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RESPONSE OF ANAEROBIC CONSORTIUM POPULATIONS TO CARBON/NITROGEN (C/N) VARIATION DURING ANAEROBIC DIGESTION OF OLIVE MILL WASTEWATER

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Abstract- This paper is a study of the relationship between increasing Carbon/Nitrogen (C/N) ratio and the stability of the anaerobic digestion, which could be considered as a factor of process imbalance during anaerobic digestion of non-diluted and diluted Olive Mill Wastewater (OMW) in batch mode at mesophilic conditions. Volume of methane, methane yield, pH, volatile fatty acids concentration (VFAs), and chemical oxygen demand (COD) removal were followed to evaluate response to C/N ratio change. Effect of increasing organic load rate (OLR), as a second perturbation on optimal C/N ratio was also studied. The decrease in the C/N ratio led to a significant increase in methane production rate without any sign of perturbation. The decrease in the C/N ratio from 25 to 13 was accompanied by a significant increase in the methane production rate as it can be deduced from methane yield values recorded during the fermentation of ND-OMW and D-OMW with C/N ratio of 13. Thus, values as 0.62 and 0.78 l CH_4 per gram of introduced COD were recorded with C/N ratio of 13 when organic load rate of 4 $g.l^{-1}.day^{-1}$ was applied.

Keywords- Carbon/Nitrogen ratio, Olive mill wastewater, anaerobic digestion, methane yield, organic load rate

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Introduction

Olive Mill Wastewater is a by-product of a three phase process of olive oil extraction from olive. These liquid residues are 100-150 times more heavily loaded with pollutants than ordinary domestic wastewater [1]. The toxic effect of this black liquid is largely attributed to their high content of phenolic compounds (up to 15 $g.l^{-1}$) with different molecular weights [2-4], and other organic molecules, such as nitrogen compounds, sugars, pectins and organic acids make it heavily loaded with organic matter (COD: 80-200 $g.l^{-1}$; Biological Oxygen Demand (BOD_5): 50-100 $g.l^{-1}$). Besides organic molecules, these acids liquid contain high concentration of potassium, magnesium and phosphate salts [5].

Important quantities of this wastewater are produced especially in the Mediterranean region (98%) with up to 30 million m^3 of OMW produced every year. During the last years, many researches related to the study of OMW treatment were conducted, such as aerobic treatment, concentration, evaporation, lime precipitation, lagooning, incineration, co-digestion, ultrafiltration/reverse osmosis, etc. None of them led to industrial applications [6].

Therefore, anaerobic digestion was considered as one of the principle treatments which could be reducing OMW organic load with energetic valorization by methane recuperation. However, problems due to the inhibitory effect of polyphenols and to the low alkalinity of OMW have also been observed. Several approaches have been applied to overcome these problems, such as dilution of OMW [7, 8]. Nevertheless, dilution of OMW with water results in unnecessary large effluent volumes. Addition of chemicals is not economically and environmentally desirable. OMW contains low nitrogen sources which could be affected the anaerobic digestion performance, one of the most solution proposed to overcome this problem is mixing and digesting OMW with other effluents containing high organic nitrogen, such as municipal solid waste and young landfill leachate [9]. Moreover, co-digestion offers several advantages such as: (a) reduction of feed COD and total phenols concentration, (b) no need to add nutrients if OMW was mixed with effluents rich innitrogen and phosphorous,and (c) the opportunityof running a year round treatment plant based on the co-digestion of seasonally generated effluents [10]. In fact, these effluents (using in co-digestion) contain high concentrations of free ammonia (NH_3) (not ammonium (NH_4^+)) which is responsible for inhibiting the methanogenic activity during the anaerobic digestion [11].

This research is aimed at studying anaerobic digestion of crude and diluted OMW by means of lab-scale experimentations in order to optimize the C/N ratio by using urea as nitrogen source. Methane production, methane yield, pH and VFAs were monitored to evaluate process imbalance.

Material and Methods

OMW Characterization

Fresh olive mill wastewater was obtained from a press unit of olive oil production (BouaayadetFils manufactory, an industrial district of Dokkarat, Fez, Morocco). Crude OMW (ND-OMW) was diluted in order to obtain (D-OMW); and it was stocked at 4°C for future utilization. pH was measured by using a HANNA 9142 pH meter, COD was determined according to standard micromethod with a COD meter HACH and BOD_5 with the manometric method using a respirometer OXI TOPIS6 [12]. Total phenols were determined by using the colorimetric method with Folin-Ciocalteau reagent [13]. Total nitrogen, suspended solids and volatile suspended volatiles were determined according to Rodier (1996) [12]. The standard method of Soxhlet solid/liquid (organic solids of OMW/hexane) was utilized to determine total fats.

Analytical Methods

Total Organic Ccarbon Determination

The total organic carbon (TOC) was determined with a Shimadzu 5000 TOC analyzer equipped with an autosampler Shimadzu (ASI-5000A Autosampler) as described elsewhere [14]. The OMW sample was acidified by sulfuric acid addition in order to obtain pH<4 (just below) and to give no free carbonate anions in solution. The catalyst used to carry out the combustion reaction was platinum. The carrier gas was oxygen with a flow rate of 150 $ml.min^{-1}$. The detection was carried out by using an infrared detector NDIR (Non-Dispersive Infra-Red). Calibration of the analyzer was achieved with potassium hydrogen phthalate standards.

GC Analysis

Volatile fatty acids such as acetate, propionate, butyrate, and valerate were analysedby chromatograph GC. 500 µl of samples was centrifuged for 15 min at 15000 rpm, filtered through 0.22 µm filter (Millipore) and acidified with 10 µl of H_3PO_4 (50% V/V). Gas chromatograms were obtained on a gas chromatograph, with a Nukol Silica capillary column (30 m × 0.32 mm ID× 0. 25 m film thickness), equipped by a FID detector and CR6A SHIMADZU integrator. The injection port and detector temperature were 150 and 200° C, respectively. The initial oven temperature was 70°C, increasing by 15°C/min to 170°C and then 30°C/min to 190°C after which the temperature was held for 3 min. Nitrogen was used a carrier gas.

CH_4, CO_2 and N_2 were measured using a gas chromatograph GC11 (Delsi instruments) equipped with a HayeSepQ 60/80 (SUPELCO) column (maintained at 60°C), a thermal conductivity detector (current intensity of 160 mA) and a servotrace integrator (SEFRAM). Helium was used a carrier gas at a pressure of 1.3 bar.

Anaerobic Digestion of Olive Mill Wastewaters with Different C/N ratio

The C/N ratio was adjusted at 25, 20 and 13 using urea as nitrogen source, taking account the nitrogen content (0.56 $g.l^{-1}$) and the value of total organic carbon (21.80 $g.l^{-1}$) of OMW. The anaerobic digestion tests were performed in glass reactors (500 ml total volume). Reactors A13, A20, A25 used for ND-OMW, and B13, B20, B25 for D-OMW. These reactors were inoculated from a 100 liter anaerobic digester operating with the same type of substrate and fed with ND-OMW (reactors "A") and D-OMW (reactors "B"). The reactors were incubated in BINDER incubator under mesophilic conditions at 35°C ± 2°C. The results are averaged with two experimental measures. Sampling was carried out manually using syringes through two sampling ports located at the top of the reactor whereas the produced biogas was measured according to the water displacement principle. The experiment continues until observing the zero production of biogas,the produced volume of biogas and the other parameters were measured once per two days. Reactors were then feed with increasing organic load rate such as4, 6 and 8 g $COD.l^{-1}day^{-1}$in order to investigate the effect of OLR on optimal C/N ratio.

Results

OMW Characterization

OMW used in this study was obtained from a three-phases press unit of oil production. OMW's physicochemical characterization is presented in [Table-1]. Value of COD obtained is lower (48.67 $g.l^{-1}$) than the one studied by the traditional system of extraction, generally exceeding the 100 $g.l^{-1}$[15]. Higher COD/BOD_5 ratio (above 4) reflect less biodegradability of some organic substances represented essentially by phenolic compounds whose concentration is up of 13 $g.l^{-1}$. Moreover, OMW contains high concentration of residual oils, acidic character (pH of 4.98), and low nitrogen content (NTK of 0.56$g.l^{-1}$). Consequently, the results of the pollution's indicators imply that the industrial effluents of OMW are highly toxic and are much more difficult to treat by biological process only.

Table 1- OMW characterization

Parameters	Average value
pH	4.98 (±0.09)
COD ($g.l^{-1}$)	48.67 (±1.04)
BOD_5 ($g.l^{-1}$)	8.95 (±0.51)
COD/ BOD_5	5.41(±0.44)
Total polyphenols ($g.l^{-1}$)	13.04 (±0.61)
Fats ($g.l^{-1}$)	6.24 (±0.39)
Total solids ($g.l^{-1}$)	49.70 (±3.00)
Total volatiles ($g.l^{-1}$)	41.27 (±1.71)
Total organic carbon ($g.l^{-1}$)	21.80 (±0.05)
Total nitrogen ($g.l^{-1}$)	0.56 (±0.03)
C/N ratio	39.03 (±1.90)

Effect of C/N Ratio on Anaerobic Digestion of no Diluted OMW (ND-OMW)

The results of anaerobic digestion of no diluted OMW (ND-OMW) were examined for a period of one month with C/N ratio of 13, 20 and 25. Data related to methane production rate, methane yield, pH, and volatile fatty acids

[Fig-1a] shows the methane production rate at loading rate of 2 g $COD.l^{-1}day^{-1}$ corresponding to hydraulic retention times of 28 day. The stationary state was attained slowly and the volume of methane was up of one liter with a high value of 1.6 l recorded in the case of C/N ratio of 13. The methane yield is expressed as liter of CH_4 produced per gram of COD introduced. As can be seen from the [Fig-1b], methane yield rate increased until 0.40, 0.49 and 0.57 $l.g^{-1}$ for C/N ratio of 25, 20 and 13 respectively after 28 days. In other words, the increase of the nitrogen source in the medium leads to a significant increase in methane yield [Fig-1b]. For C/N of 13, this increase was apparent from the second day and continues on a regular basis during 28 days.

[Fig-1c] and 1d show variations of pH and VFAs production respectively. On the one hand, VFAs analysis of samples taken from reactors during 30 days of fermentation does not present any sign of perturbation and accumulation of VFAs in the medium, thus concentrations drop immediately to1.5 $g.l^{-1}$, and does not exceed the value of 2.5 g.l-1 during 30 days period of fermentation. On the other hand, the introduction of a nitrogen source as urea could have a positive effect on VFAs metabolism. Furthermore, the profile of pH of the digestion at different C/N ratio presented in [Fig-1c], in the medium-term of the experiments the buffer system was stable. So, the pH changed a little though the concentration of VFAs was higher. The pH dropped slightly at the fourth day and ranged between 7.5 and 8 until 16th day. Thus, pH variation rises to the optimum range (8, 8.4), but does not affect the methane production in mesophilic digestion conditions [Fig-1].

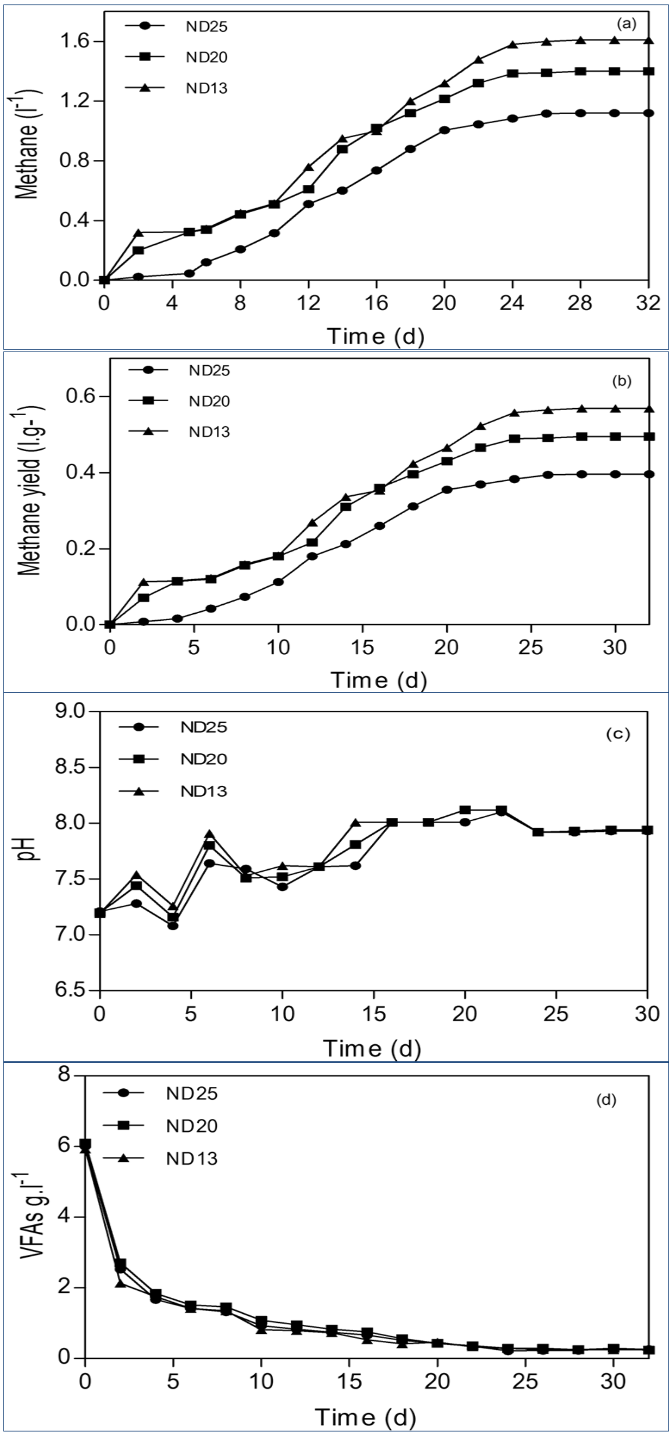

Fig. 1- Effect of C/N variation (13, 20 and 25) on anaerobic digestion during 30 days of no diluted OMW (ND-OMW): (a) methane production rate; (b) methane yield ($l.g^{-1}$); (c) pH; and (d) VFAs.

Effect of C/N Ratio on Anaerobic Digestion of Diluted OMW (D-OMW)

Anaerobic digestion of diluted OMW (D-OMW) was monitoring during a period of one month with varying the C/N ratio (13, 20 and 25). All results related to biogas production rate, methane yield, pH, and volatile fatty acids concentrations VFAs are shown in [Fig-2].

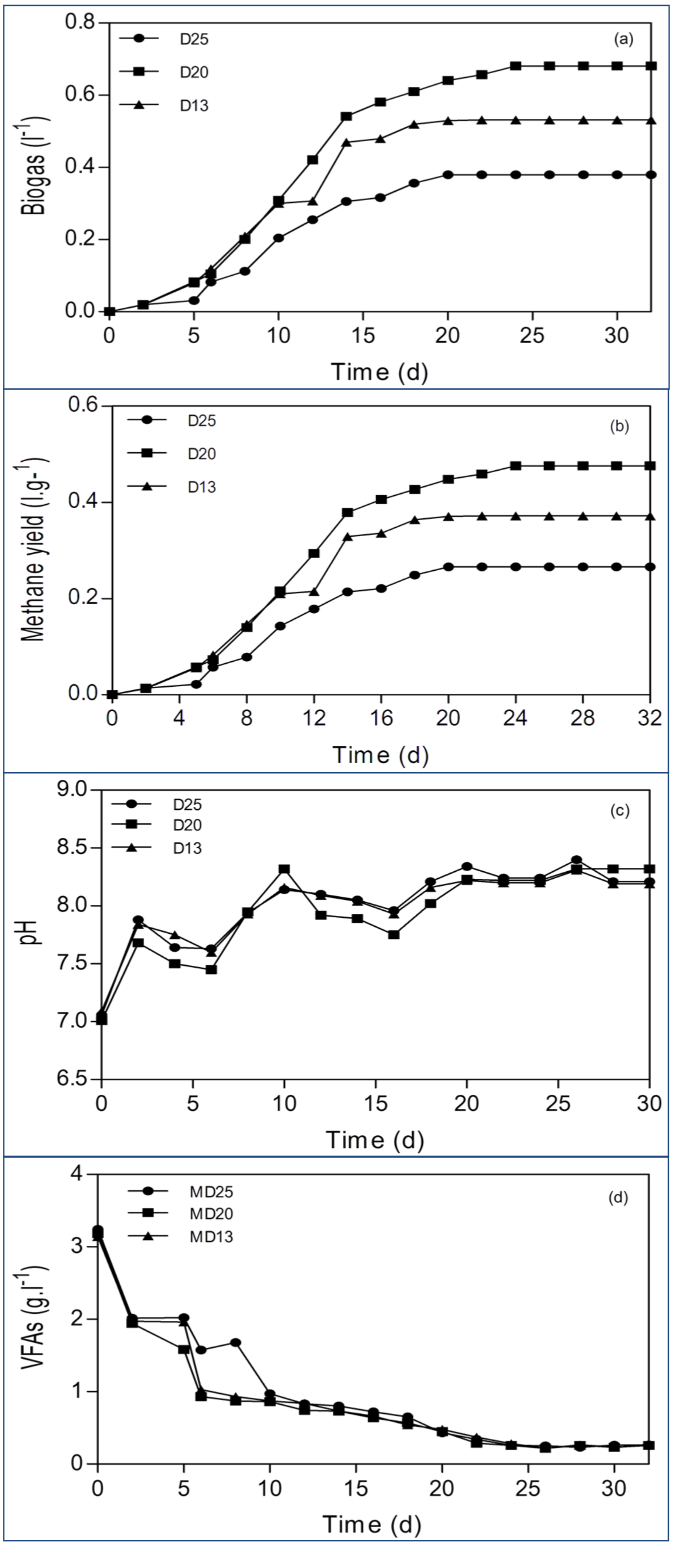

Fig. 2- Effect of C/N variation (13, 20 and 25) on anaerobic digestion during 30 days of diluted OMW(D-OMW): (a) biogas rate; (b) methane yield; (c) pH; and (d) VFAs.

The optimum C/N ratio for anaerobic digestion of D-OMW rises to 20 instead of 13 obtained with ND-OMW. However, the bacterial consortium grows slowly when rectors were fed with diluted OMW and the high volume of methane produced was 0.7 l. Analytical data obtained for methane yield [Fig-2b] shows that at the higher C/N ratio (20 and 13), the yields obtained were widely up to 0.35 $lCH_4.g^{-1}$ COD introduced. The higher values of yields, 0.47-0.37 $lCH_4.g^{-1}$ COD introduced, were obtained from C/N ratio 20 and 13 respectively. In addition, [Fig-2b] show that the methane yield does not increased with the increase of C/N ratio when the D-OMW was used as a substrate to anaerobic consortium bacteria.

pH variation illustrated in [Fig-2c] shows no signs of perturbation during the fermentation process, pH was higher than 7.0 for all C/N ratio applied. However, we observed an abnormal increase in pH from the 20th day and reached 8.4 in all anaerobic digestion in different reactors. The VFAs concentrations [Fig-2d] were low, and no sign of accumulation of these metabolites was detected.

Effect of Increasing Organic Load on Optimum C/N Ratio of Anaerobic Digestion: ND-OMW

In order to study the response of the C/N ratio face of the variation of applied organic load during the anaerobic digestion of ND-OMW with C/N ratio of13, the reactors were feed by an increasing load of COD, such as 4, 6, and 8 g $COD.l^{-1}day^{-1}$ with an hydraulic time retention of 16 day.

As it can be deduced by analyzing results of [Fig-3], methane yield rate shows a significant increase [Fig-3b] after increasing of applied organic loads compared to previous results. Methane yield reached a maximal value of 0.78 l of CH_4 per g of introduced COD with organic load 4 g $COD.l^{-1}day^{-1}$ only after 14 days of digestion. Moreover, we note an impressive yields obtained with a relatively high organic loads applied, such as 6, and 8 g $COD.l^{-1}day^{-1}$.

Response of bacteria consortium to increase of organic loads has also been evaluated by following pH and VFAs concentration. [Fig-3c] and 3d show that the values registered for pH and VFAs were all in the optimum range for anaerobic digestion.pH values rose rapidly from 7.2 to 8.21 in firsts three days when C/N ratio of 13 and 20 was applied. Few days later the value of pH ascended subsequently the VFA fell. However, the increasing pH to 8.35 could not be attributed only to the decrease of VFAs concentration in the mesophilic digestion medium.

Effect of Increasing Organic Load on Optimum C/N Ratio of Anaerobic Digestion: D-OMW

Results of anaerobic digestion of D-OMW with the C/N ratio of 13 in presence of several organic loads, such as 4, 6, and 8 6 g $COD.l^{-1}day^{-1}$were presented in [Fig-4].

The analysis of the results obtained after 16 days of fermentation shows that high values of methane yield were registered during the application of organic loads 4 and 6 g $COD.l^{-1}day^{-1}$ with a high value of 0.78 l of CH_4 per g of introduced COD. Besides this observation, methane yield obtained with organic load 8 g $COD.l^{-1}day^{-1}$ has increased compared to the results obtained with the ND-OMW.

The data analysis of pH and VFAs obtained during the fermentation of D-OMW are presented in [Fig-4c] and 4d. No signs of toxicity have occurred at reactors as it can be deduced by analyzing pH and VFAs variation.

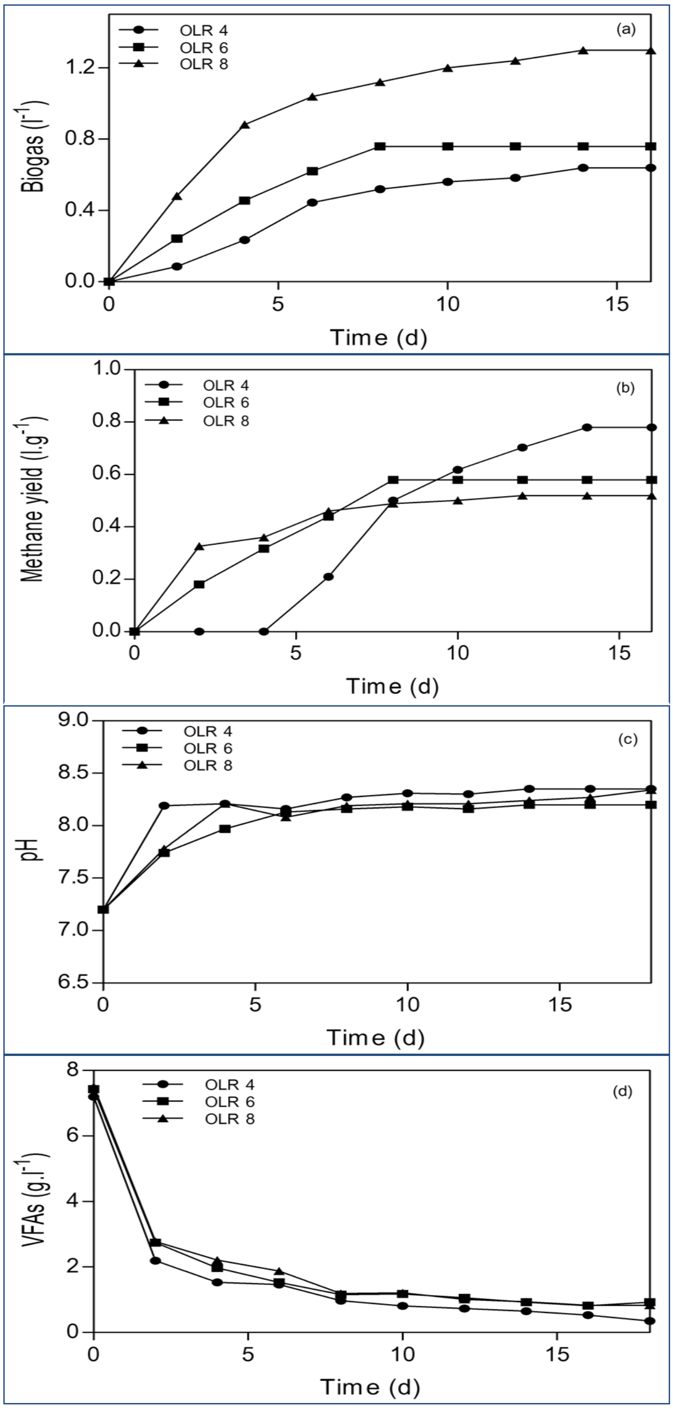

Fig. 3- Effect of organic load on C/N ratio variation of ND-OMW: (a) methane production rate; (b) methane yield ($l.g^{-1}$); (c) pH and (d) VAFs.

Discussion

The objective of this study was to determine a relationship between the variation of C/N ratio and the stability of the anaerobic digestion, which could be considered as an indicator of process imbalance during the fermentation of raw and diluted OMW in batch mode. Four parameters are volume of methane, methane yield; pH and volatile fatty acids concentration were followed to evaluate response of bacterial consortium to C/N ratio change. Thereafter, the effect of organic load, as a second perturbation, on optimal C/N ratio was also studied. Results shows that increase in C/N ratio led to a significant increase in methane production rate without any sign of perturbation. We also reported the effect of applied organic load on C/N ratio and anaerobic digestion of OMW. The decrease in C/N ratio from 25 to 13 was accompanied by a significant increase in the methane production rate as it can be deduced from methane yield values recorded during the fermentation of ND-OMW and D-OMW with C/N ratio of 13 and 20 respectively. Thus, values as 0.56 and 0.68 l CH_4 per gram of introduced COD were recorded with C/N ratio of 13 and 20 respectively. These values greatly exceeded the theoretical optimal methane yield of 0.35 l CH_4 per gram of introduced COD described by Sayadi et al., (2000) [16].

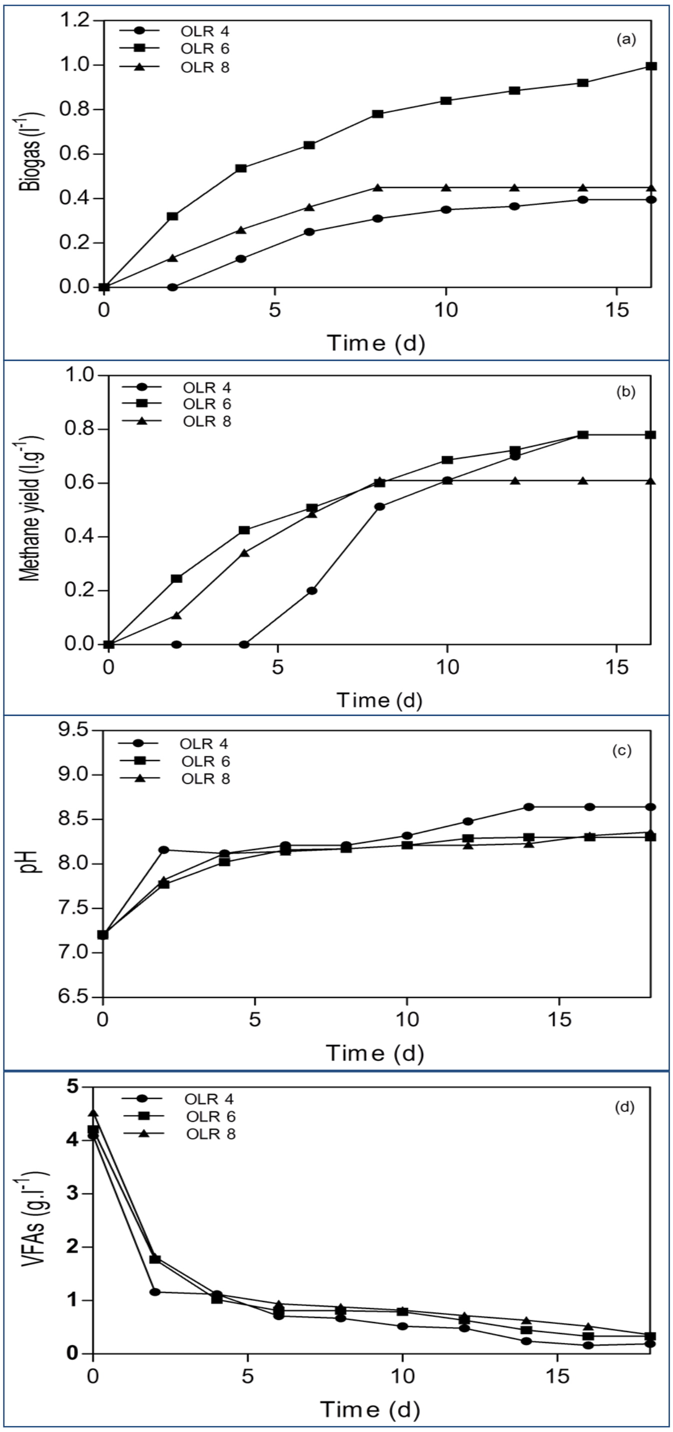

Fig. 4- Effect of organic load on C/N ratio variation of D-OMW: (a) methane production rate; (b) methane yield ($l.g^{-1}$); (c) pH; and (d) VAFs.

OMW represents a complex medium containing various assimilable carbon sources, such as sugars, lipids and organic acids at different proportions [17]. OMW dilution reduces not only their toxicity but also the availability of this variable carbon source, which decreases due to the presence of purifying biomass in anaerobic conditions. This could be explained by the difference in the optimum C/N ratio for anaerobic digestion of ND-OMW and D-OMW since the carbon source is low available to the bacterial consortium in the case of D-OMW compared to the ND-OMW. Several published researches on anaerobic digestion treatment of OMW rated this black liquid among of strong wastewaters containing high concentrations of phenolic compounds (up to 10 $g.l^{-1}$ depending on the type and origin of the effluent) that are difficult to be biodegrade and inhibit methanogenic bacteria [4,16-18]. The relative effect of this toxic molecules was manifested in a decrease in the value of methane yield for D-OMW (0.68 $l.g^{-1}$) compared to ND-OMW (0.56 $l.g^{-1}$) an increase in the value of removal COD [Fig-5] as the phenolic compound concentration increased (case of ND-OMW).

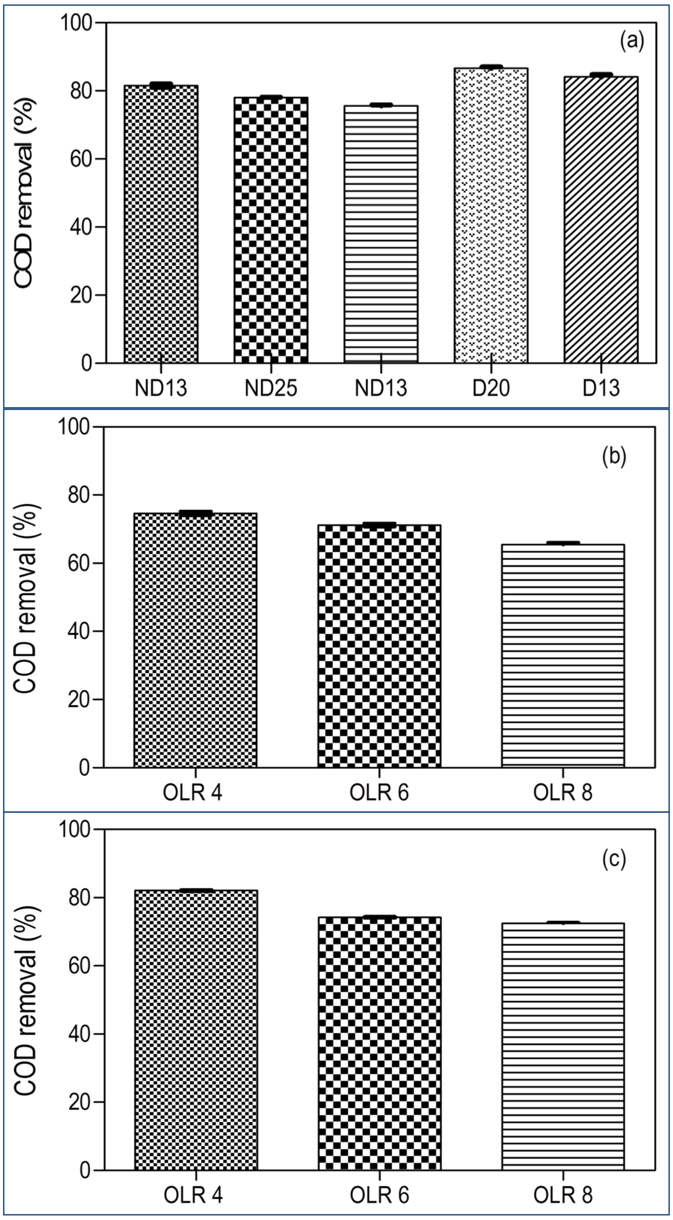

Fig. 5- COD removal: (a) organic load rate of 2 $COD.l^{-1}.day^{-1}$; (b) ND-OMW with C/N ratio of 13; and (c) D-OMW with C/N ratio of 13.

The increase of applied organic load rate from 2 g COD.$l^{-1}day^{-1}$ to 8 g COD.$l^{-1}day^{-1}$ led to a decrease in optimum C/N ratio for anaerobic digestion of D-OMW from 20 to 13, this value joins the same value than the fermentation of ND-OMW which undergoes no change. The phenomenal increase in methane yield rate reached 0.78 l.$g^{-1}$with ND-OMW and D-OMW is respectively obtained when OLR of 4 g COD.$l^{-1}day^{-1}$ was applied. This is in accordance with what we have already issued (previous paragraph) concerning the interrelationship between the carbon and nitrogen sources necessary for the growth of bacterial consortium in anaerobic conditions.

In addition, an increase in OLR beyond the 4 g COD.$l^{-1}day^{-1}$ is followed by a decrease in the main parameters, such as COD removal, hydraulic retention time and methane yield rate, especially in the case on ND-OMW. Hence, the value of methane production rate dropped to 0.38 l.g^{-1} with OLR of 8 g COD.$l^{-1}day^{-1}$. It could due to an increase in the concentrations of the VFAs with a consequent decrease in pH [19].

By contrast, such results showed a high stability in the values recorded for these two parameters. Thus, this decrease could be attributed to the effect of escalated levels of inhibitory or toxic compounds, such as phenolic compounds, long-chain fatty acids, lignin, etc. In a different study, stability and performance of anaerobic digestion were observed when the OLR increased from 1.5 to 9.2 g COD.l^{-1}.day^{-1} [20]. Hence, decreasing C/N ratio could have a positive effect on anaerobic digestion of OMW, especially D-OMW and with high OLR.

Since the thermophilic conditions are more sensitive to toxicants and the temperature control is more difficult [21, 22-23]. It is interesting to note that anaerobic digestion was occurred in mesophilic conditions at 35 ± 2°C, which allows to properly studying the effect of the C/N variation on anaerobic digestion. As far as the anaerobic digestion process is concerned, it is more appropriate to discuss VFAs and pH together because these parameters are related to each other, and very promising to detect a sign of imbalance in anaerobic digesters and for successful methanogenesisprocess [24,25]. Methane producing *Archaea* are known to be strongly affected by pH and could only survive on a very narrow range of pH with an optimum at 7.6 [26, 27-28]. In this study, higher values of methane production rate were obtained when pH is in the optimum values and there is no severely accumulation of VFAs, such the methanogenic activity is maximum once the optimum pH range is met. Therefore, as a results of degradation of the organic nitrogen sources such as urea, high concentration of total ammonium NH_4^+ (an essential nutrient for methanogens growth) plus hydroxide ions OH^- is common [29]. This can plays an important pH controlling role in the anaerobic treatment process by buffering the acidity derived from the acidogenesis process (VFAs) which explains high values of pH recorded during this study. Therefore, high concentration of ammonia generated by using urea as nitrogen source could not inhibit methanogens bacteria, since this bacterial group supports high concentration of ammonia nitrogen up to 6000 mg.l-1 at temperature of 35C° [30].

Conclusion

The anaerobic treatment process of OMW has long been known to be influenced by pH and VFAs accumulation. An increase in C/N ratio will increase the anaerobic degradation rate of the organic substances; and the methanogenic activity was higher. It was suggested that use of urea to adjust C/N ratio at mesophilic temperature gives us the opportunity to apply a high and/or desired OLR without affecting the growth of methanogens in the bioreactor by providing them with higher substrate concentrations and the buffering capacity.

The results obtained in this study could be operated at the pilot scale in the case of co-digestion of OMW with other nitrogen source effluent, such as liquid manure and some types of landfill leachate. Thus, this work could be contributed to OMW management in order to overcome one of the most challenging environmental problems in Mediterranean countries.

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Abbreviations

OMW- Olive Mill Wastewater,

C/N- Carbon/Nitrogen,

ND-OMW- Non Diluted Olive Mill Wastewater,

D-OMW- Diluted Olive Mill Wastewater,

OLR- Organic Load Rate,

TOC- Total Organic Carbon,

VFAs- volatile fatty acids, CH_4: Methane

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