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Methane production enhancement from *Tetraselmis* biomass co-digestion using frying oil residue as co-substrate and ultrasonication as pretreatment

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ABSTRACT

Tetraselmis sp. microalgae biomass co-digestion with frying oil residue was investigated without or with an ultrasonication pretreatment to enhance methane production. Interestingly, compared to untreated *Tetraselmis* biomass, sonication pretreatment using specific energy of 1.6 kJ/g VS significantly improved specific methane production by 60.5%. However, increasing specific energy sonication to 3.2 kJ/g VS decreased the positive energy balance of whole process from 8.6 to 5.5 ± 0.3 kJ/g VS. Furthermore, it was demonstrated that the correction of the C/N ratio with frying oil waste coupled to the sonication of *Tetraselmis* biomass resulted in the highest methane yield of 443.7 ± 6.4 ml CH_4/g VS and highest energy gains of 14.1 ± 0.2 kJ/g VS. This study proved the presence of a complementary effect between the co-digestion of *Tetraselmis* biomass with frying oil and the sonication pretreatment on specific methane production and energy recovery. Low ultrasonication specific energy is recommended as pretreatment process before *Tetraselmis* biomass anaerobic digestion.

1. Introduction

Anaerobic digestion (AD) is a well-known biological process for renewable energy production. It represents a proven technology that has been used at industrial scale to sustainably produce methane-rich biogas from crops, organic wastes and microalgae (Brémond et al., 2021; Khoufi et al., 2007; Uddin and Wright, 2022). The produced methane could replace fossil energy and the produced digestate can be used as fertilizer. Many studies reported the suitability of *Tetraselmis* biomass as a feedstock for AD process which does not require specific macromolecule extraction such as lipids, proteins, or carbohydrates and can be processed with wet biomass (Bohutskyi et al., 2014; Jankowska et al., 2017; Paul et al., 2023; Vargas-Estrada et al., 2022). Harun et al. (2011) demonstrated that the theoretical *Tetraselmis* sp. methane yield for the whole cell can reach 410 ml CH_4/g VS. The same authors reported that methane yield is between 200 and 340 ml CH_4/g VS when defatted *Tetraselmis* biomass is used during the digestion. According to Park et al.

List of abbreviations: AD, Anaerobic digestion; C/N, carbon to nitrogen ratio; STRs, Stirred Tank Reactors; VS, Volatile Solid; TS, Total solids; TOC, Total soluble Organic Carbon; TN, Total soluble Nitrogen; VFAs, Volatile Fatty Acids; VFAs/alkalinity, Volatile Fatty Acids and alkalinity ratio; OD, Optical Density; SPSS, Statistical Package for the Social Sciences; RSM., Response Surface Methodology.

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(2011), methane production from microalgae can generate up to 3.6 kJ/g VS of electricity.

However, low methane production from microalgae AD was also reported and was attributed essentially to two major reasons. The first is the low carbon to nitrogen (C/N) ratio, which leads to ammonia accumulation and consequently inhibits methanogenic bacteria's growth (Vargas-Estrada et al., 2022). The second reason is the thick cell walls of microalgae which prevent anaerobic microorganisms from accessing and degrading the organic matter (Jankowska et al., 2017; Vargas-Estrada et al., 2022).

To enhance energy recovery from microalgae biomass, many studies reported that co-digestion needs to be implemented and different carbon-rich substrates like lipid-rich biomass, cooking oil, corn stalks, cow manure, etc. were tested (Park and Li, 2012; Peng et al., 2012; Rétfalvi et al., 2016; Saleem et al., 2020). Indeed, feedstock mixing process balances the C/N ratio and nutrients in the digester. It improves the synergistic effect of microorganisms by diluting toxic compounds. Furthermore, it enhances buffer capacity and alkalinity of the media, and stabilizes VFAs concentration. Among different co-substrates, frying oil residue is considered as cheap carbon waste and attractive co-substrate since it can produce a higher methane yield compared to carbohydrates (Long et al., 2012). Over 24 million tons of vegetable oils were used in Europe annually but only small amount is effectively collected (Lee et al., 2013). Thus, it represents a sustainable co-substrate source which should be exploited and valorized. Frying oil residue was successfully used in co-digestion with sewage sludge to enhance methane production in batch and continuous bioreactors (Long et al., 2012; Oliveira et al., 2018). Similarly, it was found that *Chlorella vulgaris* microalgae co-digestion with cooking oil resulted in higher methane yield than with maize silage or with mill residue (Rétfalvi et al., 2016). For these multiple reasons, frying oil will be selected in the current study as potential feedstock for *Tetraselmis* sp. biomass co-digestion.

Tetraselmis sp. biomass vulnerability to AD also depends on its recalcitrant algal cell walls. It was reported that the glycoprotein enclosing membrane wall cells are protected from surrounding attacks such as temperature elevation, osmotic shock or alkaline lysis (Delran et al., 2023a; Kermanshahi-pour et al., 2014). Indeed, it was proved that the five layers forming a complex network enclosing *Tetraselmis* sp. cell requires extreme conditions to cause cell wall destruction (Azma et al., 2010; Kassim and Bhattacharya, 2016). In order to enhance microalgae biomass components availability to anaerobic bacteria and to increase methane production, different physical, chemical or biological pretreatments were suggested such as ultrasonication, microwave and thermal hydrolysis, acid and alkali treatments, and enzymatic treatment (Jankowska et al., 2017; Klassen et al., 2016). Ultrasonication is a promising technique for cell disruption which has a great potential for industrial scale-up. It was reported that ultrasonication physical pretreatment promotes cell lysis of *Tetraselmis suecica*, *Nannochloropsis sp.*, and *Chlorella sp.* and gets over membrane cells' hindrances (Natarajan et al., 2014). For instance, sonication pretreatment of *Chlorella vulgaris* increased methane production by 90% (Park et al., 2013). However, other authors (Paul et al., 2023) reported that ultrasonication pretreatment slightly decreased the specific methane production from biomass residues such as *Tetraselmis suecica* and *Nannochloropsis oceanica.* In addition, this pretreatment technique has relatively high energy requirements. To the best of our knowledge, the integration of microalgae biomass pretreatment by ultrasonication and co-digestion with frying oil waste for circular economy has never been evaluated and therefore represents a potential research topic to investigate.

The current study aimed to evaluate the potential of *Tetraselmis sp.* QUCCCM (Das et al., 2016; Saadaoui et al., 2016) as a novel biomass source for biogas production via AD. The first objective of this work was to study methane yield from *Tetraselmis sp.* biomass digestion as mono-substrate and its co-digestion with frying oil in Stirred Tank Reactor (STR) with or without sonication as pretreatment. The second objective was to assess the energy balance of the integrated process in order to evaluate the economic feasibility of energy recovery from *Tetraselmis* biomass digestion.

2. Materials and methods

2.1. Materials

A locally isolated halotolerant marine *Tetraselmis* sp. (Saadaoui et al., 2016) was used in this study. The raw *Tetraselmis* biomass was collected from a large-scale outdoor cultivation raceway of 25000 l (Das et al., 2016), divided in small quantities of 500 g and stored at

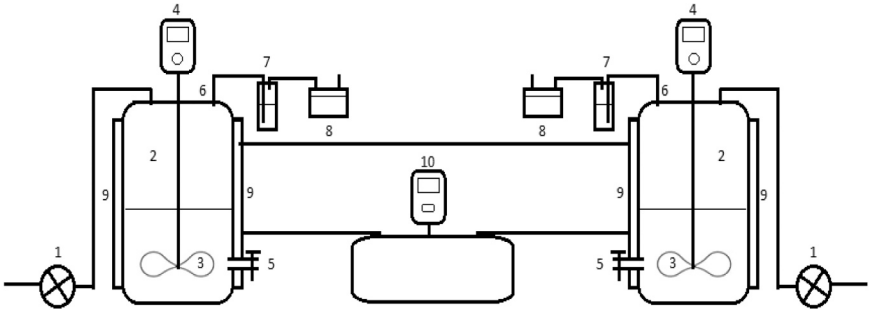

Fig. 1. Duplicated lab-scale STRs for the anaerobic treatment of microalgae; 1: feeding pump, 2: cylindrical glass reactor, 3: helix, 4: stirring device, 5: sampling valve, 6: biogas exhaust, 7: trap bottle, 8: biogas flowmeter, 9: double jacket for temperature regulation, 10: temperature-controlled water circulating bath.

− 80 °C. Before use, microalgae biomass defrost is carried out overnight at 4 °C. Frying oil residue was collected from a local restaurant. The anaerobic sludge obtained from an anaerobic digester at LBPE-CBS in Sfax, Tunisia, was used as inoculum to decompose the *Tetraselmis* sp. microalgae in the STR batches. All chemicals used in the present work were of analytical grade.

2.2. Configuration and operation of anaerobic reactor

Two lab-scale stirred tank reactors (STRs) were installed for the anaerobic treatment of *Tetraselmis* microalgae. Fig. 1 shows the STR configuration. Two cylindrical glass reactors, with an effective volume of 6.7 l for each, were equipped with an internal stirring device (Heidolph Hei-Torque 100) and a biogas flow counter (milligas counter-1 V3.0 PMMA, Ritter Inc., Germany), as well as a sampling port at the bottom. Temperature was maintained by circulating water from a thermo-regulated bath through the double jacket. Each reactor was equipped with a peristaltic feeding pump to ensure hermetic conditions. After anaerobic sludge preparation, the inoculum was divided equally into both STRs with a working volume of 3.5 l and volatile solid (VS) of 15 g/l. For each batch, the same experimental conditions were applied on both reactors in order to duplicate results for statistical analysis.

During the different experiments, the substrate was mixed with the anaerobic sludge at a substrate to inoculum ratio (S/I) = 1 (VS basis), and the reactors were operated in batch mode. The temperature was maintained at 37 °C, and the rotation speed of mixing devices was adjusted at 200 rpm to ensure sufficient mixing and an optimal contact substrate-inoculum contact.

2.3. Anaerobic digestion parameters determination

Methane content in biogas was measured using an Agilent Gas Chromatograph with a TCD detector (Soliman, 2020). Total solids (TS), VS, pH, were determined according to the APHA Standard Methods (Rice et al., 2012). The total soluble organic carbon content (TOC) and total soluble nitrogen (TN) were determined by high-temperature oxidation using a Shimadzu TOC-VCPH analyzer. The volatile fatty acids (VFAs), the alkalinity and their ratio (VFAs/alkalinity) were determined according to the method developed by Nordmann as described by (Sun et al., 2017).

2.4. Sonication pretreatment

The sonication pretreatment of *Tetraselmis* microalgae and *Tetraselmis* mixed with frying oil was performed using an ultrasonication apparatus (Qsonica Q700, Cole-Parmer) with titanium probe 4220. According to volatile matter content of 52 g and C/N ratio of 14 and 20, 250 ml of pre-defined masses of *Tetraselmis* and frying oil were placed into glass beakers of 500 ml and were sonicated under two specifics energies of 1.6 or 3.2 kJ/g VS. Different substrate contents and ultrasonication pretreatment conditions of each anaerobic batch were mentioned in Table A.1 as supplementary material.

2.5. Total carbohydrate extraction and quantification

The total carbohydrate was extracted using the phenol sulfuric acid colorimetric method with some modifications (DuBois et al., 1956). After sonication pretreatment of *Tetraselmis* biomass and centrifugation at 4500 rpm for 15 min, the supernatant was collected and lyophilized. A dry extract of known weight (usually 10 mg) was boiled in a water bath for two hours after being treated with 4 M HCl. The acid mixture was added to an equivalent amount of water. Then, the supernatant of the above centrifuged mixture was combined with sulfuric acid and phenol, and the mixture was heated for 20 min. The carbohydrate content in the sample was determined using a spectrophotometer at 490 nm; glucose standards were used to make the calibration curve of carbohydrate vs. optical density at 490 nm.

2.6. Total protein extraction and quantification

Total protein content of lyophilized supernatant of sonicated *Tetraselmis* biomass was determined according to the Lowry assay (Lowry et al., 1951). Briefly, 2 ml of NaOH (0.1 M) was added to 10 mg of dried extract. The mixture was incubated overnight at 60 °C in order to extract the total protein. After 24 h of hydrolysis, the mixture was centrifuged (10 min/14000 rpm), and protein content was calorimetrically determined using Folin ciocalteau reagent and BSA bovine serum albumin as a standard.

2.7. Chlorophyll content determination

To determine the chlorophyll content of lyophilized supernatant of sonicated *Tetraselmis* biomass, the Porra et al. (1989) method was used. 10 mg of dried extract and 1 ml of 90% methanol solution were mixed and heated in a water bath at 60 °C until it became colorless. After that, the tube was cooled to room temperature and centrifuged at 15,000 rpm for 5 min. The optical density (OD) of the supernatant at two different wavelengths (650 and 665 nm) was determined using a spectrophotometer (Jenway, 6305, UK). The chlorophyll content was calculated according to the following equations, described by (Porra et al., 1989).

$$\text{Chlorophyll A (mg/l)} = (16{\cdot}5 * OD_{665\ nm}) - (8{\cdot}3 * OD_{650\ nm}) \quad (1)$$

$$\text{Chlorophyll B (mg/l)} = (33{\cdot}8 * OD_{650\ nm}) - (12{\cdot}5 * OD_{665\ nm}) \quad (2)$$

Chlorophyll A and B (mg/l) = (4 * $OD_{665\ nm}$) + (25·5 * $OD_{650\ nm}$) (3)

2.8. Total lipid extraction and quantification

The total lipid content was determined according to the modified Folch et al. (1957) and Arora et al. (2016) methods. First, 0.88% NaCl solution was added to 10 mg of freeze-dried extract from sonicated *Tetraselmis* biomass. Then an appropriate methanol volume was added to the tube. Two volumes of chloroform were added to the mixture after an overnight incubation at 4 °C. The supernatant fraction was put into a pre-weighed Falcon tube following five minutes of centrifugation at 13,000 rpm. After that, methanol and chloroform (1:2) were utilized in a second extraction. In order to separate the aqueous and organic phases, NaCl was added until 0.88% as final concentration and the mixture was vortexed for 10 min. Finally, the organic phase was dried after the top (aqueous) layer was removed, and the weight of the tube was then recorded to calculate the percentage of total lipid per mg of dry biomass.

Lipid content (%) = (Total lipids (g)/Dry biomass (g)) x 100

2.9. Statistical analysis

All anaerobic batches were repeated twice. All quantitative results were expressed as means ± SD. The computer statistical program Statistical Package for the Social Sciences (SPSS) version 23 for Windows (SPSS Inc., Chicago, IL, USA) was utilized to analyze data. Variance was analyzed by one-way analysis of variance (ANOVA), and Tukey's test was applied to compare each parameter at $P < 0.05$.

3. Results and discussion

3.1. Tetraselmis biomass characterization

Before the AD experiments, *Tetraselmis* microalgae biomass was characterized, and the obtained results are shown in Table 1. In terms of total carbon, *Tetraselmis* biomass contained 18.5 ± 0.9%. The total nitrogen was 1.3 ± 0.1%. According to these results, the C/N ratio of *Tetraselmis* biomass was 14.3. C/N ratio is an important parameter for optimal methane production (Uddin and Wright, 2022). It is recommended to control the C/N ratio between 20 and 35 to maximize the AD process efficiency (Schwede et al., 2013; Uddin and Wright, 2022). For that reason, we investigated in this study the co-digestion of *Tetraselmis* biomass with a carbon-rich co-substrate (Frying oil residue). The dry matter of microalgae biomass was 36.7 ± 2.0%, reflecting that the *Tetraselmis* biomass was well harvested through several steps such as settling, microfiltration, and centrifugation. *Tetraselmis* biomass dry matter was made up of 57.9 ± 2.7% volatile solids, and the remaining was ash (42.1 ± 3.1%). The studied *Tetraselmis* biomass had an ash content of 15.45 g per 100 g of fresh biomass. This value is similar to values reported for other marine strains and higher than values of freshwater strains according to Pereira et al. (2019). In fact, microalgae biomass ash content depends on the concentration of salt used during microalgae growth.

3.2. Anaerobic digestion of Tetraselmis biomass with or without frying oil residue

After anaerobic biomass acclimatization, two sets of experiments were investigated. During the first set, duplicated anaerobic STRs were fed with raw *Tetraselmis* biomass with a specific C/N ratio of 14.3. In the second set, frying oil residue was used as mainly carbon source (C=77%) to adjust the C/N ratio to a value of 20. The mixture of raw *Tetraselmis* biomass and frying oil was fed into both reactors for co-digestion. All experiments were operated in batch mode, and the same VS quantity of 52 g/l equivalent to *Tetraselmis* biomass was maintained as a feed per reactor to be able to compare methane production yields. The different AD parameters were monitored and compared.

The soluble TOC evolution in the digestate obtained with the first batch showed a slight increase after two days of digestion from an average of 1100 ± 52.0–1374 ± 40.3 mg/l (Fig. 1a). This could be explained by the microalgae polymers hydrolysis and release of soluble compounds in the digestate. Afterwards, the soluble TOC continually decreased to an average of 609.1 ± 1.3 mg/l and remained relatively constant after nine days, indicating that this fraction could need a longer time to be fully degraded. Compared to the C/N equilibrated batch, the use of frying oil residue for the co-digestion increased the initial soluble TOC to 1565.0 ± 21.2 mg/l. Most likely, the frying oil residue and *Tetraselmis* biomass mixture brought to the digestor higher soluble compounds content. Fig. 2a shows that the soluble TOC of the second batch continually decreases until the fifth day to an average of 640.2 ± mg/l. Compared to the first batch, the TOC degradation was faster (5 days). Higher degradability of *Tetraselmis* biomass and frying oil mixture is observed.

Table 1
Characterization of *Tetraselmis* microalgae.

	C (%)	N (%)	TS (%)	VS (%)	Ash (%)
Tetraselmis Biomass	18.5 ± 0.9	1.3 ± 0.1	36.7 ± 2.0	57.9 ± 2.7	42.1 ± 3.1

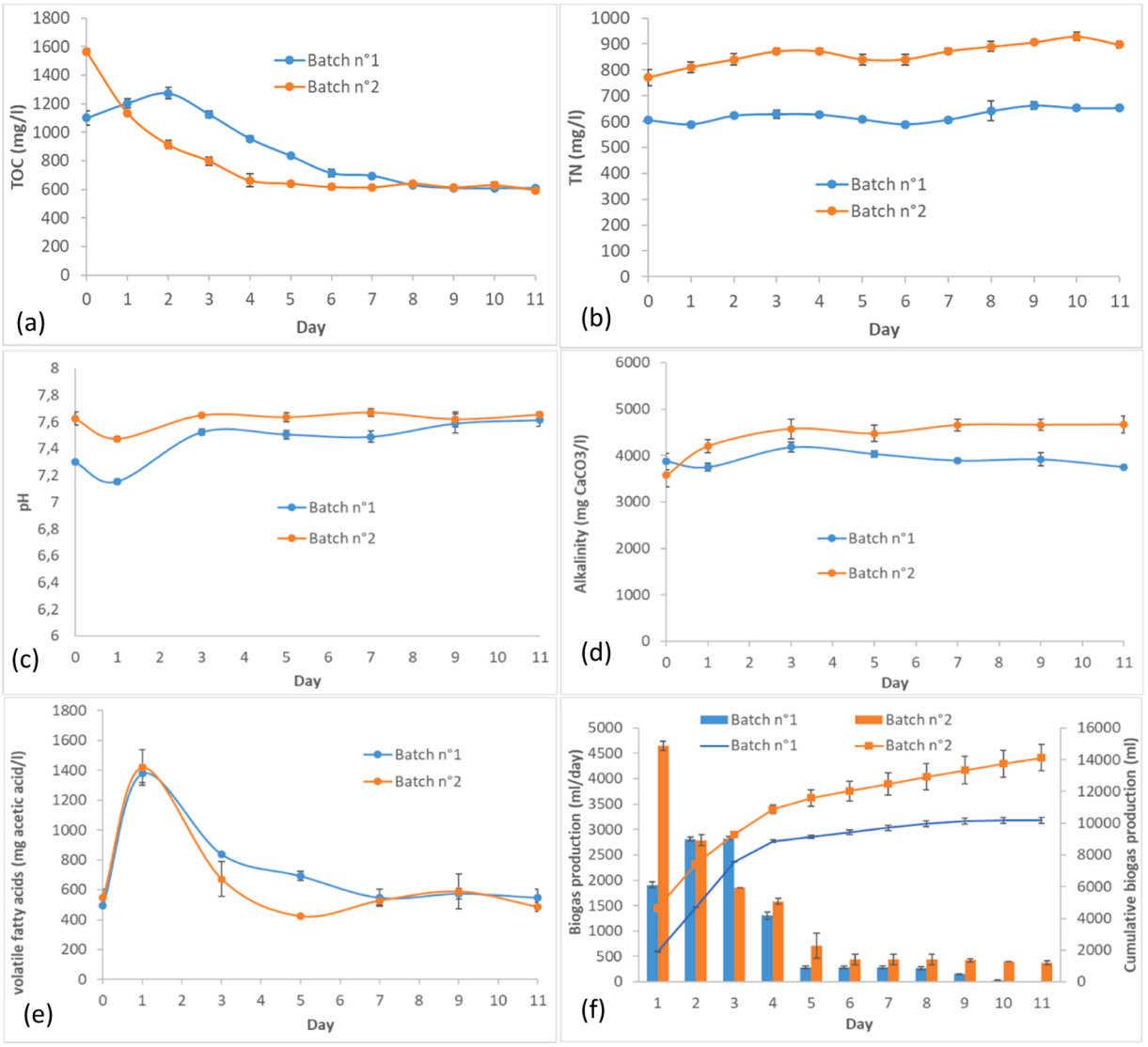

Fig. 2. TOC (a), TN (b), pH (c), alkalinity (d), and volatile fatty acids (e) variation during raw *Tetraselmis* biomass AD (Batch n°1) and *Tetraselmis* biomass anaerobic co-digestion with frying oil residue (Batch n°2), (f) daily and accumulated biogas production after raw *Tetraselmis* biomass AD (Batch n°1) and *Tetraselmis* biomass anaerobic co-digestion with frying oil residue (Batch n°2).

The added frying oil residue substituting the microalgae biomass fraction could be better anaerobically biodegraded than *Tetraselmis* itself. Indeed, it was reported that methane yield of used oil was 649 ml/g VS (Uddin and Wright, 2022) which is higher than the theoretical methane yield of *Tetraselmis* microalgae (410 ml CH_4/g VS) reported by Harun et al. (2011). These reported methane yields prove the higher frying oil biodegradability than *Tetraselmis* microalgae. Furthermore, the C/N ratio equilibration to 20 allowed a well-balanced AD process and could consequently improve the organic matter degradation as reported by Yen and Brune (2007).

Fig2.b shows the soluble total nitrogen evolution in the digestates. A slight increase is observed from an average of 606.5 ± 4.9–653.5 ± 8.6 mg/l and from 770.2 ± 32.0–897.4 ± 12.5 mg/l for batch n°1 and batch n°2, respectively. These results reflect a continual nitrogen liberation and accumulation in digestates. In fact, *Tetraselmis* proteins were degraded, under the effect of anaerobic consortium hydrolytic enzymes, to amino acids. Then, amino acids were transformed into organic acids such as acetate and butyrate, leading to ammonia liberation (NH_4^+/NH_3) in the digestate. The relative concentrations of ionized ammonia (NH_4^+) and nonionized ammonia (NH_3) forms are in equilibrium and depend on the digestate pH (Gerardi, 2003).

The pH, alkalinity and VFAs of anaerobic reactors were measured every 2 days to monitor the AD process's overall status. The pH was mostly dependent on volatile acid and alkalinity balance in digestate. However, when methanogenesis was inhibited, pH was the last parameter indicating process alteration. For that reason, alkalinity depletion detection and changes in volatile acid-to alkalinity ratio determination are essential to avoid anaerobic process deterioration (Gerardi, 2003).

During anaerobic treatment of *Tetraselmis* biomass and its co-digestion with frying oil, the pH slightly dropped during the first day

and then increased to reach values of 7.6 and 7.7 respectively, in the third day (Fig. 2c). These values were suitable for an optimized anaerobic digestion process. Accordingly, the VFAs concentration increased rapidly during the first day and reached an average of 1378 ± 58.7 mg/l for the first batch and 1419 ± 117.4 mg/l for the second batch (Fig. 2e) without any inhibition of the anaerobic process. In fact, VFAs production decreased the pH and proved the effective hydrolytic and fermentative bacteria activities of anaerobic consortium. After that, VFAs content in both batches decreases below 550 mg/l along with pH increases, reflecting the opposite effect of VFAs on pH evolution. The alkalinity evolution for both batches is shown in Fig. 2d. The alkalinity was stabilized in the range of 4000 mg $CaCO_3$/l for the first batch and increased to 4700.0 ± 185.6 mg/l for the second batch. The alkalinity increase is probably due to reactions of released ammonia with carbon dioxide and water, producing ammonium carbonate, which provides alkalinity to the system (Gerardi, 2003). Thus, the co-digestion of *Tetraselmis* biomass with frying oil improved digestate alkalinity. Similar alkalinity enhancement was reported when *Nannochloropsis* biomass was co-digested with corn (Schwede et al., 2013). In order to evaluate the stability of the anaerobic STRs, the VFAs to alkalinity ratio was also considered. This ratio did not exceed 0.37 in both batches, reflecting a good stability of the anaerobic process (Callaghan et al., 2002). According to literature (Switzenbaum et al., 1990; Zickefoose and Hayes, 1976), digester instability would occur when the VFAs to alkalinity ratio is greater than 0.4, and a significant instability could be detected when the ratio is higher than 0.8.

All values of previously studied parameters (pH, VFAs, Alkalinity, VFAs to Alkalinity ratio) were suitable for both batches for AD *of Tetraselmis* biomass and for *Tetraselmis* co-digestion with used oil. The TOC, TN, showed that the organic matter degradation was higher during *Tetraselmis* co-digestion with used oil. In order to confirm the positive effect of *Tetraselmis* biomass co-digestion with used oil on the anaerobic process, the biogas productions were monitored and compared for both batches.

Fig. 2f shows the daily and cumulative biogas production from raw *Tetraselmis* microalgae anaerobic treatment and *Tetraselmis* biomass co-digestion with frying oil residue. The daily biogas production showed that co-digestion enhanced biogas production rate and reduced the time-lags in biogas production. After 11 days, the cumulative biogas for both batches 1&2 showed an increase from 10 to 14 l, respectively. The higher biogas production could be explained by the higher accessibility of anaerobic consortium to both used substrates than to raw microalgae biomass and confirm the better biodegradability of used oil as previously reported (Labatut et al., 2011; Park and Li, 2012; Pastor et al., 2013). However, the biogas production is still far from the theoretical value of 38 l calculated according to both fractions of *Tetraselmis* sp. microalgae (600 ml/g VS) (Bohutskyi et al., 2014) and of frying oil residue (970 ml/g VS) (Pastor et al., 2013). Thus, a pretreatment of *Tetraselmis* biomass or the mixture is needed to improve substrate accessibility to anaerobic consortium and to enhance biogas production.

To improve biogas production and energy balance from *Tetraselmis* microalgae biomass and from the mixture of microalgae with frying oil, sonication was selected as a pretreatment process. The effects of sonication pretreatment on TOC solubility, proteins, lipids and carbs contents were studied in the following sections.

3.3. *Tetraselmis sonication*

In order to improve biogas production yield from *Tetraselmis* biomass and frying oil mixture co-digestion, a sonication pretreatment of *Tetraselmis* biomass was investigated using Response Surface Methodology. The main objective was to determine the effects of the different sonication parameters on the destruction extent of *Tetraselmis* microalgae cells, allowing better TOC solubilization in bulk with the lowest energy consumption (Results not shown). The calculated specific energy consumption per volatile solids corresponding to the selected optimum was 1585 J/g VS. This specific energy was applied to ultrasonication pretreatment of *Tetraselmis* biomass needed as feed for the AD in STR. A doubled specific energy was also tested in order to be compared with the optimum and to check if it could improve the energy balance of the whole process.

Table 2 shows the lipids, proteins, carbs, total chlorophylls, and TOC, liberated in the solution after *Tetraselmis* biomass sonication using different specific energies. Without sonication pretreatment, the soluble TOC content in the *Tetraselmis* biomass solution used as raw feed for the AD was 240.5 ± 20.1 mg/l. The lipids, proteins, carbs, and total chlorophylls in the soluble phase were 17.8 ± 0.8, 1.9 ± 0.2, 17.5 ± 0.4 and 3.5 ± 0.6%, respectively. After *Tetraselmis* biomass pretreatment, the soluble TOC significantly increased to 1673.0 ± 25.5 mg/l for sonicated biomass and to 1563.0 ± 125.3 mg/l for highly sonicated biomass. Thus, the increase in sonication specific energy did not significantly improve the soluble TOC content. Similarly, after *Tetraselmis* biomass sonication, proteins and total chlorophylls content significantly increased to 3.6 ± 0.3 and 12.3 ± 0.8%, but the excessive sonication treatment has no significant effect on those parameters. In fact, after the sonication pretreatment, soluble TOC, proteins and chlorophylls content increase, indicating an effective cell membrane disruption. Cell disruption increased the soluble substrates content resulting from organics release. However, doubling the specific energy of sonication pretreatment did not further improve the same parameters. The used high *Tetraselmis* biomass concentration of 180 g/l in the sonication experiments could reduce the destructed cell percentage to 80% as reported

Table 2
Lipids, proteins, carbs, chlorophylls, and TOC contents in supernatant before and after *Tetraselmis* biomass sonication.

Specific energy (J/g VS)	Lipids (%)	Proteins (%)	Carbs (%)	Chlorophylls (%)	TOC (mg/l)
0	17.8 ± 0.8^a	1.9 ± 0.2^b	17.5 ± 0.4^b	3.5 ± 0.6^b	240.5 ± 20.1^b
1584.6	17.1 ± 1.2^a	3.6 ± 0.3^a	20.3 ± 0.4^b	12.3 ± 0.8^a	1673.0 ± 25.5^a
3169.2	16.8 ± 0.9^a	3.8 ± 0.4^a	25.5 ± 2.0^a	11.1 ± 0.1^a	1563.0 ± 125.3^a

For each run using different sonication specific energy, responses with different letters are significantly different (one-way analysis of variance, $P < 0.05$; Tukey's test), ± standard deviation (SD).

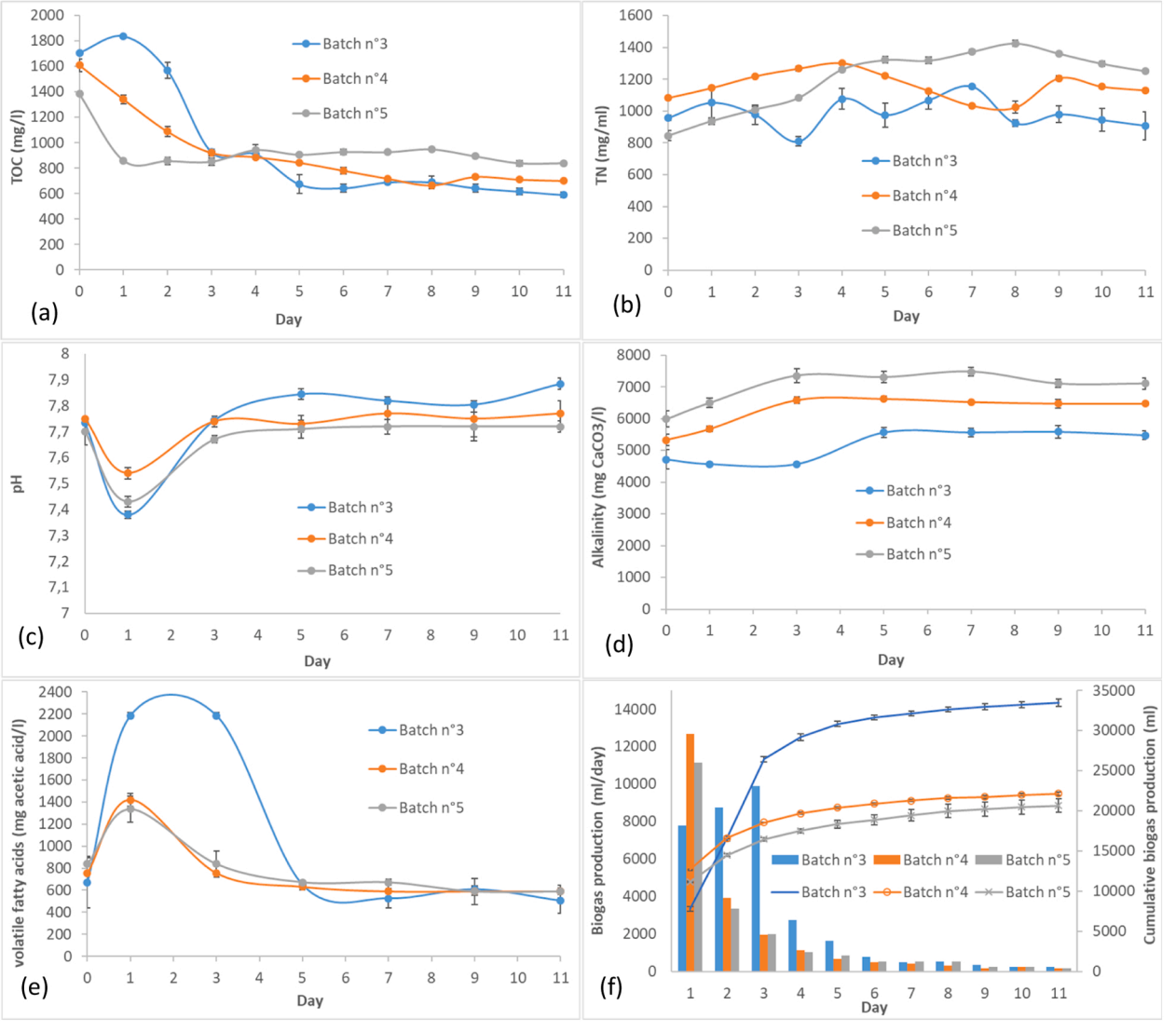

Fig. 3. TOC (a), TN (b), pH (c), alkalinity (d), volatile fatty acids (e) and daily and cumulative biogas production (f) variation after: sonication pretreatment and co-digestion of *Tetraselmis* biomass and frying oil mixture (batch n°3), sonication pretreatment and anaerobic digestion of *Tetraselmis* biomass (batch n°4) and high sonication pretreatment and anaerobic digestion of *Tetraselmis* biomass (batch n°5).

by Delran et al. (2023b). In addition, above a certain concentration, the cell population becomes excessively dense and reduces the effectiveness of shock waves created by the implosion of cavitation bubbles in the surrounding medium. As a result, the shock wave's vibration is dispersed over more cells, reducing the energy supplied to each cell (Halim et al., 2013).

Furthermore, it is observed that supernatant lipids content was not significantly affected with both specific energy sonication treatment and was around 17%. This result proves that lipids liberation in supernatant were unaffected after sonication, and almost 18% remained in the pellet. Natarajan et al. (2014) reported that *Tetraselmis suecica* has flexible cell membranes, which, after disruption, tend to coil up and retain the membrane lipids. On the other hand, carbs content values show that they were not significantly affected until doubling the sonication specific energy. Carbs content increased from 17.5 ± 0.4 for untreated biomass to 20.3 ± 0.4 and 25.5 ± 2% for sonicated biomass, and highly sonicated biomass, respectively. Higher sonication specific energy significantly improved carbohydrates solubilization. As reported previously, *Tetraselmis* carbohydrates release would be faster and more important at higher specific energy, leading to the easier release of carbohydrates into the medium (Delran et al., 2023b; Schwede et al., 2013).

Those results confirm that doubling the specific energy was ineffective in releasing more soluble compounds from *Tetraselmis* biomass that remained adsorbed to small colloids and adhered to cell debris except for carbohydrates. Results indicate that specific energy conditions of 1.6 kJ/g VS were effective to fragilize *Tetraselmis* cell wall and allow the partial liberation of soluble organic compounds to the bulk. More investigations are needed to prove the positive effect of *Tetraselmis* biomass pretreatment on its anaerobic digestion and co-digestion. In this regard, different anaerobic batches were conducted with pretreated *Tetraselmis* biomass and a pretreated mixture of *Tetraselmis* biomass and frying oil residue.

3.4. Effect of sonication pretreatment on anaerobic digestion of Tetraselmis biomass supplemented or not with frying oil

Three anaerobic batches were successively conducted in the duplicated STR. The third batch (after the first and second batches described in Section 3.2) consists on the co-digestion of the *Tetraselmis* biomass and frying oil mixture pretreated by sonication at selected specific energy conditions. The AD of sonicated *Tetraselmis* biomass as the sole substrate was investigated in a 4th batch to better understand the effect of pretreatment contribution on the biogas production yield. Finally, sonicated *Tetraselmis* biomass using a specific energy of 3.2 kJ/g VS was tested in the 5th anaerobic batch. This highly sonicated biomass was used to confirm the efficiency of the selected optimal specific energy of biomass pretreatment on biogas production yield and energy recovery. Similar to the first two batches previously developed in Section 3.2, a sonicated substrate with an equivalent total VS of 52 g of *Tetraselmis* biomass was fed to duplicated anaerobic STR. Besides, the same batch conditions were maintained, and the same digestor parameters were followed and monitored to allow a well-founded comparison of the obtained results.

The TOC evolution in batch n°3 during co-digestion of *Tetraselmis* biomass and frying oil pretreated with sonication at 1.6 kJ/g VS is shown in Fig. 3a. The soluble TOC remained relatively high in comparison to other batches, ranging between 1566.5 ± 62.9 and 1836 ± 8.4 mg/ml for the first 2 days. Compared with batch n°2 (Fig. 2a), results prove that sonication pretreatment has a positive effect on the solubilization efficiency of organic matter of both substrates contained in the reactor. It is observed that the mixture of sonicated *Tetraselmis* biomass and frying oil became more homogenous without phase separation and as an emulsion. In fact, this physical aspect of the mixture resulting from sonication pretreatment allowed higher solubilization of carbon during the first days of anaerobic treatment and better substrate accessibility compared with previous batches 1 & 2 and with ultrasonicated *Tetraselmis* biomass in batches 4 & 5. Similar results have been reported by Park et al. (2013), where ultrasound technique has been successfully used as pretreatment before microalgae anaerobic digestion, allowing an increase of organic compounds solubilization. After the first two days, the soluble TOC decreases continually to reach an average of 590.3 ± 22.8 mg/l. Thus, in addition to positive effect of ultrasonication pretreatment allowing better substrate accessibility, the C/N adjustment by frying oil residue addition could improve the anaerobic consortium degradation activity.

Fig. 3b showed a sawtooth profile of the soluble TN content in Batch n°3 digestate. TN content fluctuated around 985 mg/ml. It was no longer accumulated as previously observed in batch no.2, but it is rather decreasing, showing better nitrogen content equilibrium in the reactors. The supplementation effect of frying oil and the sonication of *Tetraselmis* biomass likely improved nitrogen metabolism during anaerobic digestion. In fact, the C/N ratio adjustment to 20 could reduce the high dissolving ammonia concentration and simultaneously reduce its inhibitory effect (Vargas-Estrada et al., 2022).

The effect of *Tetraselmis* biomass sonication on its anaerobic digestion was investigated in the 4th & 5th batches in order to understand the effect of pretreatment contribution on biogas production yield. As sole substrate, *Tetraselmis* biomass pretreated using 1584.6 and 3169.2 J/g VS as specific energy was fed to the duplicated anaerobic STR as batch no.4 and batch no.5, respectively. Fig. 3a shows that the total soluble organic carbon in the digestate was continually decreasing from an average of 1608 ± 52.0–700 ± 2.5 mg/l for batch no.4 and from an average of 1384 ± 21.2–836.5 ± 7.4 mg/l for batch no.5. After five days of anaerobic treatment, the soluble TOC slowly decreased, indicating that this fraction needs a longer time to be fully degraded. These results show that the increase in specific energy of sonication pretreatment resulted in a higher residual TOC after 11 days of anaerobic treatment. This high residual TOC could be due to the effect of pretreatment on *Tetraselmis* biomass disintegration leading to refractory compounds liberation which need longer time to be degraded.

Furthermore, it is observed that during the first day of digestion, the soluble TOC was 1608 mg/l for batch n°4 and it was lower (1384 mg/l for batch no.5 (Fig.5b). Similarly, the soluble total nitrogen in the digestate for the 4th batch was higher (1080 mg/l) in comparison with batch no.5 (844 mg/ml). The reduced values in TOC and TN in batch no. 5 in comparison with batch 4 could be explained by an increase in the adsorption capacity of *Tetraselmis* cells and debris from highly sonicated biomass inside the reactors, which decreased soluble organic carbon and nitrogen. Natarajan et al. (2014) reported previously that the *Tetraselmis* sp. cell membranes disruption through ultrasonication tend to coil up and could retain some compounds. Besides the possible adsorption

mechanism of highly sonicated *Tetraselmis* cells and debris resulting from physical microalgae disintegration, the generation of highly oxidative reactive radicals such as hydroxyl ($OH^{\bullet}$), hydroperoxyl ($HO_2^{\bullet}$) and hydrogen ($H^{\bullet}$), and hydrogen peroxide (H_2O_2) during ultrasound pretreatment, could oxidatively break down the soluble compounds into simpler forms (Khanal et al., 2007). After four days of anaerobic treatment, soluble total nitrogen (TN) in the digestate (Fig. 3b) showed an increase from 1083 ± 4.9–1300 ± 6.9 mg/l for batch no.4, while it showed an increase from 844 to 1425 mg/l after 8 days for batch no.5, which could be due to possible nitrogen-rich substrate solubilization and degradation under the effect of fermentative and hydrolytic bacteria. Then, TN decreased to 1130 ± 8.6 ml/g for batch no. 4 and to 1250 ± 12.5 ml/g for batch no.5 indicating its possible consumption for amino acids, nucleic acids, and proteins enzymes synthesis as explained in the previous section. These results show that the increase in specific energy of sonication pretreatment without C/N adjustment didn't reduce the residual soluble TN after the anaerobic treatment. Contrarily, TN is accumulated in batch no. 5 compared to batch no. 4. In fact, the higher *Tetraselmis* microalgae cells walls' fragmentation, caused by the increase of sonication specific energy as demonstrated by Delran et al. (2023b), leads to better degradation of those microalgae fragments by the anaerobic consortium. Thus, the resulting higher liberation of nitrogen-rich compounds could augment the risk of ammonia nitrogen inhibition.

Fig. 3c shows the pH evolution during the three anaerobic batches no. 3, 4, and 5. The pH of the 3rd batch decreased from around 7.7–7.4 after one day of anaerobic digestion, then it increased to 7.9 after 11 days. Indeed, the VFAs highly increased in the first 3 days to reach a value of 2186.7 ± 29.34 9 mg/l (Fig. 3e). Compared to first and second batches, these values are much higher. Under the effect of sonication, the substrate was more accessible to hydrolytic and fermentative bacteria, as reported previously (Arman et al., 2023). This better accessibility to substrate improved the anaerobic consortium activity, resulting in higher VFAs liberation in the digestate. After five days, VFAs decreased to an average of 506.0 ± 60.4 mg/l, reflecting high degradation rates of VFAs and good performance of anaerobic reactors. Furthermore, the digestate alkalinity increased after 11 days to the range of 5475.0 ± 141.4 mg $CaCO_3$/l (Fig. 3d) which mitigated the possible VFAs toxicity. In fact, VFAs/alkalinity ratio reached 0.48 and no instability in the digestion process was observed. Compared to previous batches no. 1&2, VFAs, alkalinity, and their ratio reached higher values during co-digestion with frying oil residue preceded by sonication pretreatment. These results reflect the highest achieved degradability during the batch no. 3. Similar results were reported by Park and Li (2012) in which the sonication pretreatment and the C/N ratio adjustment using a lipid-rich substrate enhanced the degradability of microalgae residue resistant cells and increased the anaerobic consortium activities and their metabolite.

During the anaerobic treatment of sonicated and highly sonicated *Tetraselmis* biomass as sole substrate, the pH slightly dropped during the first day and then increased to reach values around 7.7 and 7.8, respectively. Accordingly, the digestate VFAs concentration increased rapidly during the first day and reached values of 1419.0 ± 58.9 for batch no. 4 and 1336.0 ± 117.4 mg/l for batch no. 5. Then, VFAs concentration decreased below 590 mg/l for both batches. According to all previous batches, when the VFAs content increases, the pH oppositely decreases and vice versa. The alkalinity increased and stabilized at a value of 6475.0 ± 0.5 and 7100.0 ± 185.6 mg $CaCO_3$/l for batch no. 4 and batch no. 5, respectively. Compared to previous batches, the alkalinity highly increased. In fact, sonication pretreatment enhanced the fragmentation of *Tetraselmis* biomass cell wall which is composed of carbohydrates and proteins. The smaller size of fragments enhances proteins (nitrogen-rich compounds) release during anaerobic digestion process. The hydrolyzed ammonia from amino group of amino acids highly increased the alkalinity as reported previously by Gerardi (2003). Without C/N ratio correction, accumulation of TN content (Fig. 3b) and alkalinity (Fig. 3d) is observed which affect the anaerobic process and contribute to ammonia toxicity. Frying oil residue was an effective co-substrate with high carbon content as demonstrated during batch 3. As discussed previously, the co-digestion reduced total nitrogen content and alkalinity which reduce the inhibitory effects of alkalinity. Similarly, Park and Li (2012) reported that, the carbon and nitrogen imbalance in algal biomass residue as well as the lack of alkalinity in the lipids would be simultaneously countered by the co-digestion of algal biomass residue and a lipid-rich substrate, promoting a synergistic effect.

Batch no. 4 & 5 in Fig. 3f shows the beneficial effect of *Tetraselmis* biomass sonication on biogas production. The biogas production during the first day was around 12,000 ml against only 1856 ml for untreated *Tetraselmis* biomass (Fig. 2f). Then, the daily biogas production decreased drastically below 2000 ml after two days. Indeed, the cumulative biogas production of both batches no. 4 & 5 after 11 days was around 21 l and was much higher than the produced biogas from *Tetraselmis* raw biomass, during batch no.1 (11 l). Thus, the ultrasonication pretreatment improved the digestion of *Tetraselmis* biomass by microalgae cell wall fragilization. However, these results are still far from the theoretical cumulative biogas production value of 31 l calculated based on *Tetraselmis* biomass biogas yield of 600 ml/g VS (Bohutskyi et al., 2014). This could be due to the low C/N ratio. Moreover, after C/N ratio adjustment and 11 days incubation, the co-digestion of *Tetraselmis* biomass and frying oil sonicated mixture (Batch no. 3) showed the highest cumulative biogas production of 35 l when compared with all different batches. This value is close to the theoretical value of 38 l calculated according to both fractions considering that frying oil residue can produce up to 970 ml biogas/g (Pastor et al., 2013). These results confirm the beneficial effect of C/N ratio adjustment with frying oil residue coupled to ultrasonication pretreatment. In fact, the pretreatment of the mixture (microalgae-frying oil) allowed better accessibility of anaerobic consortium to substrates which explain the produced biogas improvement from batch no. 2 to batch no. 3. Furthermore, the higher cumulative biogas of batch no.3 compared to no.4 & 5 batches reflect not only the frying oil contribution in biogas production but also the positive effect of C/N ratio on anerobic consortium activities. Indeed, without C/N adjustment (batches no. 4 & 5) the biogas produced from *Tetraselmis* biomass (21 l) corresponds to 67.7% of theoretical value (31 l). After C/N adjustment in batch no. 3, the resulted biogas production from *Tetraselmis* biomass increased to 83.0%. These results confirm the positive effect of C/N adjustment on *Tetraselmis* biomass degradability enhancement when ultrasonication is used as pretreatment.

Fig. 4 shows biogas and methane yields. The average biogas yields in batch no.1 and batch no.2 were 195.3 ± 3.6 and 301.6 ± 15.8 ml/g VS, respectively. Based on methane percentage of 58.6% for the batch no. 1% and 63.5% for the batch no. 2, the methane

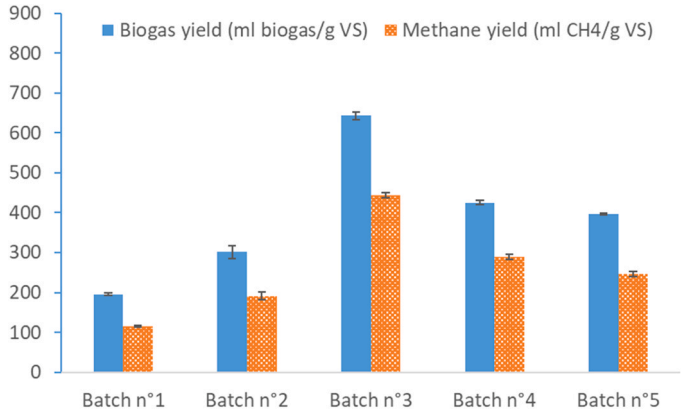

Fig. 4. Biogas and methane yields after: raw *Tetraselmis* biomass (Batch n°1), raw *Tetraselmis* biomass co-digested with frying oil residue (Batch n°2), sonicated and co-digested *Tetraselmis* biomass and frying oil mixture (batch no. 3), sonicated and digested *Tetraselmis* biomass (batch no. 4) and highly sonicated and digested *Tetraselmis* biomass (batch no. 5). * Responses with different letters are significantly different (one-way analysis of variance, $P < 0.05$; Tukey's test), ± standard deviation (SD) of two measurements.

yields increased from an average of 114.4 ± 2.1–191.4 ± 10.0 ml CH_4/g VS, respectively. Similar methane yield from raw *Tetraselmis* biomass was reported (Paul et al., 2023). *Tetraselmis* biomass co-digestion with residual frying oil showed methane yield improvement by 40.2%. Many authors reported that methane yield can be improved when C/N ratio is adjusted in the range of 20–25 using different co-substrates as fats, high carbon paper waste or glycerol (Park and Li, 2012; Santos-Ballardo et al., 2015; Yen and Brune, 2007). However, these methane yields remained under the theoretical value of 410 ml/g VS as reported previously by Harun et al. (2011).

After ultrasonication pretreatment using a specific energy of 1.6 kJ/g VS, biogas yields significantly increased from 195.3 ± 3.6 for untreated *Tetraselmis* biomass (Batch no. 1) to 425.3 ± 5.5 ml/g VS for sonicated *Tetraselmis* biomass (Batch no. 4) and to 643.0 ± 9.3 ml/g VS after C/N adjustment (Batch n°3). Based on methane percentage of 68% for Batch n°4% and 69% for Batch n°3, methane yields significantly increased from 114.4 ± 2.1–289.4 ± 7.4 and to 443.6 ± 6.4 ml CH_4/g VS, respectively. These results demonstrated the efficiency of sonication pretreatment of *Tetraselmis* biomass before the anaerobic process. The pretreatment fragilizes membrane cells of *Tetraselmis* sp. and facilitates the liberation of biodegradable compounds from the algae matrix allowing better degradation. However, the pretreatment was not enough to approach theoretical values without C/N ratio adjustment. The integration of co-digestion by mixing *Tetraselmis* biomass with frying oil residue and the ultrasonication pretreatment enhanced the methane yield during the methanization process. The obtained biogas and methane yields were close to theoretical and practical values previously reported (Bohutskyi et al., 2014; Harun et al., 2011).

When comparing batch n°4 to batch n°5, the highly sonicated *Tetraselmis* biomass produced significantly 14.8% lower methane yield than sonicated biomass with low specific energy (based on methane percentage of 62.1% for batch no. 5). The increase in specific energy of *Tetraselmis* biomass sonication during the pretreatment step, has a negative effect on its anaerobic digestion. Similarly, Paul et al. (2023) reported that higher ultrasonication pretreatment intensity of 10 kJ/g TS negatively impacted *Tetraselmis* biomass methanization. Results of the present work proved that the use of low sonication specific energy of 1.6 kJ/g VS, corresponding to 2.8 kJ/g TS, improved the anaerobic digestion process and significantly enhanced biogas yield when compared to doubled ultrasonication specific energy.

3.5. Analysis of energy balance

To assess the economic feasibility of different treatment, the output and input energy of each batch with and without sonication were calculated according to Cho et al. (2013) method. In this paragraph, the different energy balances were evaluated and compared. The results analysis indicates that low energy ultrasonic pretreatments of 1.6 ± 0.1 kJ/g VS resulted in higher positive energy balance of 8.6 ± 0.5 kJ/g VS when compared to non-pretreated *Tetraselmis* biomass (4.0 ± 0.1 kJ/g VS); while doubling the pretreatment specific energy to 3.2 ± 0.1 kJ/g VS resulted in a decrease of energy balance to 5.5 ± 0.3 kJ/g VS. Furthermore, co-digestion with frying oil increased the energy balance to 6.8 ± 0.4 kJ/g VS. When *Tetraselmis* biomass and frying oil mixture was sonicated before anaerobic co-digestion, a positive energy balance of 14.1 ± 0.2 kJ/g VS was obtained, which was far superior than that obtained from the non-pretreated mixture. All these findings allow us to conclude that *Tetraselmis* biomass co-digestion with frying oil and sonication pretreatment of the mixture highly promote methane production and consequently generate a positive energy balance.

4. Conclusion

- The combination of sonication pretreatment and co-digestion of *Tetraselmis* biomass and frying oil residue represents a useful and profitable way to upgrade waste products and generate renewable energy. Main conclusions drawn from this study are:

- Combination of ultrasonication and *Tetraselmis* biomass C/N ratio adjustment using frying oil residue improved significantly the anaerobic process and gave highest TOC solubility and best methane yields of 443.6 ± 6.4 ml CH_4/g VS.
- Ultrasonication pretreatment using Low specific energy of 1.6 kJ/g VS led to superior positive output energy recovery from *Tetraselmis* microalgae AD than 3.2 kJ/g VS.

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CRediT authorship contribution statement

Firas Feki: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft, Writing – review & editing. **Maroua Cherif:** Investigation, Methodology. **Mohamed Ali Masmoudi:** Software, Writing – review & editing. **Mohamed Chamkha:** Resources. **Imen Saadaoui:** Resources. **Probir Das:** Resources, Writing – review & editing. **Sami Sayadi:** Funding acquisition, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2023.103478.

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