Research Article

Anaerobic Co-Digestion of *Caulerpa prolifera* and *Gracilaria gracilis* from the Nador Lagoon: Evaluation of Biogas Production Potential

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Abstract
The growing global energy demand, depletion of fossil fuel reserves, and escalating impacts of climate change highlight the urgent need for alternative renewable energy sources. In this context, the present study investigates the potential of biogas production from the co-digestion of two marine macroalgae, *Caulerpa prolifera* and *Gracilaria gracilis*, which are naturally abundant and renewable in Morocco's Mediterranean Marchica Lagoon. Beyond conventional mono-digestion approaches, which are generally less exploited for marine macroalgae and often limited by process instability, this study demonstrates that combining algal substrates can induce synergistic or antagonistic interactions that strongly influence process performance, leading to enhanced biogas yield and improved process stability when appropriate mixing ratios are applied. Three co-digestion tests (T_1, T_2, and T_3) were conducted with varying substrate proportions (25–75%) to assess the influence of algal composition on biogas yield and process stability. The mixture dominated by *Caulerpa* (75%, T_1) produced the highest biogas yield (78 mL/gVS) and showed minimal digester acidification, whereas the test with a higher *Gracilaria* content (T_3) resulted in reduced biogas production and process inhibition. The balanced mixture (T_2) displayed intermediate performance and moderate acidification. These results reveal a dual interaction between the two algae: *Caulerpa* exerts an antagonistic effect on *Gracilaria* co-digestion, while *Gracilaria* enhances *Caulerpa* digestion efficiency. Overall, the study demonstrates that optimizing substrate ratios in algal co-digestion can significantly improve biogas production, providing a sustainable approach to energy recovery from local marine biomass and supporting the renewable energy transition in coastal regions.

Keywords: Alga, Biofuel, Biogas, Methane, Microbes, Sustainable energy

1 Introduction

Our planet is facing massive overexploitation of non-renewable resources, particularly fossil fuels, which still account for approximately 84% of global primary energy consumption [1]. Excessive energy use from combustion, such as fossil fuels, causes significant environmental damage [2], [3]. Growing demand, rapid depletion of fossil fuel reserves, global climate change, and rising crude oil prices, combined with the energy dependence of producing countries, are raising concerns. In response to these challenges, researchers are developing an alternative and sustainable energy source to build upon previous problems [4].

In this context, Morocco has historically relied heavily on fossil fuel imports (over 90%) to satisfy its energy needs [5]. This dependence on imported fossil fuels, especially petroleum products and natural gas, exerts pressure on the country's energy security and economic stability [6]. Fossil fuel imports have caused trade deficits and increased exposure to international fuel price swings. To address these challenges, Morocco has invested heavily in developing its energy sector, with a particular emphasis on renewable energy sources. Additionally, Morocco has set ambitious targets to increase the proportion of renewable energy in its overall energy mix [7].

Thus, our country invested in several renewable energy projects. For example, the Noor solar complex in Ouarzazate aims to become one of the world's largest solar power plants upon full operation [8]. Wind farms are located in various regions of the country, including Tangier and Taza, with projects to develop this sector currently in the planning stage. Morocco's coastal geography offers favorable conditions for wind energy generation, as seen in the Dakhla wind farm [9]. Despite these efforts, Morocco continues to rely heavily on fossil fuel imports, highlighting the need to diversify renewable energy pathways further.

Among emerging renewable energy options, biogas production from algae-based biomass is emerging as a new investment focus in Morocco, because this biomass is accessible, sustainable, renewable, and has a long lifespan [10]. The decision to use algae for biogas generation relies on three key factors: availability of this bioresource, high production, rapid growth rate, minimal development space, and low algae-farming costs [11]. Therefore, converting algae into biogas opens a new pathway for Morocco's energy strategy and development. One local opportunity to adopt this technology is the Nador lagoon, which serves as an extensive reservoir of macroalgae.

Nador lagoon contains 110 species of macroalgae divided into three groups: 60 Rhodophyceae, 31 Chlorophyceae, and 19 Phaeophyceae [12]. In several of our studies, we have confirmed that many algae from the Nador lagoon can be converted into biogas. Therefore, algae from the Nador lagoon are especially in high demand for biogas production. Now, Morocco has significant potential to regenerate algae as a renewable energy resource [13]. Therefore, it is vital to promote this and support decision-makers and entrepreneurs in using this organic material to produce green energy, reducing our dependence on imported fossil fuels and helping the sustainable development of our planet [14].

However, most existing studies on algal anaerobic digestion in lagoon environments have focused primarily on mono-digestion, in which a single algal species serves as the feedstock [15]. While mono-digestion is technically manageable, it often requires a continuous, homogeneous biomass supply and may be prone to process instability, nutrient imbalance, or inhibition. In contrast, co-digestion, which combines multiple algal substrates, offers the potential to improve biodegradability, nutrient balance, and process stability [16]. Despite these advantages, the literature on the co-digestion of dominant macroalgal species in lagoon ecosystems remains scarce, particularly regarding interaction effects, optimal substrate ratios, and process stability under batch anaerobic digestion conditions.

Previous studies on algal anaerobic digestion have mainly focused on monodigestion or co-digestion with terrestrial substrates, limiting their applicability to marine biomass systems. Generic digestion studies cannot be directly transferred to marine macroalgae due to their specific biochemical characteristics, including salinity, complex polysaccharides, and inhibitory compounds. Moreover, the co-digestion of two marine macroalgae from the same ecosystem but with contrasting biochemical properties has not been systematically investigated. More specifically, there is a clear lack of studies examining the co-digestion of *Caulerpa prolifera* and *Gracilaria gracilis*—two of the most abundant macroalgae in the Marchica lagoon—and evaluating how their combined digestion influences biogas yield and anaerobic digestion performance. Recent studies have highlighted the importance of optimizing algal combinations and digestion strategies for sustainable bioenergy production, yet lagoon-specific co-digestion systems remain underexplored, especially in Mediterranean contexts.

Based on these identified gaps, the present study aims to evaluate the anaerobic co-digestion of *Caulerpa prolifera* and *Gracilaria gracilis* collected from the Nador lagoon. The specific objectives are to assess how different substrate ratios affect biogas production and process stability, and to elucidate potential synergistic or antagonistic interactions between the two macroalgae during anaerobic digestion. We hypothesize that an optimized co-digestion ratio can enhance biogas yield and process

stability relative to mono-digestion, thereby improving energy recovery from lagoon-derived algal biomass. This work aims to contribute to sustainable bioenergy strategies and to support Morocco's national energy transition and circular bioeconomy goals.

2 Materials and Methods

2.1 *Study Area: Nador Lagoon*

Morocco is a coastal country with 3,500 kilometers of coastline, bordered by the Mediterranean Sea on one side and the Atlantic Ocean on the other.

This coastline hosts 60% of the population and 90% of its industrial units [17]. Along the Mediterranean coast lies the Nador Lagoon, also known as Marchica, which is considered one of the most important lagoons nationally and internationally in terms of surface area, biodiversity, and socio-economic benefits [18]. Our research took place in this lagoon.

Nador Lagoon is the largest in Morocco, covering 115 km² with a maximum depth of 8.2 meters. This lagoon is considered the second-largest lagoon ecosystem along the southern coast of the Mediterranean [19] (Figure 1). It is located on the northeastern Moroccan coast, between Cap de Trois Fourches and Cap des Eaux, specifically at (02°55'-02°45' and 35°16'-35°06') [20]. Semi-elliptical in shape, it is separated from the sea by a 25 km long dune belt, oriented NW-SE [21]. Its connection to the Mediterranean is secured by a pass established in 2010. This opening aims to reduce the residence time of lagoon waters to just 25 days. Thus, the Nador lagoon will transition from an "obstructed lagoon" to a "restricted lagoon." The Nador lagoon offers an artisanal fishing area and serves as a nesting and breeding site for many fish, mollusks, and crustaceans (131 macrofauna taxa) [22]. Ramdani *et al*., identified 110 macroalgae species divided into three groups: 60 Rhodophyceae, 31 Chlorophyceae, and 19 Phaeophyceae [12]. This lagoon environment has been the focus of numerous research studies across various fields, especially to understand its hydrology, sedimentology, and biological organization [23], [24]. However, research on the valorization of algae from this lagoon remains limited.

2.2 *Sampling strategy*

The goal of the sampling strategy is to carefully plan the sampling process to accurately estimate the available macroalgal biomass. The sampling stations were selected carefully to collect only easily recoverable algae. Sampling occurred along the lagoon's edges from October 2023 to October 2024. Thus, we established six sampling points, each marked by its geographic coordinates (Figure 1).

This study examines the algae *Caulerpa prolifera* and *Gracilaria gracilis*. These macroalgae were selected for their high natural abundance in the Nador (Marchica) Lagoon, ensuring readily available biomass and facilitating easy, selective harvesting [25]. When carefully managed and focused on removing excess biomass, especially during bloom periods, harvesting can help mitigate eutrophication while preserving biodiversity and habitat integrity.

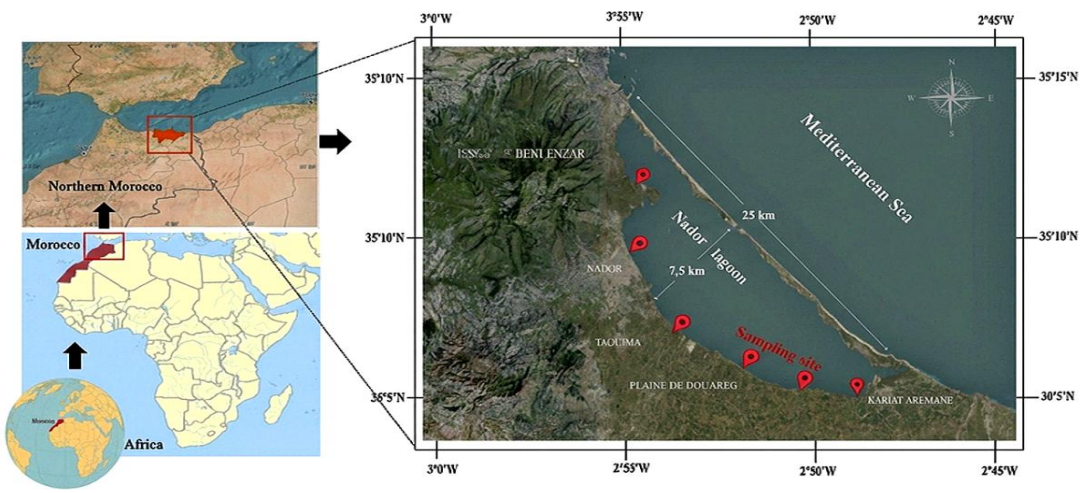

Figure 1: A representative map of the sampling network in Nador Lagoon.

Integrated into a regulated lagoon management framework, this approach minimizes ecological disturbance and enables macroalgal harvesting to serve as both an environmental remediation strategy and a sustainable, renewable feedstock for biogas production, thereby supporting long-term ecological balance and energy sustainability. *Caulerpa prolifera* is known for its high primary productivity, which helps clarify water, retain suspended minerals, and provide habitat for various animals, including crustaceans and polychaetes [26]. *Gracilaria gracilis* shows a cluster of tangled cylindrical axes, measuring 1 to 2 mm in diameter. This species tolerates salinity fluctuations and is commonly found in lagoons, where its abundance indicates moderate water eutrophication. It is a type of alga that often floats near the bottom, forming mats. Individuals attached to the substrate can tolerate having their base covered with sand (Figure 2).

Figure 2: Two algae used in this study in both fresh and dried states. (a_1) *Caulerpa prolifera* in fresh form; (a_2) *Caulerpa prolifera* after drying at 105°C; (b_1) *Gracilaria gracilis* in fresh form; and (b_2) *Gracilaria gracilis* after drying at 105 °C.

2.3 *Anaerobic digestion: Preparation of algal substrate and inoculum*

After harvesting, we cleaned and sorted the two algae we studied (*Gracilaria gracilis* and *Caulerpa prolifera*). Then, they underwent two physical pretreatments: the first was thermal, involving heating at 105 °C for one day [27]. Drying at 105 °C for 24 h was selected as a physical (thermal) pretreatment to ensure complete and reproducible dehydration of algal biomass, which is characterized by very high moisture content (85–95%). *Caulerpa prolifera* has 95% of water [28], but *Gracilaria gracilis* has 85.6% [29]. This step allows accurate determination of dry matter, volatile solids, mass balance, and biogas yields, minimizing biases associated with residual moisture. Although minor alterations of heat-sensitive compounds may occur, the temperature remains below the threshold for significant degradation of major algal macromolecules (polysaccharides, proteins, lipids), thus preserving the overall biodegradability of the substrate. Compared to lower-temperature drying or freeze-drying, drying at 105 °C is standardized, simple, cost-effective, and scalable, making it suitable for both experimental reproducibility and practical biogas applications [29]. This pretreatment represents a balanced compromise between dehydration efficiency, analytical reliability, operational feasibility, and limited impact on algal organic matter. Mechanical pretreatment was performed using a grinder (Retsch) to decrease the algae size, making anaerobic digestion easier [30]. After crushing the algae, we sieved them using a stainless steel sieve (0.25 mm diameter) to standardize the size of the algal substrate intended for the anaerobic digestion process. The inoculum used in this study was anaerobic digestate collected from a full-scale mesophilic biogas digester treating poultry manure. The digester operated under stable mesophilic conditions (35 ± 2 °C). The inoculum had a pH of 7.3 ± 1 and a volatile solids content of 67 ± 1 % of total solids. Notably, the inoculum showed high microbial activity, as evidenced by stable biogas production during blank assays and a methane content of approximately 72 % CH_4. This methane concentration is characteristic of high methanogenic activity and a well-functioning anaerobic microbial community, confirming the inoculum's suitability for anaerobic digestion. To activate this inoculum, we kept it under anaerobic conditions at 35°C in a 1-liter reactor sealed with a waterproof silicone stopper. We pierced the reactor at the top by a biogas discharge pipe for 15 days [31]. This ensured that all residual organic matter was exhausted and the bacterial population in the inoculum was activated, preparing it for anaerobic digestion.

2.4 *Anaerobic digestion test of algae*

We constructed three types of 100 mL digesters (T_1, T_2, and T_3). Each digester type contains 20 g of dry

algal matter and 80 g of inoculum, leading to a substrate-inoculum ratio of 1:4. This choice aligns with previous studies on the anaerobic digestion of algal biomass, which recommended a low substrate-to-inoculum ratio (1:4) to improve the process [32], [33]. Several studies have confirmed that this ratio enhances buffering capacity and microbial adaptation, resulting in more efficient digestion and higher biogas yields [34], [35]. So, we selected this ratio because it is considered optimal [36], [37]. Thus, we fixed all the digesters in this study with a constant inoculum amount (80 g) (Table 1). Nevertheless, the three tests are characterized by variations in their algal fraction; each test includes both species of algae but with different proportions (50% 50%; 25% 75%; 75% 25%) (Table 1).

T_1 contains a dominance fraction of *Caulerpa prolifera* (75% C), while T_3 has a dominant quantity of *Gracilaria gracilis*. In contrast, T_2 has equal 50% shares of each algae type. Thus, this variation in algal substrate allows the study of the effects of algal combinations on anaerobic digestion, measured by the amount of biogas produced.

The digesters are filled based on a scale that enables a mass balance in grams directly in the reactors, assuming an inoculum density of 1 [38]. Each test is repeated three times. After filling the digesters, the gas spaces of all six digesters were flushed with nitrogen for 5 minutes to remove oxygen and establish anaerobic conditions, and then the digesters were sealed. Hence, all digesters are batch-type. The final test type, T_4, serves as a control containing only the inoculum to measure the biogas produced by the residual organic fraction of the inoculum, which is then subtracted from the biogas generated by the algal organic fractions. The 12 digesters were incubated in a water bath at 35 ± 1 °C for 40 days [39]. Biogas production was monitored daily by water displacement using an inverted burette connected to each digester. The measured biogas volumes were corrected and reported at standard temperature and pressure (STP; 0 °C and 1 atm), following the method described in [10].

2.5 *Monitoring of physicochemical parameters*

We also measured total solids by drying fresh algae at 105 °C until it reached a constant weight. Subsequently, we measured volatile solids (VS) using a gravimetric method based on the mass loss of dried seaweed from the TS determination in a muffle furnace at 550 °C for 6 h [40]. We measured the digesters' pH using a Symphony-type pH meter, calibrated with two pH standards (4 and 7). The pH is determined in two stages: at the beginning of the manipulation (pH_i) to confirm proper initiation of anaerobic digestion, and at the end, after 40 days of incubation (pH_f) to verify completion of the process. In the present study, pH was measured at the beginning and end of each batch anaerobic digestion assay to assess the overall degree of acidification and compare process stability across the different co-digestion conditions. Intermediate pH monitoring was not feasible because the experiments were conducted in sealed batch digesters, which could be opened only at the end of the digestion period without disrupting anaerobic conditions.

3 Results and Discussion

3.1 *Biogas production kinetics*

After 35 days of incubation, we observed that the three substrate mixtures, labeled as Test 1 (T_1) (25% G + 75% C), Test 2 (T_2) (50% G + 50% C), and Test 3 (T_3) (75% G + 25% C), produced biogas but in different ways. Generally, all groups show characteristic kinetics with three phases: an initial rapid production phase, a more moderate growth phase, followed by a plateau (Figure 3). All tests indicate that there is no latency phase, meaning biogas production begins from the first day of incubation.

Table 1: Experimental protocol for the proportions of algal substrates, inoculum, and water used in this study.

Tests	Inoculum mass (g)	Alga mass *Gracilaria gracilis* (g)	Alga mass *Caulerpa prolifera* (g)	Mass of water (g)	Mass of digester (g)
T_1	80	5	15	0	100
T_2	80	10	10	0	100
T_3	80	15	5	0	100
T_4	80	0	0	20	100

The T_1 mixture (25% G + 75% C) shows the best performance, with biogas production reaching about 79 mL/gVS on the 34th day. So, T_1 demonstrates significant potential for biogas production compared to other tests. Additionally, the kinetics show a rapid rise in the early days, resulting in a significant initial output (40 mL/gVS), indicating that nearly half of the biogas is produced within the first two days. The T_2 mixture (50% G + 50% C) shows intermediate production, yielding approximately 50 mL/gVS. Therefore, the start-up production is slower than that of T_1, which reaches about 30 mL/gVS on the second day. This indicates a less pronounced initial growth phase compared to T_1. Then, we observe a steady, continuous increase; however, a cap of around 50 mL/gVS after day 10 suggests a halt in biogas production. On the other hand, the T_3 test (75% G + 25% C) shows the lowest biogas production, limited to about 28 mL/gVS. Thus, it demonstrates the lowest biogas production efficiency among the tests. The initial production is relatively low at 15 mL/gVS on day 2. After this phase, we observe a slight increase, followed by stabilization, with minimal growth continuing through day 30.

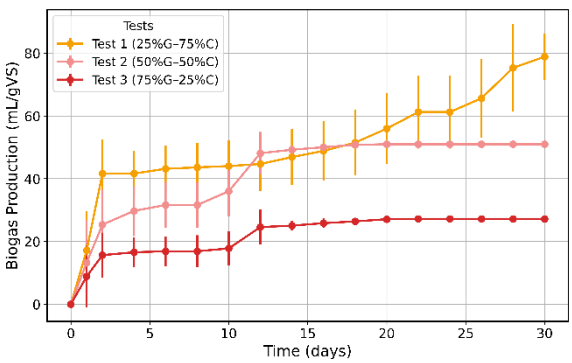

Figure 3: Biogas production kinetics of the three algal tests (0-35 days, 2-day intervals).

We used the modified Gompertz model to improve the description of biogas production kinetics in the three tests [41]. This is the best mathematical model for this strain based on the following equation:

$$B(t) = Bmax \times \exp\{-\exp[((Rm \times e)/Bmax)(\lambda - t) + 1)]\}$$

where : B(t): Cumulative biogas production at time t (mL/gVS),
Bmax: Maximum potential biogas production (mL/gVS),
Rm: Maximum rate of specific production (mL/gVS/day),
λ: Latency period before production begins (days),
e: The base of the natural logarithm (~ 2.718).

The modified Gompertz model was selected for its ability to accurately represent cumulative biogas production in batch anaerobic digestion by explicitly incorporating the lag phase, the maximum biogas production rate, and the ultimate biogas potential. Compared with first-order and Cone models, it better accounts for microbial adaptation and substrate complexity and has demonstrated superior goodness-of-fit in macroalgal co-digestion systems.

Additionally, adjusting the modified Gompertz model to the experimental data enabled us to estimate the kinetic parameters listed in Table 2. The T_1 mixture has a maximum specific production rate (Rm) of 6.5 mL/gVS/day, which is significantly higher than that of T_2 and T_3 (4.3 and 2.7 mL/gVS/day, respectively). Therefore, the conversion rate of organic matter into biogas is also faster in this case. The maximum methanogenic potential (Bmax) follows the same hierarchy; it is significantly higher for Test 1, indicating better overall biodegradability of the mixture. The lag phase (λ), linked to microbial adaptation (1.2 days), appears shorter in Test 1, while it is slightly longer in Test 3, suggesting a slower initial activity. Thus, the algal ratio (25% G + 75% C) allows for faster production and a quicker start to anaerobic digestion.

Table 2: The kinetic parameters of the Gompertz model for the three study tests.

Tets	B_{max} (mL/gVS)	R_m (mL/gVS/day)	λ (day)
T_1 (25% G + 75% C)	75	6.5	1.2
T_2 (50% G + 50% C)	50	4.3	2.8
T_3 (75% G + 25% C)	32	2.7	4.5

To improve the analysis, we combined the curves fitted by the Gompertz model with the linear regressions (Figure 4). Consequently, we observed that the curves fitted by the Gompertz model accurately represent the sigmoidal dynamics typical of anaerobic fermentations. At the same time, the linear regressions highlight average growth trends over time. Visually, Test 1 demonstrates the highest production throughout the entire period, followed by Test 2, while Test 3 has the lowest values. Linear regressions

confirm these trends: the slope, which indicates the average growth rate, is higher for Test 1 and lower for Test 3. The coefficients of determination (R^2) indicate strong consistency between the experimental kinetics and the linear or sigmoidal models used. Therefore, Test 1 shows the highest slope, indicating a significantly greater average biogas production rate. This result indicates increased microbial activity and a more suitable environment for converting organic matter into biogas. The high rate observed indicates improved availability of easily hydrolyzable substrates and consistent metabolic activity during the linear phase [42].

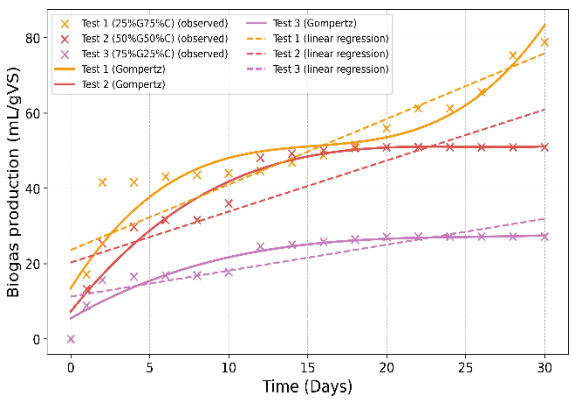

Figure 4: Comparison of biogas production from different substrate ratios using Gompertz and linear regression models.

We presented the experimental cumulative biogas production data for three co-digestion conditions using a boxplot distribution analysis combined with a scatter diagram (swarmplot). This approach displays each experimental point, providing a more detailed view of the distribution of results (Figure 5). This analysis helps us evaluate statistical spread, central tendency (median), and variability within groups, while also highlighting potential extreme values. Kasinath *et al.*, used this technique to visualize the distribution of biogas yields across substrate proportions quickly and to evaluate experimental reproducibility and process stability [43]. Test 1 (25%G, 75%C) has the highest median (46,9 mL/gVS) and the widest variation—the highest among the three configurations—indicating greater and more variable production, typical of the conversion of organic matter into very active biogas. This variability could result from intense microbial activity and a substrate rich in easily fermentable compounds [44]. The majority of the data are concentrated around this median, suggesting that the process has reached a robust stationary phase and that inter-sample variability is under control. The proximity of the median and the mean in this case indicates a narrow distribution at low values, suggesting a stable process. This confirms a synergistic effect in co-digestion, encouraging microbial stability and ongoing biogas production.

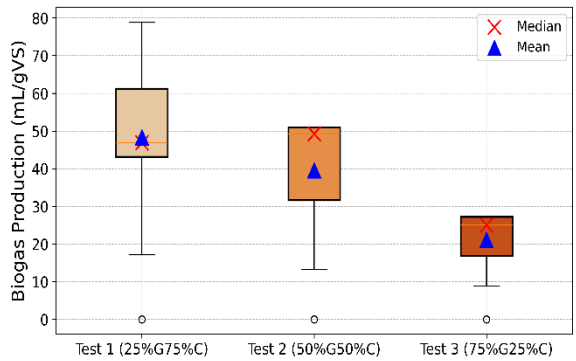

Figure 5: Distribution of biogas production per test. ANOVA (one-way analysis of variance) indicates that the *p*-value is much lower than 0.05 (*p*-value < 0.05), showing a highly significant difference among the three groups (T_1, T_2, T_3) in biogas production.

Test 2 (50% G, 50% C) shows a slightly higher median but with more variation. This result shows that, while the central value is similar, the data distribution is less homogeneous. In other words, some samples reach production levels similar to those of Test 1, while a smaller group remains below, indicating greater operational variability. This behavior might be linked to a local imbalance in organic loads or less effective microbial interactions between the two substrates [45].

In contrast, test 3 (75%G, 25%C) has the lowest median (21 mL/gVS) and a reduced amplitude, leading to limited production, a less dynamic conversion process, and slower degradation kinetics. This result is explained by the dominance of the G substrate, which limits the conversion of organic carbon into biogas. Thus, the high and stable median position in Test 1 demonstrates consistent results and good experimental reproducibility—two key criteria for assessing co-digestion systems [46]. On the other hand, the lower median in Test 3 suggests an imbalance in the process, often linked to the substrate's composition or partial inhibition of the methanogenic

microorganisms. Therefore, test 1 (25% G + 75% C) proved to be the most favorable condition, combining high yield with effective biological performance.

Therefore, this analysis reveals an inverse relationship between the proportion of substrate *Gracilaria gracilis* and biogas production. Conversely, increasing the amount of substrate *Caulerpa prolifera* enhances the overall biodegradability of the mixture and boosts biogas production. These results confirm that the composition of the algal mix is a crucial factor in anaerobic digestion performance. The box plot analysis highlighted the importance of optimizing the co-digestion ratio to maximize synergy between substrates, enhance process stability, and increase biogas productivity.

The differences observed in our graphs are therefore statistically significant. In other words, the substrate ratio (G/C) has a statistically significant effect on the total biogas produced. To advance the analysis, we employed a one-way ANOVA followed by a Tukey Honestly Significant Difference (HSD) post hoc test to evaluate significant differences between group means (Figure 6). The Tukey HSD test accurately identifies pairs of groups with statistically significant differences. The differences between Test 1 and Test 3, as well as between Test 2 and Test 3, are significant. However, the difference between Test 1 and Test 2 is small, indicating similar performance in both conditions. These results validate the strong influence of the co-digestion ratio on methanogenic performance, confirming that the proportion of substrate C is a key factor in maximizing yield.

In other words, the average biogas production decreases linearly with increasing substrate G proportion. So, test 1 (25% G + 75% C) provides the best balance between yield, stability, and experimental reproducibility. However, test 3 (75% G + 25% C) shows potential inhibition of digestion. Statistical analysis (ANOVA + Tukey HSD) confirms the significance of differences and the robustness of the results (Figure 6). These observations are consistent with previous studies on synergistic co-digestion, which show that a ratio dominated by a biodegradable, nutrient-balanced substrate maximizes biogas production from organic matter [47].

Statistical analysis from the radar chart reveals significant kinetic differences between the three tests (Figure 7). The mean and regression slope values indicate that Test 1 has the most efficient methanization process, indicating a rapid conversion of organic matter into biogas and a favorable environment for methanogenic communities. This behavior indicates active hydrolysis, sustained acidogenesis, and efficient methanogenesis, resulting in a significantly higher cumulative production, which aligns with findings in the co-digestion literature [48].

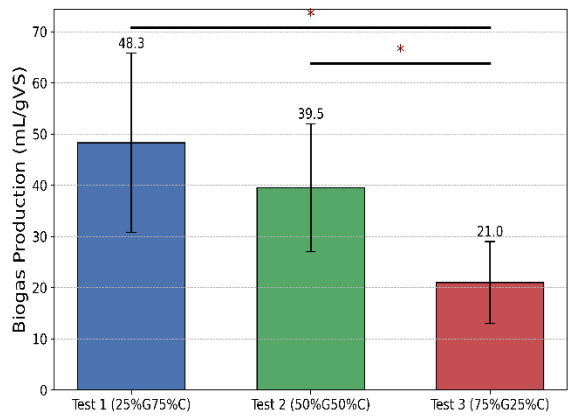

Figure 6: Mean biogas production + standard deviation (SD) for the group (ANOVA and Tukey HSD significance). The bars represent the mean ± SD of biogas production. The numbers at the top indicate the mean values (mL/gVS). The red asterisks mark theTukey HSD test, *p*-value < 0.05.

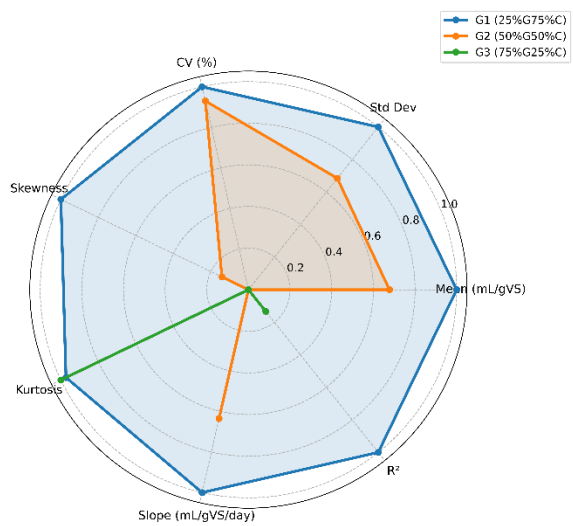

Figure 7: Comparative statistical radar chart of biogas production tests.

The coefficient of determination (R^2) supports this view: higher values in Test 1 suggest consistent, predictable kinetics, indicating a continuous,

undisturbed production phase. In contrast, the decline in R^2 observed in Test 3 suggests more irregular production, likely caused by hydrolytic limitations and lower substrate biodegradability. This slowdown agrees with the work of Qu *et al.,* (2021), who highlight the strong influence of hydrolysis rate on anaerobic system performance [49]. Dispersion parameters reinforce these trends. Although Test 3 has a low coefficient of variation indicating a certain homogeneity, this result mainly reflects slow methanization, with a stable but low-yield microbial regime. In contrast, Test 1 combines high yield and controlled variability, reflecting an optimal metabolic environment with a better nutrient balance, which promotes the joint activation of hydrolytic and methanogenic pathways [50].

The study of the asymmetry (skewness) and kurtosis parameters also shows that Test 1 tends to maintain high daily production levels. At the same time, Test 3 remains focused on low values, confirming its weaker methanogenic potential [51]. Therefore, the convergence of statistical indicators clearly indicates that Test 1 is the most effective, Test 2 is intermediate, and Test 3 is the least efficient. This hierarchy results directly from the biodegradability of the mixture and the availability of the substrate for the key microbial groups (Figure 7).

All observations confirm that the co-digestion ratio is a key parameter for optimizing the kinetics and efficiency of the methanization process, as it simultaneously influences microbial dynamics, system stability, and conversion efficiency. Therefore, Test 1 shows the best production kinetics, followed by Test 2 and then Test 3, confirming that the co-digestion ratio directly influences the algal methanogenic performance.

2.2 *Comparison of biogas production between co-digestion and mono-digestion of algae*

A later study demonstrated that *Gracilaria gracilis* is much more effective than *Caulerpa prolifera* as a bioresource for biogas production. Monodigestion of *Gracilaria gracilis* yields 86.3 mL/gVS, which is significantly higher than the biogas from *Caulerpa prolifera* (24.5 mL/gVS)[40]. Mono-digestion tests were conducted under experimental conditions strictly identical to those of the co-digestion assays (same substrate-inoculum ratio, temperature, inoculum, and batch protocol). These conditions were previously validated in a published study using the same algal biomasses and experimental setup, ensuring the robustness and reliability of the comparison. To further examine biogas production based on the ratios of these two algae, we compared the total biogas output of mixtures T_1 (25% G + 75% C), T_2 (50% G + 50% C), and T_3 (75% G + 25% C) with the biogas produced from each algae's monodigestion separately (Figure 8).

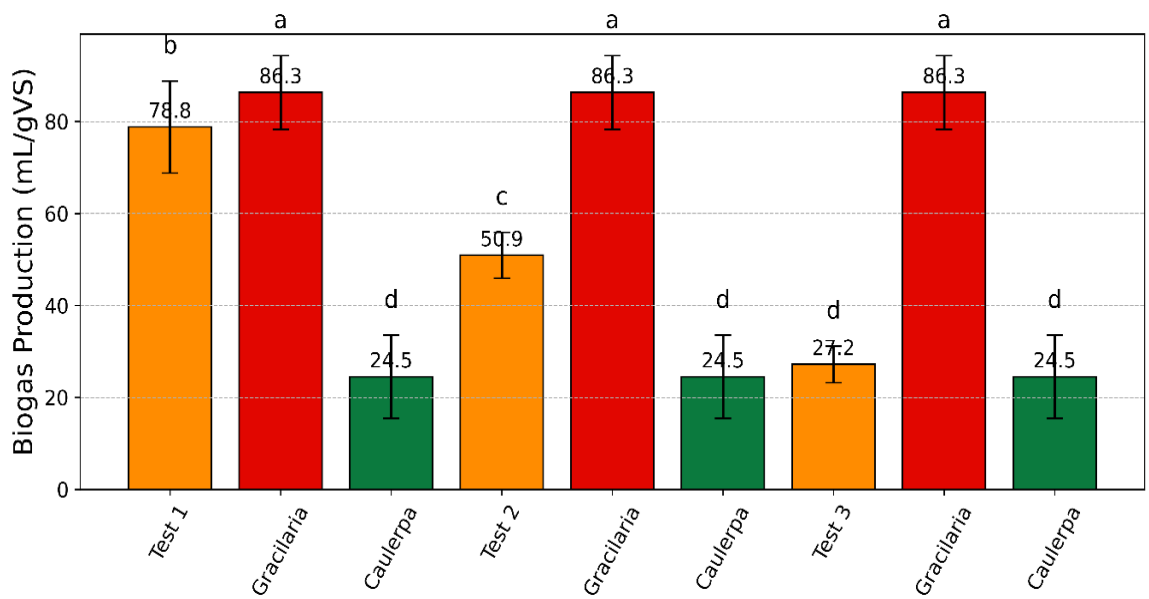

Figure 8: Comparison of biogas production from *Gracilaria* and *Caulerpa* under various test conditions.

Therefore, we found that the three mixtures (T_1, T_2, and T_3) generated more biogas than the monodigestion of *Caulerpa prolifera* alone (24.5 mL/gVS). This suggests that adding *Gracilaria gracilis* as a co-digestion substrate is an effective approach for anaerobic digestion of *Caulerpa prolifera*. Furthermore, the T_1 mixture, which contains a low 25% fraction of *Gracilaria*, helped increase the conversion of *Caulerpa prolifera* by more than three times compared to its monodigestion. On the other hand, we observed that the three mixtures (T_1, T_2, and T_3) produced less biogas than *Gracilaria gracilis* monodigestion (86.3 mL/gVS). Therefore, co-digestion of *Gracilaria gracilis* with *Caulerpa prolifera* does not benefit the red algae. Additionally, a high 75% content of *Gracilaria gracilis* combined with a low 25% of *Caulerpa* decreased biogas production from *Gracilaria* by more than threefold compared to monodigestion. Therefore, we observe a negative impact on *Gracilaria gracilis* conversion when *Caulerpa prolifera* is present. In summary, *Gracilaria* appears to have an antagonistic effect on *Caulerpa*'s conversion, whereas *Caulerpa* shows a synergistic effect when *Gracilaria* is present.

To evaluate the variability of biogas production across different experimental tests, we performed three statistical analyses (ANOVA, Levene, and Effect Size) (Figure 9). The ANOVA results (F = 22.86) indicate a highly significant difference between the groups, confirming that the observed variations are not due to chance but result from the experimental conditions and the type of substrate used.

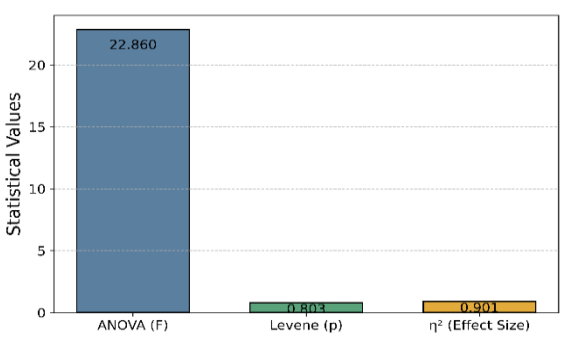

Figure 9: Global statistical results of the group comparison.

The Levene test (*p*-value = 0.803) is significantly higher than the significance threshold (α = 0.05). This indicates that there is no significant difference between the groups' variances: the data therefore demonstrate homogeneous dispersion. So, the variances between groups are homogeneous. This homogeneity increases the credibility and robustness of the statistical results, ensuring that differences in biogas production means are not due to excessive or unbalanced variability within groups. Levene's test thus confirms the reliability of intergroup comparisons and supports the use of ANOVA to evaluate the effect of algal substrate and experimental conditions on biogas production.

Finally, the effect size ($\eta^2 = 0.901$) indicates that differences in substrate and operating conditions explain 90.1% of the total variance in biogas production, while only 9.9% results from random variability. This result demonstrates that the experimental effects are not only statistically significant but also highly meaningful from both a practical and biological perspective. So, 90% of the total variability in biogas production is explained by differences in algal ratio and operating conditions. These results demonstrate the statistical robustness of the analysis and confirm that the algal ration in the digester and the experimental parameters are crucial in the observed anaerobic digestion performance.

The Tukey HSD matrix illustrates the *p*-values from multiple comparisons among the five experiments: Test 1, Test 2, Test 3, *Gracilaria*, and *Caulerpa* (Figure 10). This post hoc test, performed after the ANOVA, shows that *Gracilaria* has very low *p*-values (*p*-value < 0.05), indicating a highly significant difference from all other groups. This confirms that *Gracilaria* produces significantly more biogas across all tests. The groups Test 1, Test 2, and Test 3 also show significant differences, reflecting a decreasing progression in biogas production (Test 1 > Test 2 > Test 3). In contrast, the comparison between *Caulerpa* and Test 3 shows no significant difference (*p*-value > 0.05), indicating that their biogas yields are statistically similar and low. This result reflects the unfavorable experimental conditions in Test 3, due to the increased Glacilaria load. Overall, the Tukey HSD matrix highlights two distinct groups: a high-yield group dominated by *Gracilaria* and Test 1, and a low-yield group including *Caulerpa* and Test 3, with Test 2 positioned in between. This analysis confirms the trends observed in other statistical tests (ANOVA, effect size η^2), demonstrating that the algal ratio, substrate type, and operating conditions have a significant and decisive impact on biogas production.

3.3 *Analysis of pH evolution*

We are examining the pH changes in three mixtures of *Gracilaria* and *Caulerpa* algae with different proportions. In all tests, the initial pH was the same across the three mixtures, at 7.0 ± 0.2. This indicates similar starting conditions and confirms that no initial chemical bias could affect subsequent microbial dynamics, which are considered optimal [52]. By the end of the process, clear acidification was observed, as indicated by the final pH (pHf), which gradually decreased to 6.8, 6.4, and 6.1 for Test 1, Test 2, and Test 3, respectively. This trend shows that the pH decline becomes more pronounced as the proportion of *Gracilaria* substrate in the mixture increases. The ΔpH values (−0.2, −0.6, and −0.9) support this effect quantitatively, demonstrating that mixtures with more G undergo greater acidification during the process. Thus, this trend indicates that increasing the proportion of "G" in the mixture led to greater acidification, likely due to higher production of acidic intermediates during the process.

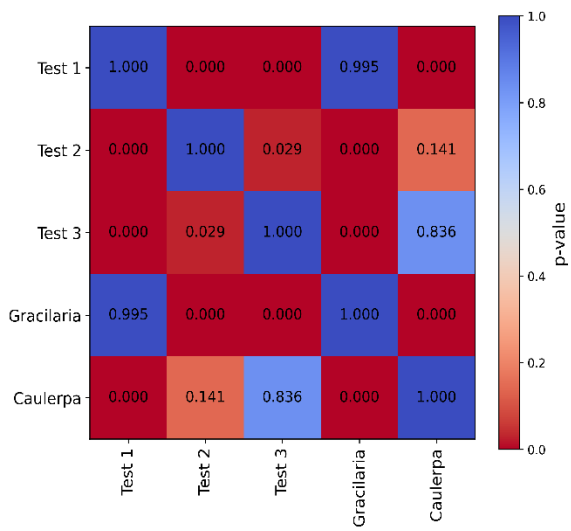

Figure 10: Tukey HSD significance matrix (Tests 1-3 and algal species).

The regression plot illustrates the relationship between the percentage of *Gracilaria* (G) in the mixture and the corresponding ΔpH (Figure 11). The data indicate a linear decline, with ΔpH becoming more negative as the *Gracilaria* proportion increases from 25% to 75%. This shows that higher *Gracilaria* levels lead to more intense acidification of the medium during the process. The regression line closely matches the experimental data points, indicating a strong linear relationship between %G and ΔpH, which suggests that substrate composition is a key factor influencing pH changes in this context. The high proportion of *Gracilaria* promotes a more active fermentative metabolism due to the increased availability of fermentable substrates, such as easily degradable polysaccharides that characterize this alga. This linearity indicates a strong link between algal composition and acidification levels, suggesting that the proportion of *Gracilaria* plays a key role in pH changes.

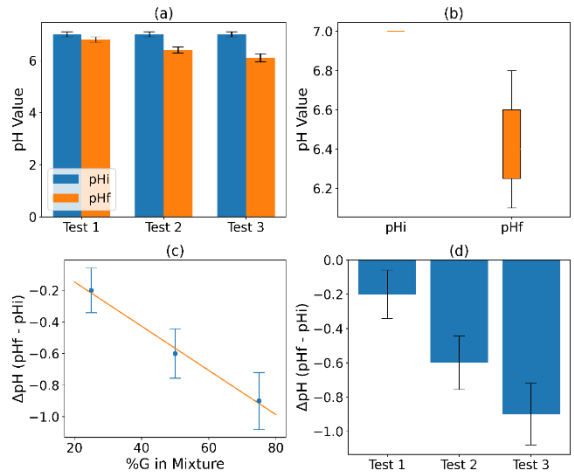

Figure 11: pH evolution across tests: (a) pHi and pHf, (b) Boxplot distribution, (c) ΔpH regression, (d) ΔpH comparison.

From a microbiological point of view, this behavior results from increased production of acidic intermediates—particularly volatile fatty acids (VFAs)—during the hydrolytic and acidogenic stages of the process [53]. A higher proportion of G likely enhances fermentative activity, accelerating the production and accumulation of these metabolites. Since methanogenic archaea are very sensitive to pH and perform best near neutral conditions, increased acidification can inhibit their activity, potentially destabilizing the system and reducing biogas production if not managed. So, this trend indicates that mixtures richer in G may need more stringent pH control to prevent excessive acidification, which could inhibit downstream microbial activity, particularly methanogenesis [54].

Consequently, pH decreased as the proportion of *Gracilaria* increased (from 25% to 75%) or as the *Caulerpa* content decreased (from 75% to 25%), confirming the antagonistic effect of the algae on each

other within a digester. Overall, the combined analysis of pHi, pHf, ΔpH, and the regression trend indicates that substrate composition directly influences the biochemical balance of the process (Figure 12). Controlling the G proportion or enhancing buffering capacity is crucial for maintaining pH stability and optimal microbial performance during anaerobic digestion.

3.4 *Correlation of conversion into biogas with physicochemical parameters*

The combined analysis of pH variation and biogas production emphasizes the significant role of acidification in anaerobic digestion performance [55]. The experimental results demonstrate a gradual decrease in final pH as the proportion of substrate G increases, with average ΔpH values of −0.19, −0.60, and −0.89 for Tests 1, 2, and 3, respectively. This trend of acidification is associated with a significant decrease in cumulative biogas production, which drops from approximately 78–80 mL/gVS to 50–52 mL/gVS, and then to 26–27 mL/gVS across the same tests. This contrasting evolution clearly illustrates an inverse relationship between pH stability and methanogenic efficiency.

Statistical analysis confirms this relationship. A strong, negative, and statistically significant correlation (high R^2 and p-value < 0.05) indicates that pH variation is a key factor affecting differences in biogas yields. The ANOVA reveals significant differences among the three experimental groups, and the Tukey post hoc test confirms that each condition differs significantly from the others. These results demonstrate that pH decline is not random but a fundamental causal factor governing process efficiency.

Mechanistically, this behavior is explained by the accumulation of VFAduring the acidogenic phase, which increases when the proportion of substrate G is high. The accumulation of VFAs lowers the pH and creates selective pressure that inhibits methanogenic archaea, whose enzymatic systems are very sensitive below pH 6.8. Consequently, the conversion of VFAs to CH_4 is hindered, slowing the entire methanogenic process.

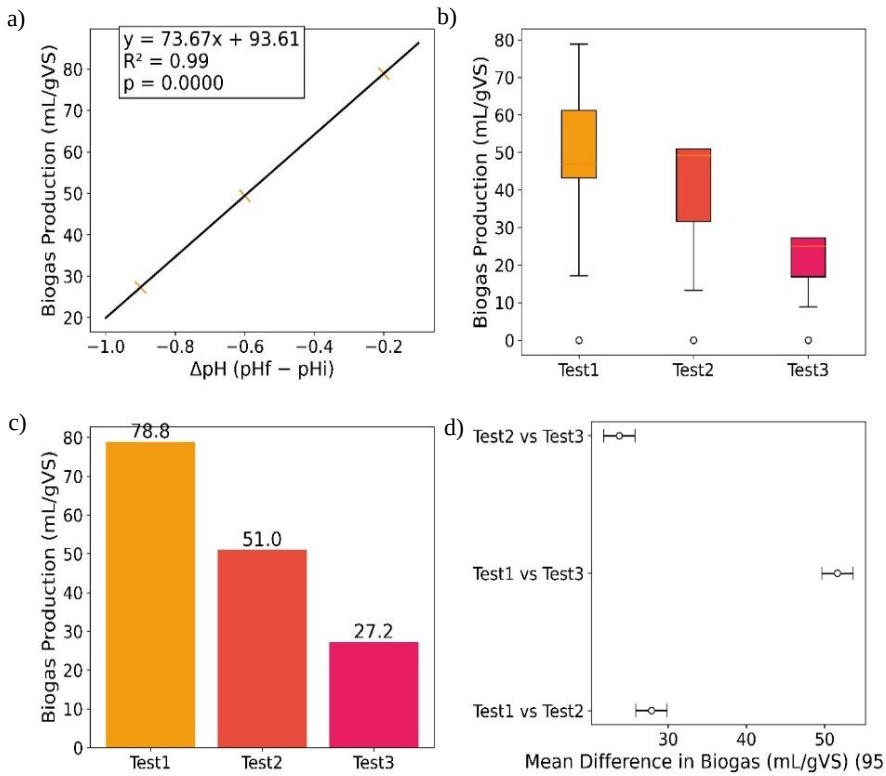

Figure 12: Linking biogas production to pH variation: (a) Correlation between ΔpH and biogas production, (b) Biogas distribution, (c) ANOVA results, and (d) Tukey HSD comparison.

Conversely, Test 1, which exhibits limited acidification, maintains a physicochemical environment closer to methanogenic optima, explaining its higher biogas yield. From a process engineering perspective, these results emphasize the importance of strict pH control to maintain system stability. Maintaining buffering capacity, adjusting the organic loading rate, and supplementing alkalinity can all be achieved through co-digestion strategies with *Caulerpa* species substrates. Therefore, this alga acts as a crucial lever for reducing VFA accumulation and preventing methanogenic inhibition. In summary, the experimental and statistical results indicate that acidification is a major limiting factor for biogas production. The negative relationship between ΔpH and biogas yield, supported by correlation, ANOVA, and Tukey analyses, confirms that pH optimization using *Caulerpa* must be incorporated as a key variable in the design and operation of anaerobic digestion systems.

5 Conclusions

This study highlights the promising potential of co-digesting *Caulerpa prolifera* and *Gracilaria gracilis* as a valuable Moroccan strategy to enhance biogas production from the marine biomass of the Nador (Marchica) lagoon. The experimental results clearly demonstrate that the substrate ratio plays a decisive role in determining both the efficiency and stability of the anaerobic digestion process.

Among the tested combinations, the mixture T1 (25% *Gracilaria gracilis* and 75% *Caulerpa prolifera*) was identified as optimal, yielding the highest biogas production while maintaining stable digestion conditions. Thus, the highest proportion of Caulerpa prolifera yielded superior biogas output. In contrast, high proportions of *Gracilaria gracilis* were associated with reduced methane productivity and signs of process inhibition.

These findings emphasize the importance of optimizing algal co-digestion ratios to promote synergistic interactions between algal substrates, thereby improving biodegradability, nutrient balance, and overall process performance. From a practical perspective, the results suggest prioritizing *Caulerpa*-dominant mixtures in biogas plants processing marine biomass, particularly in coastal lagoons affected by macroalgal proliferation. This approach offers a dual benefit by supporting lagoon management strategies to control excessive algal growth while simultaneously enhancing renewable energy recovery.

Moreover, the successful valorization of locally available algal resources from the Marchica lagoon illustrates a sustainable approach to waste-to-energy pathway, transforming an ecological challenge into an opportunity for renewable energy generation. Beyond the technical outcomes, this work contributes to the broader framework of sustainable resource management and circular bioeconomy in coastal regions. By promoting the energetic recovery of marine biomass, the process supports local energy autonomy, mitigates the environmental impacts of eutrophication, and aligns with global objectives of the energy transition and climate change mitigation.

Future research should therefore focus on conducting continuous anaerobic digestion trials to evaluate long-term process stability and resilience under steady-state conditions. In addition, we recommend pilot-scale validation to assess the operational feasibility, scalability, and practical constraints of implementing algal co-digestion under real environmental and industrial conditions. Finally, we emphasize the importance of detailed microbial community analyses to better elucidate the biological mechanisms governing substrate interactions, microbial adaptation, and overall process performance, thereby providing a stronger scientific basis for optimizing algal co-digestion strategies.

Author Contributions

Y.I: conceptualization, investigation, reviewing and editing; R.B: investigation, methodology, writing an original draft; S.F.: research design, data analysis; F.F. And O.E.: conceptualization, data curation, writing—reviewing and editing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors utilized the ChatGPT tool to enhance the language and readability of the manuscript.

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