fig. 6). The removal yield of TVFA in the first 40-d HRT tested was 83.2%. As a result, from day 150 of the assay (in the second 40-d HRT), acetic and propionic acids reached average values of 70 and 14 mg/L respectively, and the methane productivity was 0.34 $L/L_R d$. The stability of the reactor was also confirmed by the total VFA/ alkalinity ratio, which remained below the instability level (<0.1). All of these results indicated that the anaerobic digestion process had been started and stabilized efficiently for the co-digestion of 2POMW and CM.

3.2. Indirect parameters (NSC, ASC and DAC) for start-up evaluation

The analysis of parameters such as the acidogenic substrate as carbon (ASC), non-solubilized carbon (NSC) and dissolved acid carbon (DAC) can be appropriate to assess the performance of the different microbial stages involved in the anaerobic digestion process [19]. The trends of DOC and DAC and the average values of NSC are shown in Figs. 4 and 5, respectively, for the different experimental stages. As it can be seen in Fig. 4, the hydrolysis, acidogenesis and acetogenesis occur during the first 8 days (Stage 1). A fraction of DOC was transformed into VFA as indicated by ASC decrease and DAC increase. Simultaneously, the NSC levels decreased between stages 1 and 2 (Fig. 5), from 15.9 to 10.0 gC/kg, as a consequence of the hydrolysis of particulate organic carbon and its transformation into DOC.

The reinoculation with inoculum from the reactor treating ESBC was performed on day 8 of the assay (Stage 2), adding soluble organic matter to the reactor (Table 2). Thereafter, a gradual decrease in DOC, DAC and ASC was observed due to VFA degradation until the end of this phase when the observed values remained constant (Fig. 4). The NSC remained relatively constant during the stages 2 and 3, with an average value of 9.8 gC/kg (Fig. 5).

In this period (Stage 3), the fast increases of DOC and ASC observed were related to the carbon compounds supplied by the inoculum (ESBC) that had not yet degraded into VFA. Subsequently, no variations were observed in these parameters until day 81 of the assay. After day 81, a gradual decrease of DOC and DAC related to the metabolization of propionic acid was observed.

At the beginning of the semi-continuous operation, two periods related to adaptation of the microorganisms to the feeding were observed. Between days 110 and 115, the degradation of soluble compounds into VFA occurs, resulting in an increase of the soluble organic matter (DOC) measured as DAC (Fig. 4). This evolution was produced by the activity of the hydrolytic-acidogenic microorganisms on the organic matter. The fluctuations observed in the ASC during this period were due to the sequential hydrolysis of less biodegradable compounds present in the feed. During this period, a slight increase of NSC (Fig. 5), with an average value close to 15.0 gC/kg, was observed due to the less hydrolyzable fraction contained in the feedstock. From day 120 of the assay, the consumption of DAC was accomplished with an increase in the generation of methane (Fig. 6). This behaviour is indicative of the microorganism adaptation to the substrate.

From day 144 until the end of the operation period, the reactor operated at pseudo steady-state, since it was observed that the hydrolysis and acidogenesis were coupled with a constant production of methane. All the solubilized substrate, measured as DOC, was metabolized to generate acids, except a soluble fraction resistant to acidogenesis. Thus, the levels of ASC and DOC remained constant with concentrations around 3.9 gC/kg until the end of the assay for both parameters (Fig. 4). The NSC increased to average values close to 20 gC/

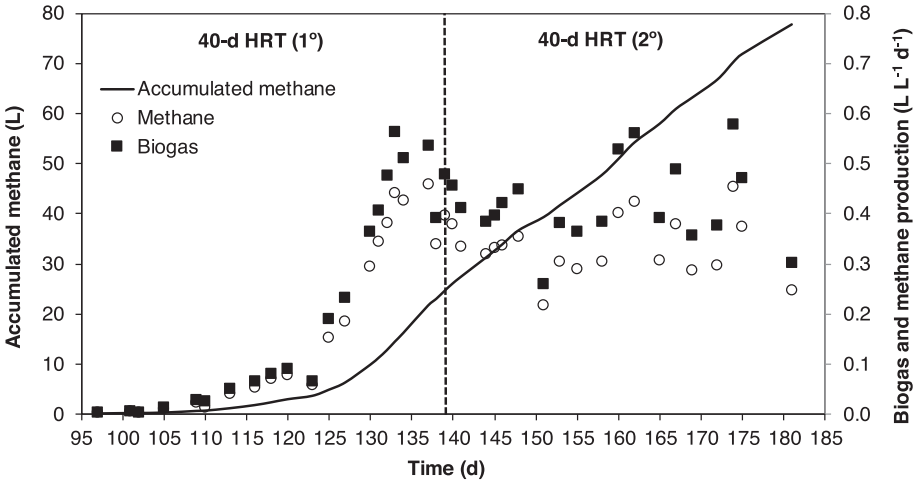

Fig. 6. Evolution of biogas and methane generation. The dashed line differentiates the period of each HRT.

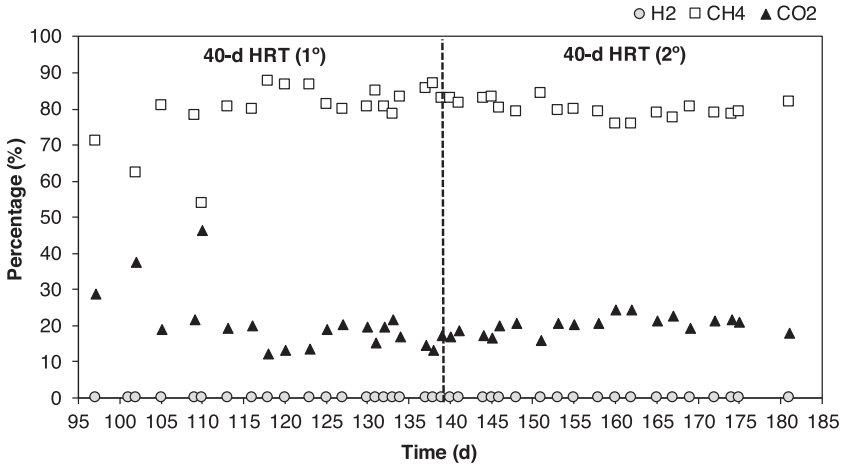

Fig. 7. Evolution of biogas composition.

kg due to the accumulation of non-biodegradable particulate organic matter. This led to stabilization of the NSC at this basal value. The evolution of these parameters agrees with the information provided by the VFA analyses, and it confirms a stable reactor operation in semi-continuous regime from day 144 of the start-up assay.

3.3. Biogas production

The monitoring of biogas evolution was realized during the semi-continuous operation period. As previously shown in section 3.1 (Evolution of VFA), the catabolism of propionic acid started on day 81. This result demonstrated the existence of an adapted microbiota and, therefore, a semi-continuous operation was started on day 98 with a 40-d HRT. In this last phase of the start-up assay, the reactor was operated for two periods of 40 days in a semi-continuous feeding regime to confirm the stability of the process. The evolution of the cumulative methane, productivity and biogas composition during the semi-continuous stage are shown in Figs. 6 and 7, respectively.

Methane production began at day 125, coinciding with the removal of acetic, propionic and long-chain VFAs. The average productivity of methane at the end of the first HRT tested (98–137 day) was 0.38 ± 0.06 $L/L_R{\cdot}d$. From this moment until the end of the assay (138–181 day), methane productivity remained relatively constant with an average value of 0.34 ± 0.06 L/L_Rd. The results obtained in this study are higher than those observed by Borja et al. [39] with a productivity of 0.240 L/L_Rd for mesophilic anaerobic treatment of 20% 2POMW with a 40-d HRT. In relation to the biogas composition (Fig. 7) during the semi-continuous operation phase, an absence of hydrogen was observed. The proportion of methane in the biogas was almost constant throughout this period. The average methane concentration was 82.5 ± 3.2% at the end of the assay. Tekin and Coşkun Dalgıç [40] observed compositions of methane in the biogas around 80% in anaerobic digestion of olive pomace (substrate similar to 2POMW). Moreover, the specific methane productivity at the end of assay (as VS removed) was 0.38 ± 0.08 $LCH_4/gVS_{removed}$. In Figs. 6 and 7, stable operation of the reactor was observed from day 144 of the assay approximately.

4. Conclusions

The start-up and stabilization stages of anaerobic co-digestion of 2POMW and CM (60:40 w/w) were analyzed. After a period of about 140 days, the start-up of a semi-continuously fed stirred tank reactor in mesophilic regime using an adapted inoculum from a reactor for digestion of ESBC was achieved. The microbiota provided by the initial inoculum from the digested sewage sludge was not effective in the start-up process. To achieve the objective of this work, the selection of an inoculum adapted to a substrate with similar characteristics to 2POMW, in relation to the lignocellulosic fraction and the tendency for propionic acid generation, was essential. The start-up process occurred in a total period around 140 days: 97 days for inoculum adaptation and 42 days for HRT stabilization (40 days). The use of the VFAs trends as a control parameter was essential in the assessment of the anaerobic process, both in the initial stage of inoculum adaptation (start-up period) and in the subsequent stage of stable operation of the reactor in semi-continuous feeding mode. The indirect parameters NSC and ASC, directly related to the VS, the content of soluble organic matter (measured as DOC) and the concentration of carbon in VFAs (measured as DAC), served to confirm the results obtained from VFA evolutions. The accumulation of propionic acid from the beginning of the assay and the imbalance in the composition of the main VFAs, led to a prolonged period of inhibition, delaying the start-up process. The methane production started around 28 days from the beginning of the semi-continuous operation stage (40-d HRT). The evolution of the different parameters (VFAs, DAC, NSC and ASC) was used to establish precisely when the stable operation was achieved under these conditions (approximately on day 144 of assay).

CRediT authorship contribution statement

J.A. Rubio: Investigation, Writing – original draft, Visualization. **L. A. Fdez-Güelfo:** Conceptualization, Methodology, Writing – review & editing, Writing – original draft, Visualization. **L.I. Romero-García:** Conceptualization, Methodology. **A.C. Wilkie:** Conceptualization, Methodology. **J.L. García-Morales:** Conceptualization, Methodology, Funding acquisition, Project administration.

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.

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