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Mesophilic anaerobic co-digestion of two-phase olive-mill waste and cattle manure: Optimization of semi-continuous process

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ABSTRACT

Anaerobic co-digestion of organic wastes is an effective technology for the management of two or more substrates with different characteristics. In this context, the main objective of this work was the optimization of biogas production for the treatment of a mixture of two-phase olive-mill waste (2POMW) and cattle manure (CM) (60:40 w/w) at mesophilic temperature range (35 °C). The effect of hydraulic retention time (HRT) on the performance and stability of the digestion process was studied. A decreasing series of HRTs in the range of 40–12 days was analyzed. The corresponding organic loading rates (OLR) were in the range 2.01–6.07 $gVS/L_R \cdot d$. Pseudo steady-state operation of the reactor was established for HRTs between 40 and 15 days. For 15-days HRT, the maximum values of methane productivity (0.94 $LCH_4/L_R \cdot d$) and specific methane yield (0.52 $LCH_4/gVS_{removed}$) were obtained while total acidity (measured as acetic acid) in the effluent were<150 mg/L, verifying process stability. In addition, the removal efficiencies of volatile solids (VS) and dissolved organic carbon (DOC) were 38 and 67 %, respectively. At 12-days HRT, decreases in methane production and organic matter removal efficiencies were observed, with values of 27 and 47 % for VS and DOC removal, respectively. Therefore, 12-days HRT was considered as inadequate for the anaerobic co-digestion of 2POMW and CM because a clear increase in volatile fatty acids was observed at the end of this period, leading to process destabilization and a decrease in biogas production.

1. Introduction

According to the International Olive Oil Council (IOC), global olive oil production in 2020/2021 was 2.9 million tons of which 1.4 million tons (48.3 %) corresponds to Spain [1]. Two-phase olive-mill waste (2POMW) is a by-product of olive production that employs a two-phase decanter system. The 2POMW generation occurs between the months of November and February when carrying out the collection and processing of olives. Approximately 800 kg of 2POMW per ton of processed olives is produced by the two-phase decanter system [2]. From these data, the estimated production in 2021 of 2POMW in the world and Spain was 2.3 and 1.1 million tons, respectively. This by-product is characterized by a high organic load, high moisture content, slightly acid pH, high C/N ratio (29.3–59.7), low alkalinity and low organic nitrogen content. It is composed mainly of lignin, hemicellulose and cellulose and contains a large amount of fats, proteins, carbohydrates and phenolic compounds [3]. Due to their high phytotoxicity, these characteristics may cause a significant environmental impact and contamination problems of soil and water if appropriate management is not performed.

On the other hand, about 44 million tons of cattle manure (CM) are produced annually in Spain [4]. The environmental problems associated with CM are caused by inadequate management (handling, storage, stabilization and land application) of animal manures [5]. Methane emissions to the atmosphere due to its fermentation and pollution of soil and groundwater are the major problems caused by CM, as well as being a source of pathogens [6,7]. The specific composition of CM depends on the type of livestock farm (high or low livestock density), diet and waste management, but generally contains material excreted by animals (feces and urine), waste feed and used bedding (straw, sand, mud, etc.) [8]. Many of these solid wastes are slowly biodegradable or recalcitrant substances [9]. Thus, most of the volatile solids (VS) in manure

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correspond to cellulose and hemicelluloses and can be readily converted to methane, but another substantial portion of the VS in CM is related to lignin compounds, which are difficult to biodegrade [8]. The CM is also characterized by high organic nitrogen content and, therefore, a low C/N ratio (15.5), and high pH [10].

The anaerobic co-digestion of food industry waste is an important alternative for its treatment [11–13]. This process involves the joint anaerobic treatment of two or more wastes of different origin and composition, obtaining a stabilized effluent potentially usable as source of biogas and/or fertilizer, a renewable energy source. The co-digestion of organic waste has been a successful technology, both in thermophilic and mesophilic temperature ranges [14]. The mixture of different substrates improves the balance of nutrients in the digestion medium, increases biogas production [15], and dilutes potentially toxic compounds and inhibitors. Ye et al. [16] obtained increases in the production of biogas by co-digestion of rice straw with kitchen waste or pig manure of 71.67 % and 10.41 %, respectively. Zarkadas & Pilidis [17] observed a 50 % increase in the specific methane yield in the co-digestion of process wastewater from table olives with cattle and pig manures, both in thermophilic and mesophilic temperature ranges, compared to digestion of the manure alone.

The physico-chemical characteristics of 2POMW and CM make them suitable for co-digestion since the mixture of both substrates would improve the C/N ratio. Moreover, it can also compensate for the low pH or alkalinity values and reduce the levels of inhibitory compounds, such as polyphenols in the case of the 2POMW. Some studies about the co-digestion of olive oil mill wastewater (substrate similar to 2POMW) with different types of manures have demonstrated the feasibility of this treatment. Angelidaki and Ahring [18] studied the co-digestion of olive oil mill wastewaters with cattle manure, household waste and sewage sludge. Gelegenis et al. [19] investigated the anaerobic digestion of olive oil mill wastewater with diluted poultry manure and Azbar et al. [20] studied the anaerobic co-digestion of olive oil mill wastewater with cheese whey and chicken manure. However, few studies have been published about the co-digestion of 2POMW and CM. Goberna et al. [21] evaluated the mesophilic and thermophilic anaerobic digestion of a mixture of CM and 2POMW. The study was performed using a 3:1 ratio (CM:2POMW) with a solids content of 11 % and at the specific hydraulic retention time (HRT) of 21.4 days. These authors observed a significant increase in biogas production (337 %) with respect to the digestion of cattle manure in both temperature regimes.

In co-digestion of substrates with high organic matter content, HRT is one of the most important control parameters. The HRT determines the mean residence time of the organic matter in the reactor and, therefore, the contact time between microorganisms and substrate. The HRT used in the start-up of suspended biomass anaerobic reactors is usually high enough to allow acclimatization of the microorganisms to continuous operation and to avoid the wash-out of the methanogenic population. Comino et al. [22] obtained stable biogas production in the co-digestion of cattle slurry and cheese whey operating at 42-days HRT. Rincón et al. [23] investigated a wide range of HRTs (between 108 and 15 days) in the anaerobic digestion of 2POMW, reaching a maximum methane productivity at 17-days HRT.

In the previous context, the main objective of this study was to optimize the co-digestion process of 2POMW and CM (60:40 ratio) in semi-continuously fed stirred tank reactors (SSTR) operated in the mesophilic temperature regime and identify the optimal and critical HRTs. The methane productivity and organic matter removal efficiency were used to evaluate the process performance.

2. Materials and methods

2.1. Semi-continuously fed stirred tank reactor

Two stirred tank reactors of 5 L total volume (4 L active volume) were used (Fig. 1). The top of the reactor allows the output of biogas and the feed inlet through two openings. The biogas produced was collected in a TEDLAR® bag (40 L volume) for each reactor.

A mechanical stirrer operating continuously at 15 rpm was connected to a stainless steel rod with a shovel-shaped end for adequate homogenization of the reactor contents. The reactor was equipped with a discharge valve that enables the output of the digestate. The temperature was maintained in the mesophilic range (35 ± 1 °C) by circulating water from a thermostatic bath (7 L volume) through the reactor heating

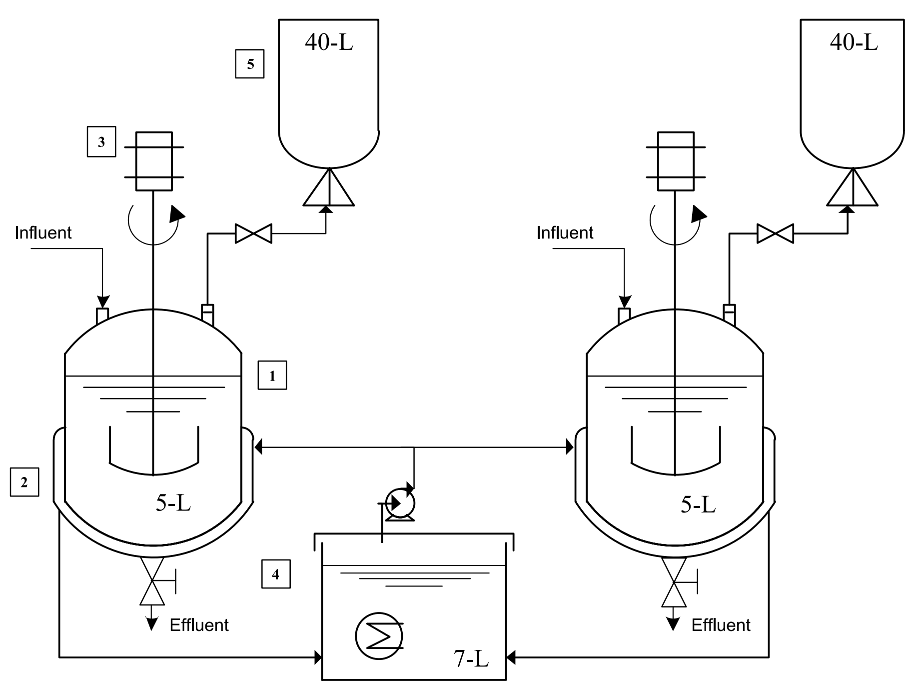

Fig. 1. Schematic representation of both SSTR utilized in this study. (1) Reactor, (2) heating jacket, (3) Motor agitation, (4) thermostatic water bath and (5) Tedlar® bag.

jacket.

2.2. Characteristics of co-substrates and feed

Fresh 2POMW was collected from an olive oil mill (Cooperativa Nuestra Señora de los Remedios) located in Olvera (Cádiz, Spain). The CM was obtained from a semi-intensive livestock farm of dairy cattle in El Puerto de Santa Maria (Cádiz, Spain). Both co-substrates were stored at 4 °C to avoid its degradation and prior to use were sieved (<5 mm) to remove some materials (residues of olive stones and livestock bedding) to avoid obstruction of the reactor discharge valve.

The co-digestion feed was composed of a mixture of both substrates at a 60:40 ratio (2POMW:CM) (w/w) with a total solids (TS) concentration around 10 %. It was maintained at 4 °C to avoid variations in its composition. The physico-chemical characteristics of 2POMW, CM and the feed are detailed in Table 1.

2.3. Experimental procedure

In this study, two semi-continuously fed stirred tank reactors (R1 and R2) were filled up to 80 % of effective volume (3200 mL) with a mixture 60:40 (w/w) of 2POMW and CM. The selected mixture results in a C/N ratio of 25.7, which is within the optimal range (20–30) for the growth of anaerobic microorganisms [24]. The TS content was adjusted to 10 % by addition of distilled water in order to dilute the high organic load, provided mainly by 2POMW. An inoculum was added at 20 % of the effective volume (800 mL) in both reactors.

The inoculum used in R1 was the effluent of a mesophilic anaerobic digester, adapted to substrates used in this study, operating in batch mode. In the case of R2, effluent of R1 was used as inoculum. Thus, in R2 the microorganisms from the inoculum were already adapted to the semi-continuous operation.

Only one dose of feed was added to the reactors each day (semi-continuous mode). The pH was maintained about 8 by using a Na_2CO_3 solution (2.8 M).

2.4. Semi-continuous operation

The semi-continuous operation was started in reactor-R1 using a high HRT (40 days), similarly to other authors with substrates of similar characteristics to 2POMW [25,26]. In the case of reactor-R2, the initial HRT was 30 days. Thus, a decreasing sequence in HRTs was established to evaluate the influence of this parameter on the organic matter removal efficiency, the daily methane generation rate and the specific methane yield. The series of HRTs analyzed was as follows: 40 days (R1), 30 days (R2), 20 days (R1), 15 days (R2) and 12 days (R1). Each HRT was maintained for three periods in order to establish stable operation in the reactor, except for 12-days HRT in which an additional period was established to evaluate its destabilization. The average values of organic loading rate (OLR) are shown in Table 2.

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

Parameter	Units	2POMW	CM	Average feeding (SD**)
pH	–	5.35	7.52	4.79 (0.19)
Moisture	%	68.50	81.70	91.31 (0.49)
Total solids (TS)	g/kg	315.00	182.95	86.91 (4.90)
Volatile solids (VS)	g/kg *	282.64	143.91	75.95 (5.08)
Soluble chemical oxygen demand (CODs)	gO_2/kg *	142.16	43.20	26.55 (2.25)
Dissolved organic carbon (DOC)	gC/L	52.55	16.46	11.04 (0.93)
Total volatile fatty acid (TVFA)	mgHAc/L	1534.46	1099.35	829.29 (66.31)
Organic matter	%	89.75	78.65	–
Carbon (C)	%	52.06	45.62	–
Nitrogen (N)	%	1.10	2.18	–
C/N ratio	–	47.28	20.90	–
Total Alkalinity	$gCaCO_3$/L	2.60	5.50	–
Total Kjeldahl Nitrogen (TKN)	g/kg *	3.47	3.99	–
Ammonia	gNH_3-N/kg *	0.38	2.16	–

*Expressed in wet weight
**Standard deviation

2.5. Analytical methods

For the substrate characterization and process monitoring and control, the following analytical determinations were used. Total solids (TS), total volatile solids (VS), organic matter (OM), pH and total Kjeldahl nitrogen (TKN) were determined directly in the effluent samples. Moreover, soluble chemical oxygen demand (sCOD), dissolved organic carbon (DOC), alkalinity, ammonia (NH_3-N) and volatile fatty acids (VFA) were measured in samples previously lixiviated with distilled water and filtered. The lixiviation was performed for 30 min and the effluent:water ratio used was 10:100 (w/v). All parameters were determined by duplicate according to Standard Methods [27] at least three times a week, except pH measurement which was daily.

The determination of DOC was conducted in a total organic carbon analyzer Shimadzu® TOC-5000 by combustion-infrared analytical method. The analysis of VFAs (from acetic acid to heptanoic acid) was performed using a Shimadzu® GC-2010 gas chromatograph equipped with a flame ionization detector (FID) and a capillary column filled with Nukol (polyethylene glycol modified by nitro-terephthalic acid). Nitrogen was used as carrier gas with a flow rate of 42.1 mL/min and hydrogen and synthetic air as gas chromatograph flame ionization. The temperatures of the injection port and detector were 200 and 250 °C, respectively. Before analysis, the samples were filtered through a 0.22 μm Teflon filter, acidified with a solution 1:2 (v/v) of phosphoric acid and phenol was added as internal standard. Total acidity (TVFA), expressed as acetic acid, was calculated by weighting the different concentrations of the individual VFAs by their molecular weights.

The biogas produced was collected in a 40 L Tedlar® bag and the measurement of the gas volume was carried out by means of a high precision gas meter (RITTER® Drum-type, 0.1 mbar). Gas volumes were expressed at standard temperature and pressure conditions. The biogas composition (hydrogen, methane and carbon dioxide) was determined using a Shimadzu® GC-14B gas chromatograph equipped with a stainless steel column packed with Carbosive® SII and a thermal conductivity detector (TCD). Operating conditions were as follows: 7 min at 55 °C; ramped at 27 °C/min until 150 °C; detector temperature: 255 °C; injector temperature: 100 °C. Helium was used as carrier gas with a flow rate of 30 mL/min. The volume of biogas and its composition were determined every couple of days.

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

The parameters non-solubilized carbon (NSC) and acidogenic

Table 2
Operational conditions in both reactors.

Reactor	HRT (day)	OLR (gVS/L_R·day)	OLR ($gDOC/L_R$·day)
1	40	2.01	0.29
2	30	2.68	0.39
1	20	3.95	0.58
2	15	5.36	0.76
1	12	6.07	0.84

HRT: hydraulic retention time
OLR: organic loading rate
VS: volatile solids
DOC: dissolved organic carbon

substrate as carbon (ASC) were used to evaluate the hydrolytic and acidogenic phase performance during the assay. The NSC is the fraction of the organic carbon that has not been solubilized in the hydrolytic step. The ASC is the fraction of solubilized organic carbon that has not been transformed into VFAs and thus serves to evaluate the evolution of the acidogenic phase.

Both parameters were calculated from the determination of VS, DOC and dissolved acid carbon (DAC), according to equations (1), (3) and (4) described by Fdez-Güelfo et al. [28]. The total organic carbon (TOC) was calculated from equation (2) as suggested by Navarro et al. [29]. DAC is a measure of the carbon content in the VFAs and it is determined by equation (4) where, A_iH represents 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.

$$NSC = TOC - DOC \quad (1)$$

$$TOC = VS \bullet 0.51 \quad (2)$$

$$ASC = DOC - DAC \quad (3)$$

$$DAC = \sum_{i=2}^{i=7} \bullet (A_i H n_i 12)/MW_i \quad (4)$$

3. Results and discussion

The analysis of process evolution in the different HRTs tested allowed the determination of the optimal and critical values of the HRT. The results of the statistical analysis performed, as it will be showed in Table 6 (subsection 3.5), indicated the validity of using the results of the operation in both reactors as a single data set for this purpose.

Optimal HRT can be defined as that which permits the digestion process to reach the maximum organic matter removal while the biogas production is also maintained stable at maximum values. Moreover, critical HRT can be defined as the HRT in which destabilization of the process occurs.

3.1. Evolution of total volatile solids

In Table 3, the VS removal efficiencies are shown. As it can be seen, a decrease in removal efficiency of VS was linked to the increase in OLR. Thus, the removal efficiencies of VS diminished from 49.4 % (40-days HRT) to 26.9 % (12-days HRT).

Trujillo et al. [30] obtained similar results in the mesophilic co-digestion of tomato-plant wastes and rabbit wastes. The decrease of VS removal percentages at lower HRTs can be related to the lower period of time available for the microorganisms to metabolize the hardly biodegradable fractions in the feed. Thus, the VS concentration in the effluent at the end of each HRT can be related to the basal levels of slowly biodegradable compounds of the feedstock as these levels showed an increase when the HRT decreased. The average VS concentration was between 40.4 ± 2.4 g/kg at 40-days HRT and 58.4 ± 0.6 g/kg at 12-days HRT. The average concentration of VS at 15-days HRT was 49.7 ± 1.5 g/kg and it can be observed that the greater value of this parameter at 12-days HRT is indicative of the process destabilization observed in R1.

Table 3
Average organic matter removal efficiencies and average values of soluble carbon parameters (DOC and ASC) and non-solubilized carbon (NSC) for each HRT.

Reactor	HRT (day)	VS (%)	DOC (%)	NSC (gC/kg)	DOC (gC/kg)	ASC (gC/kg)
1	40	49.4 (±3.1)	70.3 (±2.0)	17.68 (±1.14)	3.50 (±0.20)	3.41 (±0.23)
2	30	44.8 (±4.9)	69.8 (±1.2)	17.83 (±2.18)	3.59 (±0.15)	3.54 (±0.15)
1	20	41.2 (±3.1)	69.1 (±1.2)	19.40 (±1.14)	3.53 (±0.14)	3.49 (±0.13)
2	15	37.8 (±4.9)	66.6 (±0.6)	19.95 (±0.80)	3.76 (±0.09)	3.72 (±0.09)
1	12	26.9 (±0.8)	47.2 (±8.0)	22.46 (±0.86)	5.37 (±0.85)	4.28 (±0.08)

Note: values in parentheses correspond to standard deviation.

The hardly biodegradable compounds, that are not being solubilized, can be represented by the non-soluble carbon (NSC). As it can be seen in Table 3, the evolution of NSC was similar to the VS trend, increasing progressively from 17.68 to 22.46 gC/kg when HRTs were diminished from 40 to 12 days, respectively.

3.2. Evolution of dissolved organic matter

The analysis of the evolution of DOC and ASC provides information on the performance of hydrolytic and acidogenic stages in the reactors. An increase in the ASC, the fraction of the solubilized organic matter that has not been transformed into VFAs, may indicate a problem in the acidogenic phase. Increasing the ASC can also mean that the fraction of solubilized organic matter is recalcitrant and accumulates in the reactor.

The evolutions of the DOC and ASC at the different HRTs tested for both reactors are shown in Fig. 2. At the beginning of the assay, an increase of dissolved organic matter (measured as DOC) occurred. Maximum concentrations were reached at 40-days HRT (R1) and 30-days HRT (R2), with 8.50 and 6.59 gC/kg, respectively. This increase of the solubilized organic matter was related to the hydrolysis and acidogenesis of the feed. In the initial HRTs (40 and 30 days), the differences in the maximum DOC concentration were due to the origin of the inoculum used in each reactor. The microorganisms in R1 were not previously adapted to the semi-continuous operation and, therefore, the DOC levels achieved were higher. However, in R2 the increase of dissolved organic matter was related to the increase in the OLR because the microorganisms are acclimatized to a lower rate (40-days HRT). The evolution of ASC was similar to that observed for DOC. Initially, the rates of solubilization (hydrolysis-acidogenesis phase) and degradation (acetoclastic-methanogenesis phase) of the organic matter were decoupled. This caused an increase in ASC, which was accumulated in the reactors. Later, a net removal of DOC and ASC was observed in the reactors R1 and R2 on days 30 and 12, respectively, corresponding to the consumption of VFAs by the acetoclastic methanogenic microorganisms. Both parameters decreased to very similar values, which correspond to non-biodegradable substrate in the operating conditions, 3.41 and 3.54 gC/kg (as ASC) for the HRTs of 40 and 30 days, respectively (Table 3). Moreover, this indicated that all the solubilized organic matter, excluding the hardly biodegradable fraction of DOC, has been transformed into VFAs and did not accumulate in the reactors, because it was metabolized into methane and carbon dioxide. These evolutions of the DOC and ASC indicated the balanced performance of all the phases involved in the anaerobic digestion. The removal efficiencies of DOC are shown in Table 3 and reached values around 69 % for the HRTs of 40, 30 and 20 days.

In the intermediate HRTs (20 and 15 days), the DOC and ASC levels remained at similar values, indicating that the anaerobic digestion phases were well coupled. Despite this, in 15-days HRT (R2), a slight decrease in DOC removal efficiency was observed as a result of the increase of OLR, with a value of 67 % (Table 3). The average concentration of ASC in the HRTs 40, 30, 20 and 15 days was 3.54 ± 0.13 gC/kg. This level represents the average concentration of recalcitrant compounds in the anaerobic co-digestion of 2POMW and CM, operating under stable conditions.

Destabilization of the reactor R1 was observed on day 231 of the test operating at 12-days HRT (Fig. 2). The dissolved organic matter, expressed as DOC and ASC, gradually increased to reach concentrations of 6.22 and 4.36 gC/kg, respectively, in the last period. The differences between the concentrations of DOC and ASC in this HRT indicate a disturbance or inhibition of methanogenic and acetogenic phases. Thus, a fraction of the VFAs generated was not transformed into biogas and

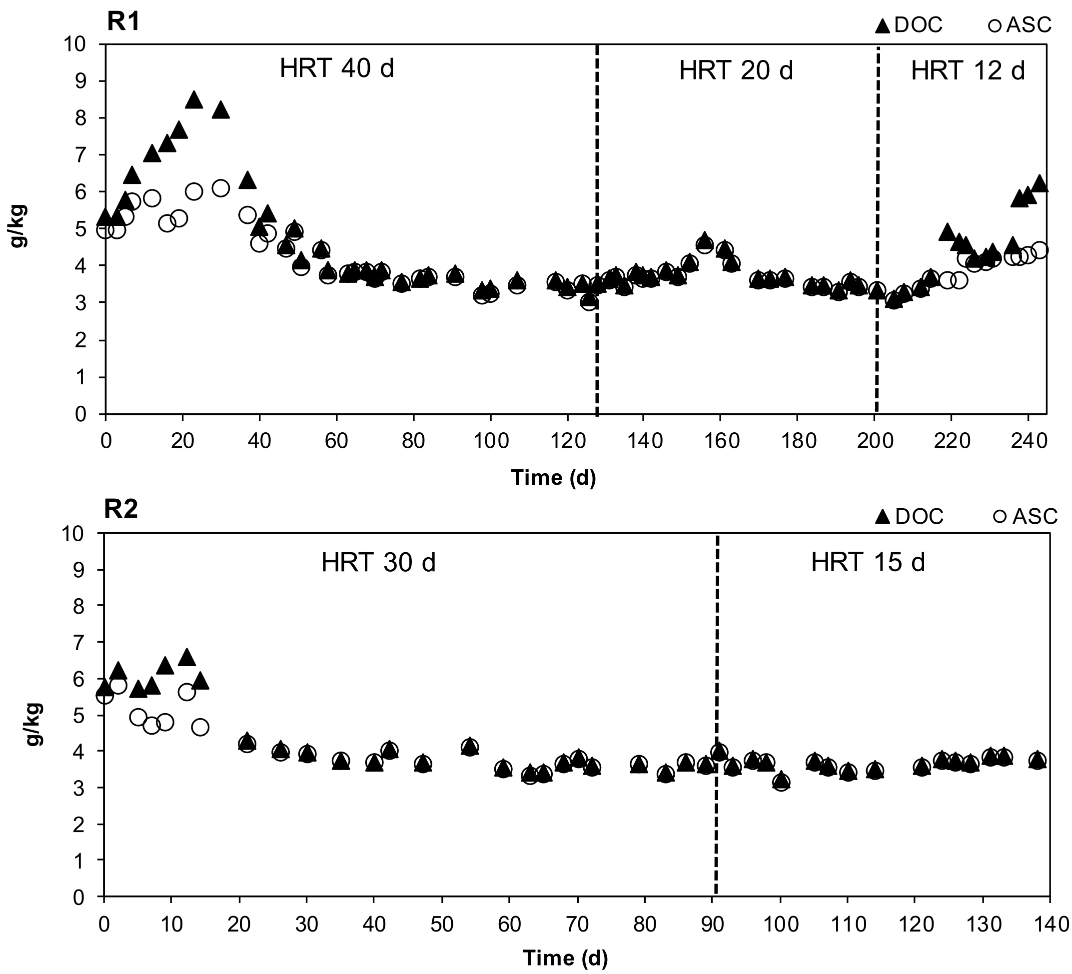

Fig. 2. Evolution of dissolved organic carbon (DOC) and acidogenic substrate as carbon (ASC) in the effluent of R1 and R2 reactors.

was accumulated in the reactor. Furthermore, the accumulation of compounds not transformed into VFAs can be seen in Fig. 2. This phenomenon was related to a gradual increase of ASC, which accumulated in the reactor. The destabilization of the process could be the result of accumulation of propionic acid, as HPr has an inhibitory effect on acidogenesis and leads to a lower removal efficiency of dissolved organic matter [31]. Thus, in this period, the percentage of DOC removal was 47 % (Table 3).

3.3. Evolution of volatile fatty acids (VFAs)

Volatile fatty acids (VFAs) are one of the most important control parameters in the anaerobic reactors because VFAs are intermediates in the degradation pathway from organic matter to methane [14]. Therefore, VFA concentrations should be as low as possible to ensure adequate performance of the anaerobic digestion process. The evolution of the main VFAs (acetic, propionic and *n*-butyric acids) and the total acidity (TVFA) for the different HRTs tested is shown in Fig. 3. An increase in the concentration of the VFAs was observed at the beginning of the semi-continuous operation mode in both reactors. This increase in the concentration of the dissolved organic matter in form of VFAs lasted for the first 33 days in R1 (40-days HRT) and for 14 days in R2 (30-days HRT) (Fig. 3). In R1, the maximum levels of acetic acid and propionic acid were 5516.07 and 788.66 mg/L, respectively. However, in R2, these concentrations were lower than in R1 despite the higher OLR. Thus, the maximum values of acetic acid and propionic acid in R2 were 3405.84 and 592.49 mg/L, respectively. Furthermore, the average levels and the evolution of *n*-butyric and the long-chain volatile fatty acids ($n > 4$) were not significant in both reactors.

Subsequently, a high degradation of the VFAs accumulated in the system was observed, leading to a final average concentration of total acidity in R1 (40-days HRT) and R2 (30-days HRT) of 95.03 (±34.60) and 114.96 (±37.06) mg/L, respectively. Thereafter, the total acidity in both reactors was maintained at low values in each of the HRTs tested. Thus, the TVFA concentrations were around 92.84 (±17.29) and 101.23 (±16.51) mg/L for the HRTs of 20 and 15 days, respectively. This implies a balanced operation of the anaerobic digestion process since these TVFA levels are indicative of the practically complete metabolization of the biodegradable organic matter provided by the feedstock.

During the first 14 days of the operation at 12-days HRT, the total acidity was below 130 mg/L. However, at the end of the test, a large increase in TVFA concentration (4540.01 mg/L) was registered. Also, as a difference with respect to previous HRTs, long-chain volatile fatty acids, namely *n*-butyric and *n*-valeric acids, were detected in this period. All these results, together with the evolution of the rest of the previously analyzed parameters, are indicative of process destabilization at this HRT.

3.4. Evolution of the biogas production

In Fig. 4, the accumulated production of methane for the HRTs tested is shown. The start of methane production occured on days 28 and 7 for reactors R1 and R2, respectively, operating at HRTs of 40 and 30 days. Furthermore, once stabilized, the methane productivities achieved were 0.37 and 0.39 $LCH_4/L_R \cdot d$, respectively (Table 4). The start of methane production was coupled with the metabolization of the VFAs, which had

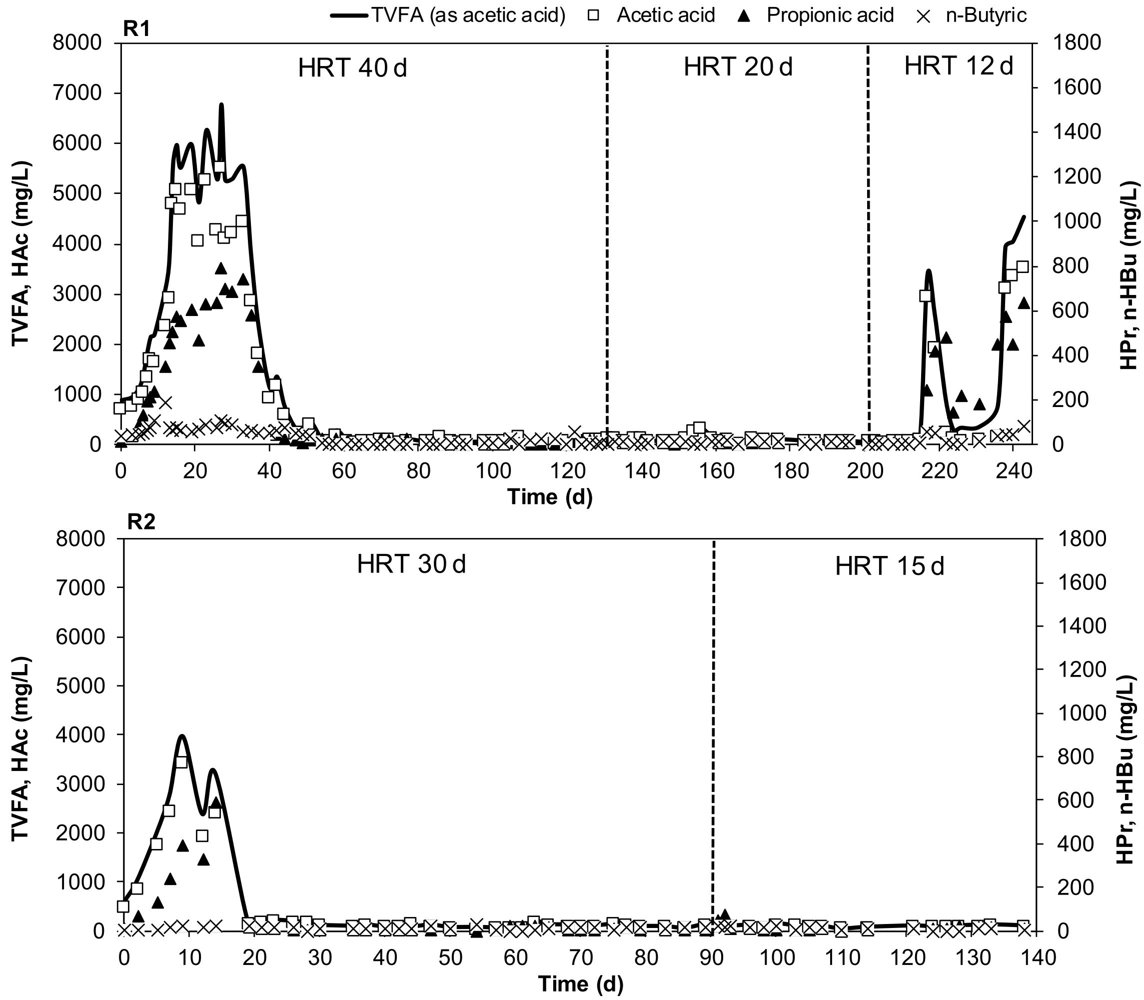

Fig. 3. Evolution of total volatile fatty acids (TVFA) and individual volatile fatty acids (HAc, HPr, *n*-HBu) in the effluent of R1 and R2 reactors.

accumulated in the reactors during the first days of the assay. In the subsequent HRTs tested, 20 (R1) and 15 (R2) days, the slope of the methane production curve versus time increased significantly due to the increase of OLR (Fig. 4). This resulted in an increase in methane production of 53 % for reactor R1 and 58 % for reactor R2.

For the 12-days HRT, the methane production was initially maintained or even slighty increased after the HRT change. However, at the end of this operational period (from day 231 of the test), as previously commented, the accumulation of VFAs in the system caused reactor destabilization and, hence, the methane production diminished. Destabilization was probably due to inhibition of the microorganisms responsible for the acetoclastic-methanogenic phase by the high concentrations of VFA [32], leading to an increase of organic matter in the reactor and a decrease in methane production.

On the other hand, the average biogas composition, in the stable period of each HRT tested, is shown in Table 4. Throughout the experimental period, H_2 was not detected in the biogas, due to its fast conversion into methane by hydrogenotrophic populations. At higher HRTs (40 and 30 days), the percentage of CH_4 was about 78 %, decreasing to 73–74 % in the following HRTs evaluated (20, 15 and 12 days).

As it is shown in Table 4, the methane productivity gradually increased from 0.37 to 0.94 $LCH_4/L_R{\cdot}d$ for HRTs between 40 and 15 days, as the OLR increased. Therefore, the observed inverse proportionality between HRT and methane productivity was related to the greater availability of metabolizable organic matter as a consequence of increasing OLR.

Moreover, as it can be seen in Table 5, decreasing the HRT from 40 to 20 days resulted in an increase in methane yield, expressed with respect to the VS added, from 0.14 LCH_4/gVS_{added} to the maximum value of 0.22 LCH_4/gVS_{added} obtained at 20-days HRT. This value is higher than obtained by Goberna et al. [21] (0.18 LCH_4/gVS_{added}), operating at 21.4-days HRT and OLR of 5.5 g COD/L·d, for the mesophilic co-digestion of cattle manure and 2POMW. In this study, a decrease in methane yield was observed at lower HRTs, with 0.19 and 0.11 LCH_4/gVS_{added} for the HRTs of 15 and 12 days, respectively.

The values of the specific methane yield for HRT 40, 30, 20, 15 and 12 days were 0.30, 0.38, 0.53, 0.52 and 0.42 $LCH_4/gVS_{removed}$, respectively (Table 5). The results obtained by Borja et al. [25] of 0.280 $LCH_4/gVS_{removed}$ in the mesophilic anaerobic digestion of diluted 2POMW (40 %) at 12.5-days HRT were lower than that obtained in this work. This demonstrates the improvement in the methane yield provided by the co-digestion of 2POMW and CM. In this study, similar results were observed in specific methane yield based on dissolved organic matter removed ($DOC_{removed}$).

3.5. Statistical analysis

The aim of this statistical study has been the comparison of the performance of the two reactors used in this work to the testing of the different HRTs. In order to consider the evolution of both reactors as a single system, the null hypothesis was that there were no significant differences in their evolutions. For the comparison, the evolutions of several parameters in the last period of each HRT (in which it is considered that the reactor operates under stable operating conditions)

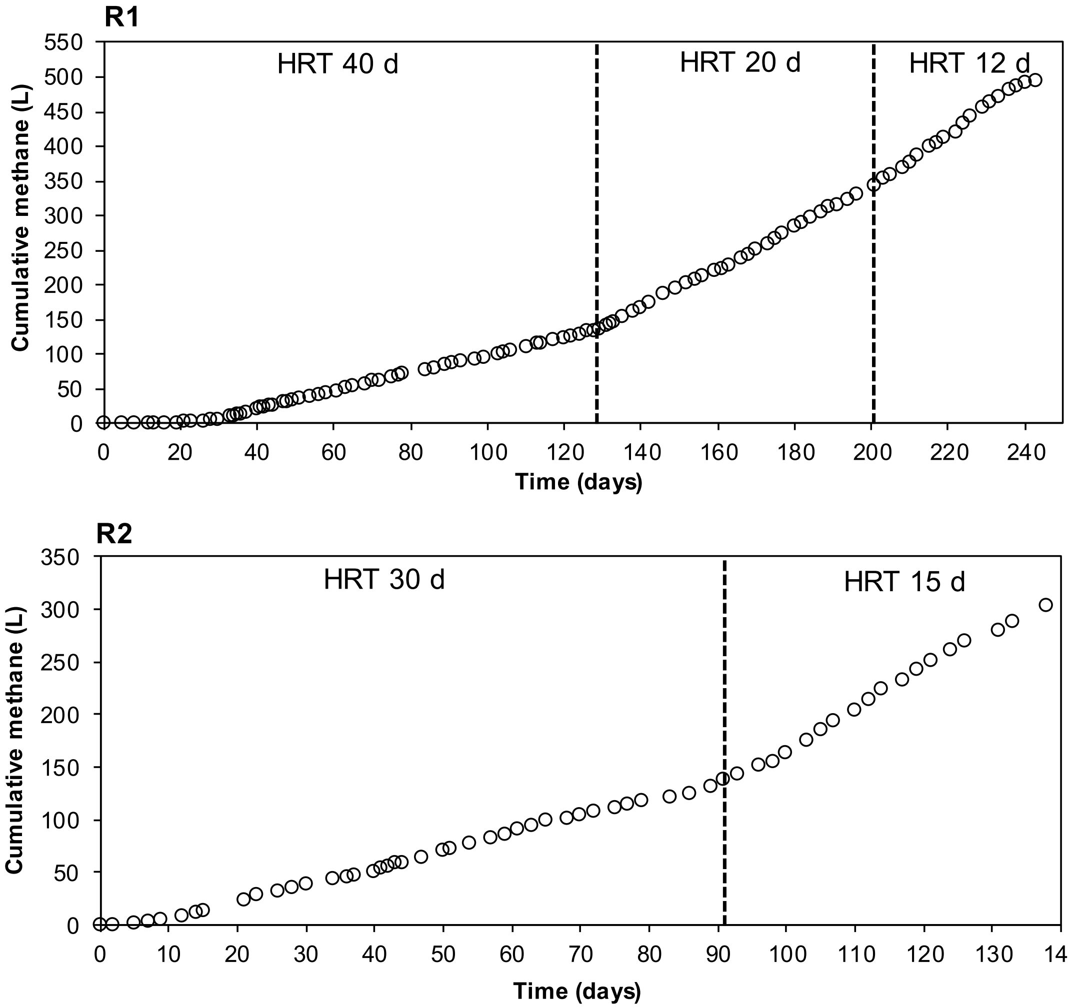

Fig. 4. Cumulative methane production in R1 and R2 reactors.

Table 4
Biogas composition and productivity for each reactor.

Reactor	HRT (day)	$LCH_4/L_R \cdot day$	$L_{Biogas}/L_R \cdot day$	CH_4 (%)	CO_2 (%)
1	40	0.37 (±0.11)	0.48 (±0.15)	78.1 (±2.9)	21.9 (±2.9)
2	30	0.39 (±0.13)	0.50 (±0.17)	78.3 (±1.6)	21.7 (±1.6)
1	20	0.80 (±0.20)	1.09 (±0.28)	73.3 (±1.9)	26.7 (±1.9)
2	15	0.94 (±0.15)	1.26 (±0.21)	74.4 (±1.3)	25.6 (±1.3)
1	12	0.71 (±0.29)	0.95 (±0.37)	73.9 (±3.0)	26.1 (±3.0)

have been used. Thus, the average values of different variables (VS and DOC removal, NSC, DOC, ASC, total acidity, methane productivity and methane yield) were compared to analyze the performance of both reactors.

The Shapiro–Wilk test was used to decide if a data set is normally distributed. The null hypothesis of this test is that the population is normally distributed. If the p-value is less than the chosen confidence level ($\alpha < 0.05$), then the null hypothesis is rejected and one concludes the data are not from a normally distributed population and therefore the Mann–Whitney U nonparametric test should be used. If the p-value is greater than the chosen confidence level ($\alpha > 0.05$), then the null hypothesis is not rejected and it is concluded that data came from a normally distributed population. In these cases, the parametric Student's *t*-test was used.

For the Mann–Whitney *U* test and Student's *t*-test, a confidence level of 0.05 was chosen. When the calculated p-value is less than the significance level ($\alpha < 0.05$), the null hypothesis is rejected, and the result

Table 5
Specific methane and biogas yield for each reactor.

Reactor	HRT (day)	LCH_4/gVS_{added}	L_{Biogas}/gVS_{added}	$LCH_4/gVS_{removed}$	$L_{Biogas}/gVS_{removed}$	$LCH_4/gDOC_{removed}$	$L_{Biogas}/gDOC_{removed}$
1	40	0.14 (±0.06)	0.17 (±0.07)	0.30 (±0.13)	0.36 (±0.16)	1.58 (±0.26)	2.00 (±0.33)
2	30	0.17 (±0.04)	0.22 (±0.06)	0.38 (±0.09)	0.49 (±0.12)	1.64 (±0.36)	2.11 (±0.47)
1	20	0.22 (±0.04)	0.29 (±0.06)	0.53 (±0.08)	0.72 (±0.12)	2.18 (±0.47)	2.95 (±0.69)
2	15	0.19 (±0.03)	0.25 (±0.04)	0.52 (±0.15)	0.70 (±0.20)	1.85 (±0.29)	2.50 (±0.40)
1	12	0.11 (±0.05)	0.15 (±0.06)	0.42 (±0.17)	0.56 (±0.21)	1.66 (±0.57)	2.25 (±0.72)

Table 6
Statistical results for the all parameters studied.

Parameter	W [a]	p-value*	T [b]	p-value*	U [c]	p-value*
VS removal (%)	0.886	0.002	–	–	222	0.522
DOC removal (%)	0.715	0.000	–	–	178	0.791
NSC (g/kg)	0.965	0.330	0.502	0.619	–	–
DOC (g/kg)	0.840	0.000	–	–	176	0.269
ASC (g/kg)	0.934	0.040	–	–	150	0.294
TVFA (mgHAc/L)	0.382	0.000	–	–	284	0.903
Methane productivity ($LCH_4/L_R \cdot d$)	0.930	0.005	–	–	296	0.739
Methane yield (LCH_4/gVS_{added})	0.978	0.696	0.037	0.970	–	–
Specific methane yield ($LCH_4/gVS_{removed}$)	0.978	0.703	0.304	0.762	–	–
Specific methane yield ($LCH_4/gDOC_{removed}$)	0.952	0.138	0.684	0.499	–	–

[a]*Statist Shapiro-Wilk's test.*
[b]*Statist Student's test.*
[c]*Statist Mann-Whitney's U test.*
**Level of significance: $P_\alpha = 0.05$.*

is said to be statistically significant. Otherwise, if the p-value calculated is greater than the significance level ($\alpha > 0.05$), the null hypothesis is accepted, and the result is said to be not statistically significant and there are no differences between the data sets. Statistical analysis of variables studied was developed with the software IBM SPSS Statistics 21. The results of the statistical analysis of the different variables are shown in Table 6.

4. Conclusions

The comparative statistical analysis of the variables used in this study indicated that there were no significant differences in the performance of both reactors. This allowed evaluating the evolution of system performance like a unique reactor and distinguishing the HRT that produced better yields.

At the higher HRTs (40 and 30 days), the removal efficiencies of VS were 49.4 % and 44.8 %. This implies a high content of slowly biodegradable organic compounds in the feedstock. These results were confirmed by the analysis of the evolution of NSC and DOC since accumulation of these compounds was observed throughout the experimental period, reaching the highest concentrations of both parameters, 23.7 gC/kg and 4.5 gC/kg, respectively, at 12-days HRT.

The reactors reached stable operation in all the HRTs tested, except at 12-days HRT. The greatest average methane productivity occurred in the HRT of 15 days (0.94 $LCH_4/L_R \cdot d$) and maximum methane yield at 20-days HRT (0.22 LCH_4/gVS_{added}). Considering the specific methane yield ($LCH_4/gVS_{removed}$), significant differences were not observed in the values obtained at HRTs of 20 and 15 days. Moreover, the DOC removal efficiency at 15-days HRT was similar to values observed for the higher HRTs with an average value of 66.6 %. Therefore, 15-days HRT can be considered as the optimum among the HRTs studied.

At the end of the stable period at 12-days HRT, a destabilization of the anaerobic co-digestion process occurred due to the accumulation of soluble organic matter. The VFA, mainly acetic and propionic acid, were increased at the end of the assay. The VS removal efficiency, with a value of 26.9 %, was the minimum reached in the series of HRTs tested and the DOC removal efficiency decreased considerably to 47.2 %. Considering these results, 12-days HRT can be considered as the critical HRT in the studied conditions.

CRediT authorship contribution statement

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

Data availability

No data was used for the research described in the article.

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References

[1] IOC, 2022. Production database. International Olive Council. Available from http://www.internationaloliveoil.org/ [accessed 23 June 2022].
[2] Rubio JA, Fdez-Güelfo LA, Romero-García LI, Wilkie AC, García-Morales JL. 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. Fuel 2022;325:124901.
[3] Morillo JA, Antizar-Ladislao B, Monteoliva-Sánchez M, Ramos-Cormenzana A, Russell NJ. Bioremediation and biovalorisation of olive-mill wastes. Appl Microbiol Biotechnol 2009;82:25–39. https://doi.org/10.1007/s00253-008-1801-y.
[4] National Institute of Statistics of Spain, 2022. https://www.ine.es/jaxi/Tabla.htm?tpx=51256&L=0 [accessed 23 June 2022].
[5] Hughes K, Wilkie AC. Cost-effective and Environmentally Beneficial Dairy Manure Management Practices. National Dairy Environmental Stewardship Council. 2005. Available online at: https://suscon.org/pdfs/dairies/pdfs/NDESCreportCostEffective.pdf.
[6] Sanchez C, Wilkie AC. Bioenergy production potential from small ruminant manure. *J Undergraduate Res* 2017;18(3):1–4. Available online at:.
[7] Cornejo C, Wilkie AC. Greenhouse gas emissions and biogas potential from livestock in Ecuador. Energy Sustain Dev 2010;14(4):256–66. https://doi.org/10.1016/j.esd.2010.09.008.
[8] Wilkie AC. Anaerobic digestion of dairy manure: design and process considerations. In: Dairy Manure Management: Treatment, Handling, and Community Relations. NRAES-176, p.301-312. Natural Resource, Agriculture, and Engineering Service, Cornell University, Ithaca, New York. 2005a.
[9] Rico JL, García H, Rico C, Tejero I. Characterisation of solid and liquid fractions of dairy manure with regard to their component distribution and methane production. Bioresour Technol 2007;98:971–9. https://doi.org/10.1016/j.biortech.2006.04.032.
[10] Bernal MP, Alburquerque JA, Moral R. Composting of animal manures and chemical criteria for compost maturity assessment. A review. Bioresour. Technol. 2009;100:5444–53. https://doi.org/10.1016/j.biortech.2008.11.027.
[11] Graunke RE, Wilkie AC. Examining the mechanisms of short-term solubilization of ground food waste for high-rate anaerobic digestion. Int Biodeterior Biodegrad 2014;86:323–33. https://doi.org/10.1016/j.ibiod.2013.10.007.
[12] González-González A, Cuadros F, Ruiz-Celma A, López-Rodríguez F. Energy environmental benefits and economic feasibility of anaerobic codigestion of Iberian pig slaughterhouse and tomato industry wastes in Extremadura (Spain). Bioresour Technol 2013;136:109–16. https://doi.org/10.1016/j.biortech.2013.02.031.
[13] Cavinato C, Fatone F, Bolzonella D, Pavan P. Thermophilic anaerobic co-digestion of cattle manure with agro-wastes and energy crops: comparison of pilot and full scale experiences. Bioresour Technol 2010;101:545–50. https://doi.org/10.1016/j.biortech.2009.08.043.

[14] Brinkman J. Anaerobic digestion of mixed waste slurries from kitchens, slaughterhouses and meat processing industries. In: Proceeding of the II International Symposium on anaerobic digestion of solid waste, Barcelona, Spain. 1999.

[15] Mata-Alvarez J, Macé S, Llabrés P. Anaerobic digestion of organic solid wastes. An overview of research achievements and perspectives. Bioresour Technol 2000;74:3–16. https://doi.org/10.1016/S0960-8524(00)00023-7.

[16] Ye J, Li D, Sun Y, Wang G, Yuan Z, Zhen F, et al. Improved biogas production from rice straw by co-digestion with kitchen waste and pig manure. Waste Manage 2013;33:2653–8. https://doi.org/10.1016/j.wasman.2013.05.014.

[17] Zarkadas IS, Pilidis GA. Anaerobic Co-Digestion of table olive debittering & washing Effluent, cattle manure and pig manure in batch and high volume laboratory anaerobic digesters: effect of temperature. Bioresour Technol 2011;102:4995–5003. https://doi.org/10.1016/j.biortech.2011.01.065.

[18] Angelidaki I, Ahring BK. Codigestion of olive oil mill wastewaters with manure, household waste or sewage sludge. Biodegradation 1997;8:221–6. https://doi.org/10.1023/A:1008284527096.

[19] Gelegenis J, Georgakakis D, Angelidaki I, Christopoulou N, Goumenaki M. Optimization of biogas production from olive-oil mill wastewater, by codigesting with diluted poultry-manure. Appl Energy 2007;84:646–63. https://doi.org/10.1016/j.apenergy.2006.12.001.

[20] Azbar N, Keskin T, Yuruyen A. Enhancement of biogas production from olive mill effluent (OME) by co-digestion. Biomass Bioenergy 2008;32:1195–201. https://doi.org/10.1016/j.biombioe.2008.03.002.

[21] Goberna M, Schoen MA, Sperl D, Wett B, Insam H. Mesophilic and thermophilic co-fermentation of cattle excreta and olive mill wastes in pilot anaerobic digesters. Biomass Bioenergy 2010;34:340–6. https://doi.org/10.1016/j.biombioe.2009.11.005.

[22] Comino E, Riggio VA, Rosso M. Biogas production by anaerobic co-digestion of cattle slurry and cheese whey. Bioresour Technol 2012;114:46–53. https://doi.org/10.1016/j.biortech.2012.02.090.

[23] Rincón B, Borja R, González JM, Portillo MC, Sáiz-Jiménez C. Influence of organic loading rate and hydraulic retention time on the performance, stability and microbial communities of one stage anaerobic digestion of two-phase olive mill solid residue. Biochem Eng J 2008;40:253–61. https://doi.org/10.1016/j.bej.2007.12.019.

[24] Yadvika, Santosh, Sreekrishnan TR, Kohli S, Rana V. Enhancement of biogas production from solid substrates using different techniques—a review. Bioresour Technol 2004;95(1):1–10.

[25] Borja R, Rincón B, Raposo F, Alba J, Martín A. A study of anaerobic digestibility of two-phases olive mill solid waste (OMSW) at mesophilic temperature. Process Biochem 2002;38:733–42. https://doi.org/10.1016/S0032-9592(02)00202-9.

[26] Tekin AR, Coşkun Dalgıç A. Biogas production from olive pomace. Resour Conserv Recycl 2000;30:301–13. https://doi.org/10.1016/S0921-3449(00)00067-7.

[27] APHA. Standard Methods for the Examination of Water and Wastewater, 22nd ed. Washington DC, USA: American Public Health Association, American Water Works Association, Water Pollution Control Federation. 2012.

[28] Fdez-Güelfo LA, Álvarez-Gallego C, Sales D, Romero LI. New indirect parameters for interpreting a destabilization episode in an anaerobic reactor. Chem Eng J 2012;180:32–8. https://doi.org/10.1016/j.cej.2011.10.091.

[29] Navarro AF, Cegarra J, Roig A, García D. Relationships between organic matter and carbon contents of organic wastes. Bioresour Technol 1993;44:203–7. https://doi.org/10.1016/0960-8524(93)90153-3.

[30] Trujillo D, Pérez JF, Cebreros FJ. Energy recovery from wastes. Anaerobic digestion of tomato plant mixed with rabbit wastes. Bioresour Technol 1993;45:81–3. https://doi.org/10.1016/0960-8524(93)90093-Q.

[31] Boone DR, Chynoweth DP, Mah RA, Smith PH, Wilkie AC. Ecology and microbiology of biogasification. Biomass Bioenergy 1993;5(3–4):191–202. https://doi.org/10.1016/0961-9534(93)90070-K.

[32] Hill DT, Gobb SA, Bolte JP. Using volatile fatty acid relationships to predict anaerobic digester failure. Trans ASAE 1987;30:496–501. https://doi.org/10.13031/2013.31977.