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Digestate dilution shapes carbohydrate and pigment production during microalgal and cyanobacterial-based biogas upgrading

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ABSTRACT

Microalgae and cyanobacteria offer a promising platform for integrating sustainable technologies aligned with circular and green economy goals. However, current studies often focus on a limited number of genera and overlook how centrate dilution influences metabolite production. This study investigates the potential of the freshwater microalga *Parachlorella hussii* N9 and the marine cyanobacterium *Cyanothece* sp. CE4 for photobiological biogas upgrading coupled with nutrient recovery from centrate, assessing the impact of centrate dilution on carbohydrate and pigment content. By varying centrate concentration (5–50 %) in tap or seawater, this research explores how the biogas-to-centrate ratio can be adjusted for biomass production, TN and CO_2 abatement, and to target specific metabolites, advancing circular bioeconomy strategies. The microalga exhibited faster growth than the cyanobacterium, achieving the stationary phase in three days, and higher cellular and soluble carbohydrate productivity (up to 237 and 75 mg $L^{-1}d^{-1}$, respectively). CO_2 abatement (almost complete in all treatments) reached ~513 ± 28 mg L^{-1} of culture, while nitrogen removal considering initial centrate concentration ranged between 32 and 250 mg N L^{-1}, but 100 % TN removal was exhibited only with the lower centrate concentrations (5–10 %). These lower concentrations also induced the highest carbohydrate content in biomass (41–44 % dw). In contrast, pigment content increased with higher centrate concentrations: the microalga reached 3.6 % dw of chlorophyll *at* 50 % centrate, while the cyanobacterium produced up to 0.6 % dw of C-phycocyanin; both strains showed similar carotenoid content (0.4–0.5 % dw). This study highlights the potential of adjusting centrate dilution to target microalgal metabolism for integrated CO_2 capture, nutrient recovery, and bioproduct generation.

1. Introduction

Despite recent efforts to mitigate the impacts of anthropogenic activities on ecosystems and climate change, significant amounts of waste and pollution are still generated, compromising the long-term ability of the planet to provide essential services and resources [1]. Therefore, the implementation and integration of multiple strategies based on cleaner production, circular economy, and nature-based solutions is needed [2].

In this context, eukaryotic microalgae and prokaryotic cyanobacteria (generally referred to as microalgae) offer a promising platform for simultaneous wastewater remediation, carbon capture and added-value product generation [3]. Microalgae exhibit multiple applications in sectors such as pharmaceutical, agricultural, energy, food and feed, and wastewater remediation due to their metabolic diversity. They are considered green biofactories for the production of pigments, proteins, polysaccharides (including exopolysaccharides - EPS), lipids, antioxidants, and fatty acids, being capable of modifying their metabolism as a response to the environmental growth conditions [4,5]. However, the feasibility of microalgae-based technologies is often constrained by high production costs (typically exceeding 5.0 € kg^{-1} DW) and environmental impact. This impact is partly due to the use of chemical fertilizers to supply essential nutrients for microalgal growth (ranging from 0.8 to 2.2 % of the total cost depending on the case study [6,7]), but a large share of the total algal biomass production costs is represented by plant depreciation and harvesting [6–8]. According to Olguín et al. [5], the main strategy to overcome these drawbacks consists of the implementation of biorefinery and circular economy concepts, which requires the integration of different technologies to achieve an economically

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competitive biomass while also meeting sustainable goals.

In this regard, wastewater represents a readily available and low-cost feedstock for biomass production compared to synthetic growth media [9]. Microalgae cultivation in wastewater not only promotes biomass and metabolites production, but also enables wastewater treatment and nutrients and water recovery [10]. Up to 100 % of the nutrients and organic pollutants present in wastewaters can be removed in microalgae-based systems, thereby reducing the eutrophication and ecological risks of wastewaters [11]. Additionally, since 1.83 kg carbon dioxide (CO_2) per kg dry biomass are typically needed as a carbon (C) source [12], the autotrophic metabolism of microalgae provides an effective tool for carbon capture and storage. Indeed, the utilization of wastewater and flue gases in open algal reactors can drop biomass production cost [6,13]. In this context, biogas represents another low-cost CO_2 source. Biogas is a renewable energy source produced by anaerobic digestion of wastewater, organic municipal or agricultural wastes, and composed of 53–70 % of methane (CH_4) and 30–47 % CO_2 [14]. Apart from CO_2, biogas contains some contaminants such as hydrogen sulfide (H_2S, 0–10,000 ppm_v), nitrogen (N_2, 0–3 %), oxygen (O_2, 0–1 %), water (H_2O, 5–10 %) [14]. The removal of CO_2, which represents the major biogas contaminant, is required to increase the specific calorific value of biogas, allowing its use in natural gas grids and as vehicle fuel in the form of biomethane [15].

Unlike most physical/chemical biogas upgrading technologies (which release CO_2 into the atmosphere), photosynthetic biogas upgrading promotes the conversion of CO_2 into valuable bioproducts through carbon fixation by autotrophic microorganisms [14]. Elevated CH_4 concentrations in biogas do not affect microalgal growth due to its chemical stability, limited reactivity under most biological conditions, and low aqueous solubility [14,16]. Nevertheless, several challenges hinder photobiological upgrading, including limited CO_2 tolerance in certain microalgal strains, CO_2 mass transfer inefficiencies, the presence of contaminants in the biogas or cultivation broth, and pH instability. The above-mentioned limitations can negatively impact microalgal growth and reduce CO_2 fixation efficiency [1]. Other benefits compared to physical/chemical processes include lower energy and chemical consumption, lower operating costs, and possible commercialization of the microalgal biomass (or its associated bioproducts) obtained at the end of the process [17]. Indeed, the biogas upgrading costs can be reduced by a factor of 7 and the energy demand by a factor of 3.8 when algal-bacterial photobioreactors are used for biomethane generation instead of physical/chemical processes [17]. Despite the vast biodiversity of microalgae [18], microalgae-based wastewater treatment and biogas upgrading has primarily relied on few genera such as *Chlorella*, *Tetraselmis*, *Scenedesmus*, *Desmodesmus*, *Nannochloropsis* and *Arthrospira*, known for their rapid growth and/or resilience to adverse conditions [10,11,19] or microalgal-bacterial consortia [20]. Indeed, many factors such as high ammonium (NH_4^+) content, suboptimal nutrient content, presence of heavy metals, turbidity, and extreme pH, can limit microalgae growth in wastewater [21]. Despite the beneficial role of using N-laden wastewaters by avoiding the supply of artificial salt, the presence of NH_4^+ as the main N source in wastewaters, can hinder algal growth. For instance, the digestate obtained via anaerobic digestion of livestock or urban solid waste typically contains NH_4^+ concentrations ranging between 2 and 4 g L^{-1} [22]. Differently, NH_4^+ concentrations drop to 0.2–1.5 g L^{-1} in the particular case of centrate (i.e., the liquid fraction obtained via centrifugation of anaerobically digested sewage sludge) [23,24]. Even if NH_4^+ promotes faster algal growth compared to other inorganic N forms (e.g., nitrate), due to lower energy consumption compared to oxidated species, the combination of high NH_4^+ concentrations and pH can cause growth inhibition or cellular death [25,26]. In this context, wastewater dilution is often necessary to support high biomass productivities [23,25]. Also, environmental conditions, such as light and temperature, impact on cellular metabolism and govern the synthesis of specific metabolites as an adaptive strategy [18]. Unfortunately, the studies devoted to biogas upgrading coupled to nutrient recovery from digestates are limited, focusing only on the most commonly used microalgae and without taking into account the effect of digestate dilution on metabolite production. Nevertheless, targeting a specific biomass composition is necessary to maximize its potential for valorization.

Thus, this study integrates biogas upgrading with nutrient recovery from centrate, while simultaneously producing carbohydrates (cellular and soluble) and pigments (Chlorophyll *a* and *b*, carotenoids, phycocyanin and allophycocyanin) using the freshwater microalga *Parachlorella hussii* N9, and the marine cyanobacterium *Cyanothece* sp. CE4. Indeed, the effect of biogas-to-centrate ratio on growth, pollutant removal, and metabolite production can be enhanced by the differences in metabolic patterns of a freshwater microalga and a marine cyanobacterium. Moreover, the use of a marine cyanobacterium enables the use of seawater for centrate dilution instead of tap water, thereby enhancing the sustainability of the process [27]. By adopting biogas and different centrate dilutions, the toxicity of ammonium can be reduced while providing different carbon-to-nitrogen ratios, thus promoting novel insights into metabolite production and resource recovery, advancing the implementation of sustainable, circular bioeconomy strategies. The results herein obtained exploiting byproducts from the anaerobic digestion of sewage sludge can support the fulfilment of some of the sustainable development goals (SDG) proposed by United Nations in 2015 [28], particularly clean water and sanitation (SDG 6), affordable and clean energy (SDG 7), and responsible consumption and production (SDG 12).

2. Material and methods

2.1. Microalgae and growth conditions

Two microalgae, namely the microalga in *senso stricto Parachlorella* sp. N9 (GenBank: PQ110317), courtesy of Guehaz Karima (Université Kasdi Merbah Ouargla, Ouargla, Algeria) and the cyanobacterium *Cyanothece* sp. CE4 (GenBank: OQ945752), both available at the Cyanobacteria and Microalgae Collection of the University of Florence (Italy), were selected based on their high carbohydrate and exopolysaccharide production, as well as the different behaviour originating from the different growth environments: freshwater (microalga) and marine (cyanobacterium) [29]. The two strains were respectively grown in BG11 medium [30] and in artificial seawater (Tropic Marine® sea salts, Switzerland) enriched as follows (g L^{-1}): NaCl, 28; ferric ammonium citrate, 0.006; citric acid, 0.006; Na_2 EDTA, 0.001 and 0.5 mL L^{-1} of BG11 trace metal solution [31]. The inoculum for the assays was obtained by cultivating the two strains first in 250-mL glass bottles and then in 1.2-L glass bottles filled with 50 and 200 mL of the corresponding growth media, respectively. The headspace of the bottles was initially flushed with N_2 before being flushed with synthetic biogas, to provide carbon and acclimatize the cells to the high CO_2 concentrations of biogas. The bottles were incubated at 35 °C under continuous magnetic stirring (250 rpm) and illumination (150 μmol photons m^{-2} s^{-1}) provided by LED lights.

2.2. Centrate and biogas

Centrate wastewater was provided by the wastewater treatment plant of Valladolid (Spain), stored at 4 °C and filtered with glass microfibers filters with a 0.7 μm pore size (Fisher Scientific, US) before use. The centrate had the following composition, expressed in milligrams per liter (mg L^{-1}): total nitrogen (TN) 705, total carbon (TC) 700, ammonium nitrogen ($N\text{-}NH_4^+$) 635, inorganic carbon (IC) 620, phosphate phosphorus ($P\text{-}PO_4^{3-}$) 138, and sulfate sulfur ($S\text{-}SO_4^{2-}$) 17. In addition, the centrate contained 8.7 and 23.0 μg L^{-1} of copper (Cu) and nickel (Ni), respectively. The synthetic biogas, purchased from Carburos Metalicos (Spain), was composed of CO_2 (30 %) and CH_4 (70 %) and was filtered through 0.2 μm pore size syringe filters before being flushed in closed bottles as detailed in the next section.

2.3. Experimental set-up

Batch assays were performed to evaluate the effect of centrate dilution (50, 20, 10, 5 % dilutions) on microbial growth and carbohydrate and pigment content of each strain. Thus, four different culture media (Table 1) were prepared by mixing the centrate with the corresponding volume of tap water for *Parachlorella hussii* N9 or artificial seawater for *Cyanothece* sp. CE4, supplemented with $MgSO_4$ to reach the final Mg concentration of the two standard growth media (7.4 and 1.3 mg L^{-1}, respectively). Additionally, a control was prepared using a modified version of the corresponding standard growth medium. Hence, NH_4Cl and a $Na_2CO_3/NaHCO_3$ mixture (1:2, w:w) were supplied as N and C sources in order to provide the same N and C source of tests conducted with 50 % centrate. Main media composition is summarized in Supplementary Table S1. 1.2 L glass bottles were filled with 200 mL of medium, closed with butyl septa and plastic caps and autoclaved (120 °C, 20 min). After cooling, the 1-L headspace was flushed with sterile N_2 and then with sterile synthetic biogas using inlet and outlet needles to replace the N_2 headspace. The biogas was provided once before microalgae inoculation. The different centrate dilutions resulted in different C/N ratios as shown in Table 1.

The electrical conductivity of *Cyanothece* sp. CE4 culture medium was measured with a Basic 30 conductivimeter (CRISON Instruments, Spain) to assess the effect of reduced salinity in centrate-containing media. The pH of the culture media for *Cyanothece* sp. CE4 was adjusted to 8.0 after biogas flushing via addition of 10–20 mM TRIS buffer and 30 mM NaOH depending on the centrate concentration. After 1 h of stabilization, the bottles were inoculated with 2 mL of concentrated inoculum to get an initial optical density at 680 nm (OD_{680}, selected according to the absorption peak of Chlorophyll *a* which is characteristic pigment for both microalgae and cyanobacteria [32]) of 0.3 and 0.5 in tests conducted with *Parachlorella hussii* N9 and *Cyanothece* sp. CE4, respectively. The bottles were incubated at 35 °C under continuous magnetic stirring (250 rpm) and illumination (400 μmol photons m^{-2} s^{-1}) provided by LED (light emitting diode) lamps. Each assay was carried out in duplicate and was stopped after reaching the stationary phase. CO_2 and O_2 concentration in the headspace, OD_{680}, the cellular and soluble carbohydrate concentration and the pH of the cultures were periodically monitored during the growth. Finally, Cu and Ni removal, TN consumption, carbohydrate and pigment concentration and CHN content of the freeze-dried biomass were determined at the end of the experiments. Detailed analytical procedures are described in the next section. Summary of the main experimental step are shown in Fig. 1.

Table 1
C and N per liter of culture supplied by the centrate and the biogas in different treatments.

Assay	Composition	TC (mg L^{-1})	TN (mg L^{-1})	C-CO_2 (mg L^{-1})	C/N ratio
ctrl[a]	Modified BG11 or enriched seawater	350	350	725	3.1
50 %	50 % centrate + tap or sea water + Mg	350	350	725	3.1
20 %	20 % centrate + tap or sea water + Mg	140	140	725	6.2
10 %	10 % centrate + tap or sea water + Mg	70	70	725	11.4
5 %	5 % centrate + tap or sea water + Mg	35	35	725	21.7

Each test was made of 0.2 L of liquid fraction (main composition in Supplementary Table S1) and 1 L of biogas-containing headspace.
[a] Control.

2.4. Analytical methods

A small aliquot of the cultures was collected twice per day using sterile needles and syringes to monitor algal growth and carbohydrate (CH_2O) concentration. OD_{680} was determined with a UV–Vis spectrophotometer (CE2021, CECIL Aurius, UK). Then, aliquots of 1 mL of culture sample were centrifuged for 7 min at 10′000 rpm (LABNET, Madrid, Spain). The soluble and cellular carbohydrate (sCH_2O and cCH_2O, respectively) concentrations were determined in the supernatant and in the cells previously resuspended in the same volume (1 mL) of ultrapure water using the phenol-sulfuric method [33], as previously described by Ciani et al. [29]. The absorbance was measured at 480 nm with a UV–Vis spectrophotometer (CE2021, CECIL Aurius, Cambridge, UK). D-glucose was used as standard. The data were used to calculate the average daily carbohydrate productivity exhibited during the growth and the maximum daily carbohydrate productivity with the following formula:

$$\text{Daily carbohydrate productivity}\ \left(\text{mg L}^{-1}\ \text{d}^{-1}\right) = \left({}_{c/s}\text{CH}_2\text{O}_f - {}_{c/s}\text{CH}_2\text{O}_i\right)/\text{n}^{\circ}\ \text{of days}$$

where ${}_{c/s}CH_2O_{f/i}$ represents the cellular or soluble carbohydrate concentration at the final sampling (*f*) and initial sampling (*i*), divided by the days of cultivation. Maximum daily carbohydrate productivity was determined by calculating the carbohydrate productivity every 24 h and selecting the highest value.

Every two days, the pH was monitored in a AB315 pHmeter (Fisherbrand™, Hampton, NH, USA). Additionally, a 100 μL sample of the headspace was collected twice per day using a gas-tight syringe to monitor gas composition. In this context, CO_2 and O_2 concentration (%, v:v) was determined by gas chromatography in a Bruker 430 GC-TCD (Bruker, Palo Alto, CA, USA) according to Posadas et al. [34]. CO_2 concentration was used to calculate CO_2 consumption (%) from the headspace, considering the initial CO_2 concentration of 30 % in the biogas. Dissolved Cu, Ni and TN were quantified at the beginning and at the end of the assay from 0.22 μm-filtered samples to assess metal and nitrogen removal. Metal quantification was carried out by inductively coupled plasma mass spectrometry (ICP-MS) from filtered samples supplemented with 1.5 % (*v*/v) of HNO_3 65 %. TN concentrations were quantified in a Shimadzu TOC-VCSH analyzer (Kyoto, Japan) equipped with a TNM-1 chemiluminescence module. TN concentrations were used to calculate TN removal efficiency (%), subtracting the concentration of N supplied by the TRIS buffer in *Cyanothece* sp. CE4 media from the calculations. The remaining cultures were centrifuged (8000 rpm for 10 min) with a Sigma centrifuge (Osterode, Germany), the pellets were washed with saline solution (NaCl 0.1 and 0.9 % for freshwater and marine microalgae, respectively), centrifuged again and freeze-dried before being weighed to obtain the final biomass yield. Freeze-dried biomass was used for carbohydrate and pigment quantification. Briefly, 2 mg of freeze-dried sample were hydrolysed in 2 mL HCl 1 M at 100 °C for 2 h. The quantification of the carbohydrate content was carried out using hydrolysed samples as described above. Chlorophyll *a*, *b* (only for the microalga) and carotenoids (Chl*a*, Chl*b*, and Car, respectively) were extracted by dissolving 2 mg of biomass in 2 mL acetone 80 % (v/v). The samples were kept at 60 °C for 20 min, vortexed, and maintained at 4 °C overnight in the dark. The absorbance of centrifuged samples was evaluated at 663, 647, and 470 nm using a SPECTROstar Nano Absorbance Reader spectrophotometer (BMG LABTECH, Germany). Lichtenthaler's equations [35] were used to calculate Chl*a*, Chl*b*, and Car concentrations. C-Phycocyanin (C-PC) and allophycocyanin (APC) were extracted from CE4 biomass as described by Papalia et al. [36]. Briefly, 2 mg of biomass were resuspended in 0.05 M phosphate buffer (pH 7.5) and subjected to two consecutive freeze-thaw cycling before being centrifuged. The absorbance of the supernatant was measured at 615 and 652 nm using a SPECTROstar Nano Absorbance Reader spectrophotometer (BMG LABTECH, Germany). Bennet and

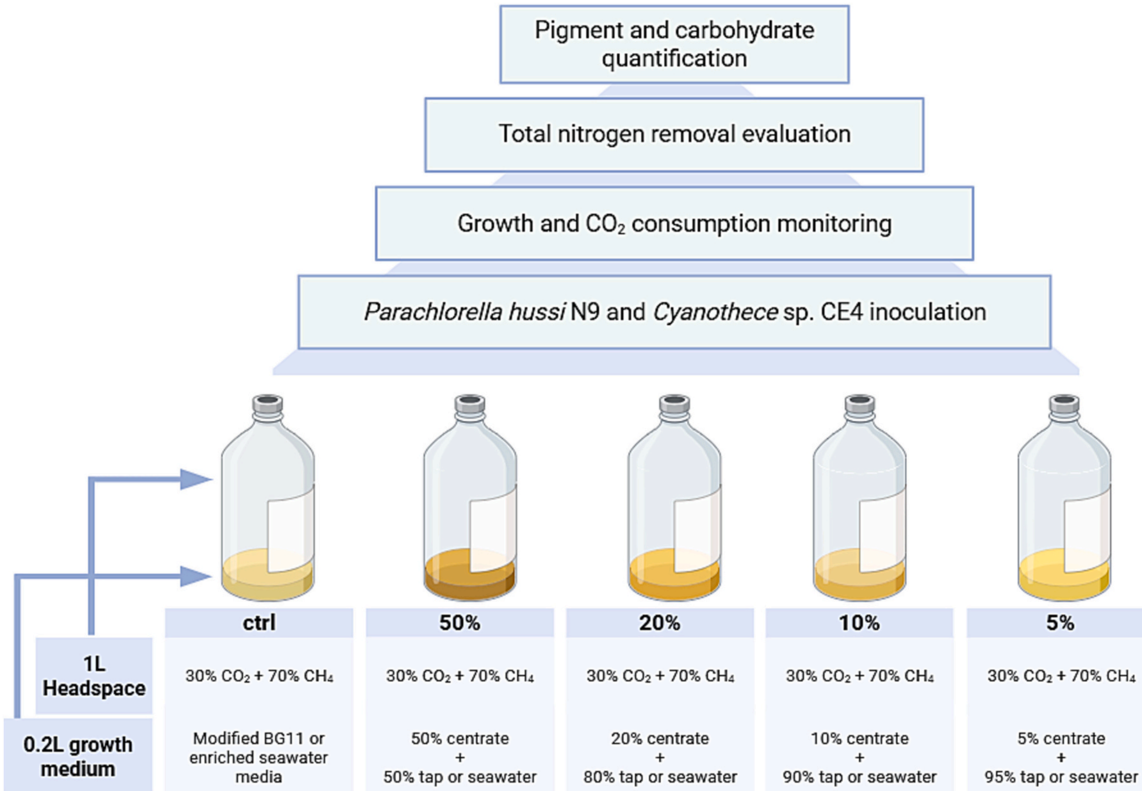

Fig. 1. Experimental set-up.

Bogarad's equations [37] were used to calculate C-PC and APC concentrations, which were expressed as % dry weight (dw). The CHNS analysis of the freeze-dried biomass was carried out to elucidate the C/N ratio of the biomass using an elemental analyzer EA Flash 2000 (Thermo Fisher Scientific) coupled with a TCD detector. The oven temperature was kept at 900 °C, and O_2 was used at a flow rate of 250 mL min^{-1}. Helium was used as carrier gas at 140 mL min^{-1} and as reference gas at 100 mL min^{-1}. Carbohydrate and pigment concentrations of the freeze-dried biomass of the marine cyanobacterium *Cyanothece* sp. CE4 were corrected by removing the percentage of salts provided by the artificial seawater and estimated by comparing the C content (% dw) from the CHNS analysis to its theoretical value of 40 % [38].

2.5. Data analysis

The effect of centrate dilution on TN removal efficiency, and carbohydrate and pigment contents was analyzed with a one-way ANOVA and Tukey's test at the 5 % significance level for comparison between groups for each strain. In addition, the carbohydrate-to-pigment ratio, herein refer to as CH_2O_{norm}/pigments$_{norm}$, was estimated by dividing the value obtained through the normalization of carbohydrate content with the value obtained through the normalization of total pigment content for each assay.

3. Results

3.1. Algal growth and biogas upgrading

Within the first 24 h, *Parachlorella hussii* N9 exhibited exponential growth under all centrate dilutions tested and in the control test, becoming more linear between 24 and 40 h and reaching the stationary phase between 42 and 63 h, according to the accumulated O_2 and OD_{680} (Fig. 2c and e). Despite the identical C/N ratios in the control and the assay conducted with 50 % diluted centrate, the OD_{680} (Fig. 2e) in the test supplied with 50 % centrate was nearly half of that in the control, which achieved the highest OD_{680} value of 5.9, followed by the assay conducted with 10 % centrate (OD_{680} 5.1). CO_2 consumption from the headspace by *Parachlorella hussii* N9 (Fig. 2a) was complete in all the assays except in the tests conducted with 50 % centrate (94 % CO_2 consumed). On the other hand, the addition of TRIS buffer and NaOH to initially increase the pH of the medium in the test series with *Cyanothece* sp. CE4 (Fig. 2b, d, f) induced a higher solubilization of the CO_2 present in the biogas, thereby reducing its concentration in the headspace during cyanobacterium inoculation as can be observed in Fig. 1b. CO_2 consumption in the headspace reached (considering the initial CO_2 content of the artificial biogas) 97–100 % in all assays except in the test conducted with 50 % centrate, where approx. 84 % of the initial CO_2 was consumed by the end of the experiment. Compared to *Parachlorella hussii* N9, the cyanobacterium *Cyanothece* sp. CE4 exhibited a slower growth, reaching the stationary phase after 73–110 h of cultivation, depending on the treatment (Fig. 2d, f). For this reason, the tests were stopped at different times from inoculation. The control showed a faster growth during the first 40 h of cultivation, before slowing down and reaching the stationary phase after 110 h. Centrate dilutions of 10 % and 20 % promoted the highest OD_{680} values (4.0 and 3.8, respectively), while a 50 % centrate dilution, characterized by the lowest electrical conductivity (Supplementary Table S2), inhibited *Cyanothece* sp. CE4 growth, resulting in an OD_{680} of 1.7 by the end of the experiment (Fig. 2f). Similarly to *Parachlorella hussii* N9, the control of *Cyanothece* sp. CE4 achieved OD_{680} values 2.3 times higher than the test conducted with a 50 % dilution, despite having the same C/N ratio and $N\text{-}NH_4$ content, indicating that other factors affected the growth.

The pH of the cultivation broths gradually increased during the growth of *Parachlorella hussii* N9 from 6.7 ± 0.5 to 9.5 ± 1.5 (Supplementary Fig. S1), with the lowest and highest increase experienced by the assays supplied with 50 % centrate and the control, respectively. The pH remained stable at 7.5 ± 0.5 during the growth of *Cyanothece* sp. CE4 (Supplementary Fig. S2).

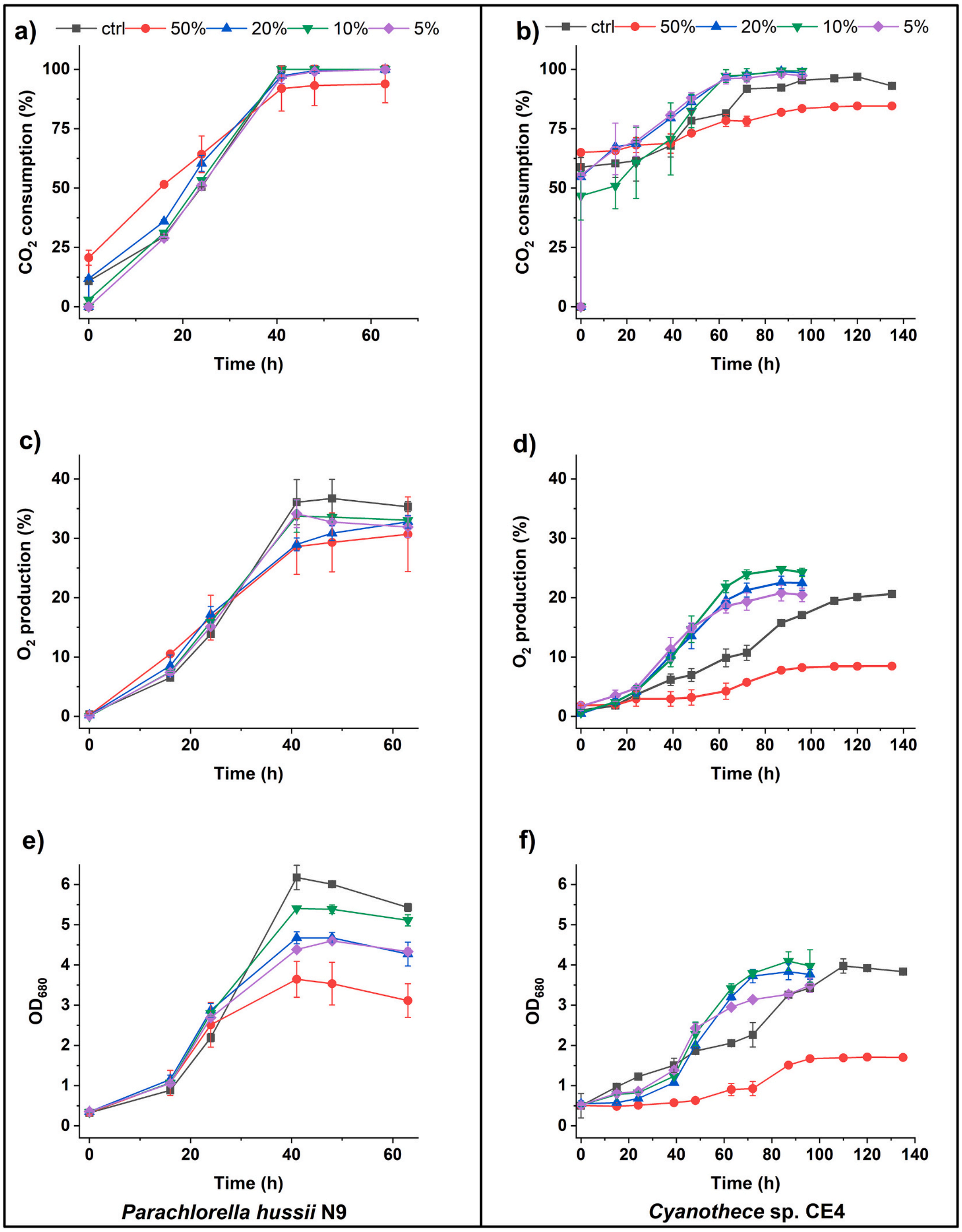

Fig. 2. Time course of cumulative CO_2 consumption from the headspace, considering the initial CO_2 concentration in artificial biogas (a, b), O_2 production (c, d) and optical density (e, f) in batch cultures of *Parachlorella hussii* N9 (left figures) and *Cyanothece* sp. CE4 (right figures) until stationary phase under biogas atmosphere and control medium (ctrl, black square) or centrate diluted at 50 % (red circle), 20 % (blue pointed-up triangle); 10 % (green pointed-down triangle); and 5 % (purple rhombus). Data shown as average ± standard deviation ($n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The highest cellular (Fig. 3a and c) and soluble (Fig. 3b and d) carbohydrate concentrations were observed in the tests supplied with the largest centrate dilutions (10 and 5 %). Cellular and soluble carbohydrate concentrations up to 960 and 200 mg L^{-1} for *Parachlorella hussii* N9, and 745 and 143 mg L^{-1} for *Cyanothece* sp. CE4, respectively, were recorded during batch cultivation. Moreover, tests supplied with the lowest centrate dilution (50 %) exhibited a decrease in cellular and soluble carbohydrate concentration in both strains (up to 10 times lower than the other assays). The highest average-maximum daily cellular carbohydrate productivities exhibited during the growth of the two strains (Supplementary Table S3) were attained when using 10 % centrate: 236.8–428.2 mg L^{-1} d^{-1} and 178.9–392.9 mg L^{-1} d^{-1}, for *Parachlorella hussii* N9 and *Cyanothece* sp. CE4, respectively. On the other hand, the highest average-maximum daily soluble carbohydrate productivities (Supplementary Table S3) were attained in tests supplied with centrate diluted at 5 %, reaching 73.8–70.3 mg L^{-1} d^{-1} and 25.2–60.7 mg L^{-1} d^{-1}, for *Parachlorella hussii* N9 and *Cyanothece* sp. CE4, respectively. The microalga exhibited higher carbohydrate productivities than the cyanobacterium regardless of the growth medium conditions.

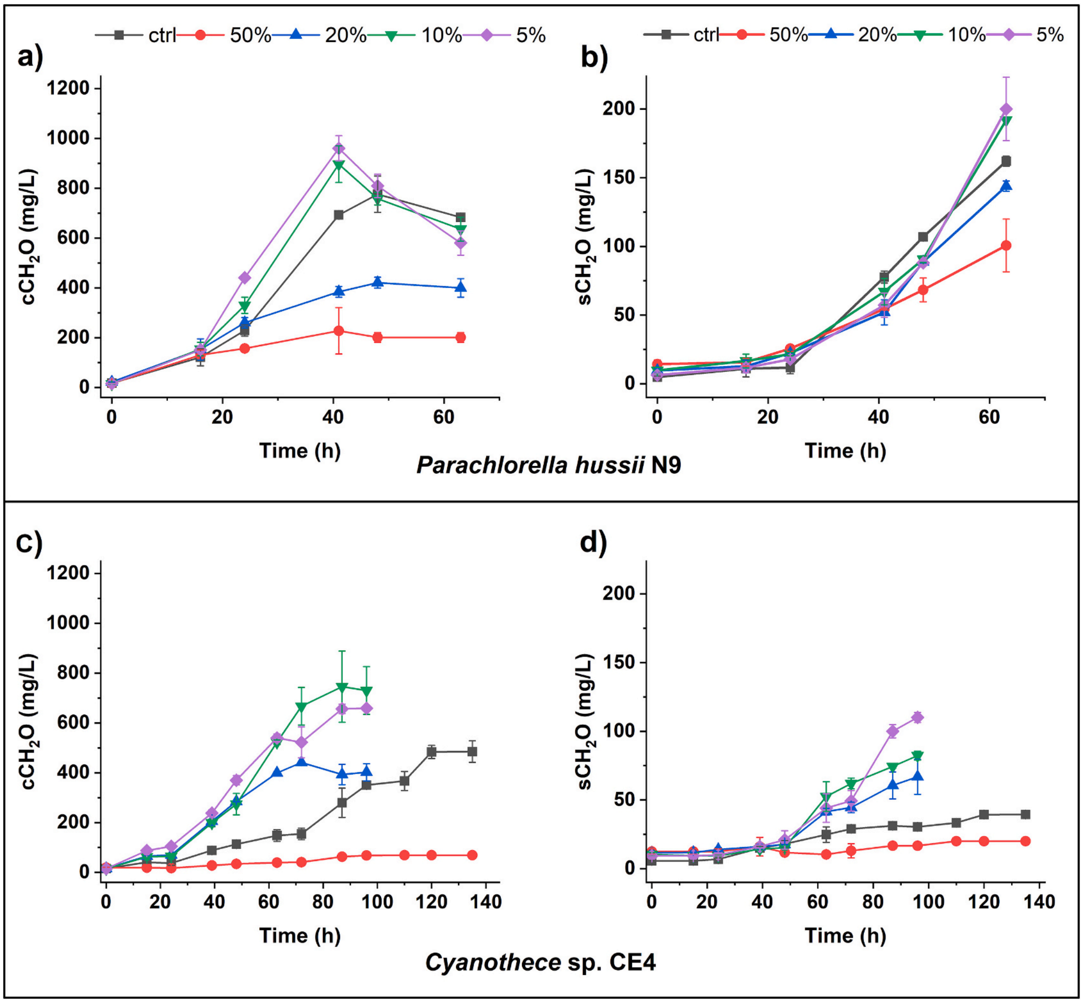

Fig. 3. Time course of cumulative cellular (cCH_2O) and soluble (sCH_2O) carbohydrate concentration in batch cultures of *Parachlorella hussii* N9 (a, b) and *Cyanothece* sp. CE4 (c, d) until stationary phase incubated under a biogas atmosphere in control medium (ctrl, black square) and centrate diluted at 50 % (red circle), 20 % (blue pointed-up triangle), 10 % (green pointed-down triangle) and 5 % (purple rhombus). Data shown as average ± standard deviations ($n = 2$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

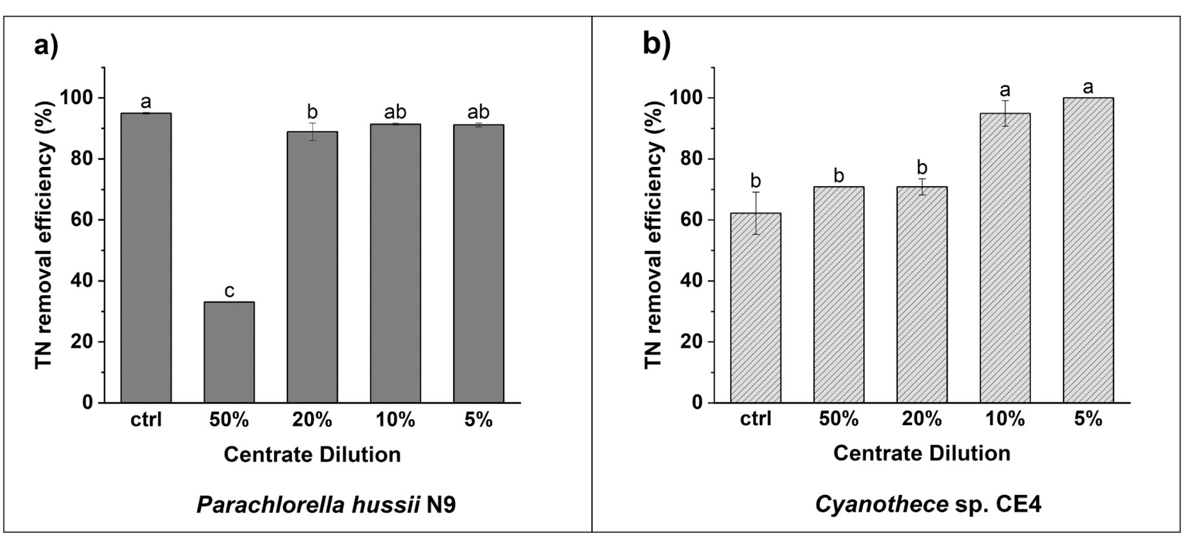

Fig. 4. Total nitrogen removal efficiency at the end of the assays carried out with *Parachlorella hussii* N9 (a) and *Cyanothece* sp. CE4 (b) under a biogas atmosphere in control medium (ctrl) or in centrate diluted at 50, 20, 10 and 5 %. Data shown as average ± standard deviations ($n = 2$). Different letters mean statistically significant differences between culture media.

3.2. Pollutant removal

At the end of the batch assays, the biomass produced was harvested and used for pigment and carbohydrate quantification, while the removal of TN, Cu and Ni from the culture media was evaluated. The quantification of TN concentration at the beginning and the end of the assays revealed that both strains exhibited an almost complete N removal in the tests supplied with 10 and 5 % centrate dilutions (Fig. 4). Additionally, any significative differences in TN removal were observed between the assays conducted with 20, 10 and 5 % centrate dilutions with *Parachlorella hussii* N9 (88–91 % N removal, Fig. 4a). On the other hand, *Cyanothece* sp. CE4 exhibited significantly higher N removal efficiencies (95–100 %, Fig. 4b) in tests supplied with 10 and 5 % diluted centrate compared to the control and tests with 20 and 50 % centrate dilutions (<71 %) ($p < 0.01$).

Parachlorella hussii N9 removed 50–61 % of the Cu without any statistically significant differences among the culture media (Supplementary Fig. S3), while no Ni removal was observed (data not shown). On the other hand, *Cyanothece* sp. CE4 did not exhibit any metal removal.

3.3. Carbohydrate and pigment production

The cellular carbohydrate contents of the freeze-dried biomass grown in 5 and 10 % diluted centrate were statistically significantly higher compared to the biomass grown in 20 and 50 % diluted centrate ($p < 0.05$), reaching a 42 and 48 % (dw) carbohydrate content in *Parachlorella hussii* N9 (Fig. 5a) and in *Cyanothece* sp. CE4 (Fig. 5b), respectively. On the contrary, the highest pigment content was recorded at the lowest centrate dilutions (Fig. 5, c–d). Thus, the content of Chlorophyll *a* and *b* in *Parachlorella hussii* N9 reached 2.0 % and 1.5 % (dw) in 50 % diluted centrate, respectively, resulting in a statistically significant difference compared to the rest of the test series ($p < 0.001$). Chlorophyll concentration gradually decreased with higher centrate dilution. On the other hand, the maximum carotenoid content in *Parachlorella hussii* N9 was reached with 20 % centrate concentration (5 %, dw) (Fig. 5c). *Cyanothece* sp. CE4 showed roughly 10 times lower chlorophyll content than *Parachlorella hussii* N9 (maximum 2.5 % dw in 50 % diluted centrate), while the carotenoid content was similar (maximum 4 % dw in 20 % diluted centrate). Indeed, a chlorophyll - to - carotenoids ratio below 1 was recorded in *Cyanothece* sp. CE4, while this ratio ranged from 4.4 to 10.2 in *Parachlorella hussii* N9. Additionally, *Cyanothece* sp. CE4 accumulated contents of C-PC and APC of 5–6 % and 5–9 % (dw), respectively, at the lowest centrate dilutions (Fig. 5d).

The C/N ratio and the normalized carbohydrate-to-pigment ratio in the freeze-dried biomass can be used as a proxy to highlight the effect of centrate dilution on specific metabolite production. Indeed, the different biogas–to–centrate ratios impacted the initial C/N ratio available for the microalga and the cyanobacterium and influenced the C/N ratio in the final biomass (Table 2). The C/N ratio remained around 5 in *Parachlorella hussii* N9 and *Cyanothece* sp. CE4 grown in 50 % and 20 % diluted centrate, but increased up to 10 in 10 % diluted centrate in both

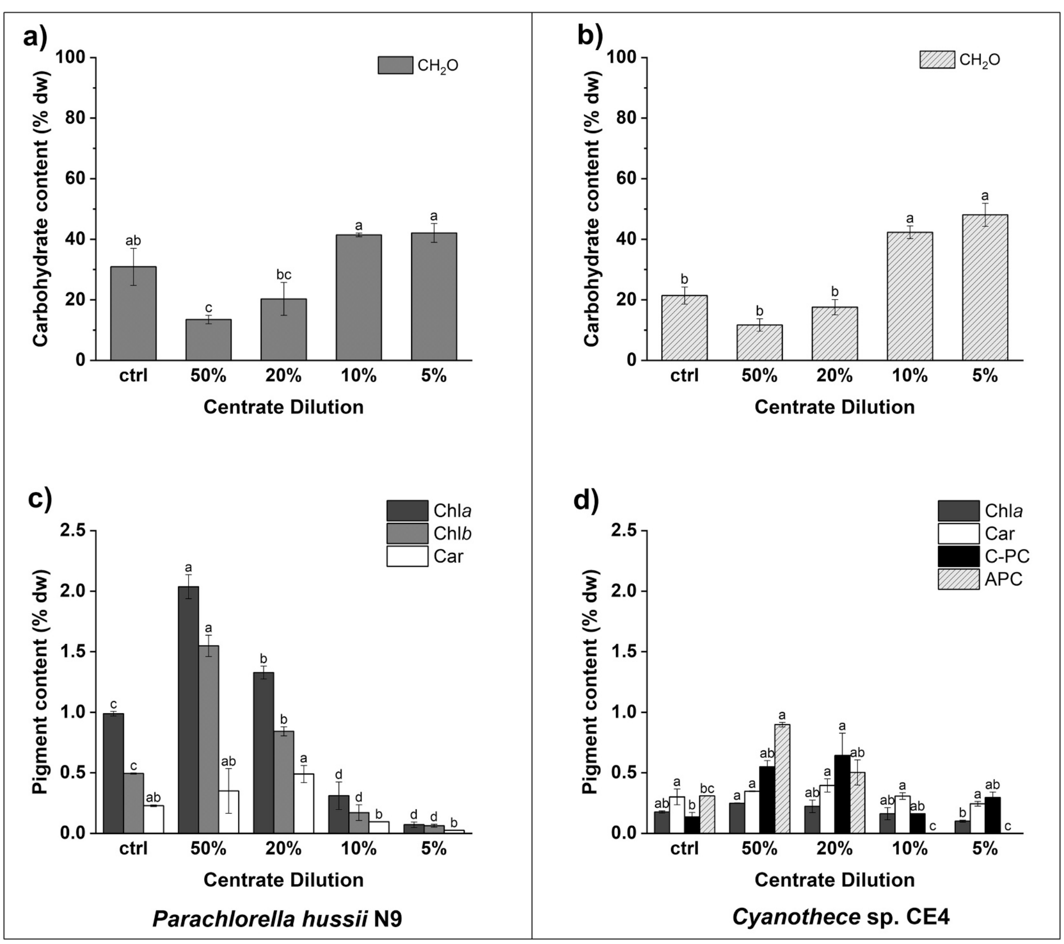

Fig. 5. Cellular carbohydrate and pigment content (% dw) of *Parachlorella hussii* N9 (a, c) and *Cyanothece* sp. CE4 (b, d) at the end of the assays under biogas and modified standard growth media (control) or different centrate dilutions. Pigments are separated in Chl *a*, Chl *b*, Car, C-PC, and APC, which stand for chlorophyll a, chlorophyll *b*, carotenoids, C-phycocyanin, and allophycocyanin, respectively. Data shown as average ± standard deviation ($n = 2$). Different letters mean statistically significant differences between culture media.

Table 2
C/N ratio in the media composed of different centrate dilutions and in the final biomass.

Centrate	C/N (medium)	Strain	C/N (biomass)
50 %	3.1	N9	5.5
		CE4	5.1
20 %	6.2	N9	5.6
		CE4	5.9
10 %	11.4	N9	10.3
		CE4	10.6
5 %	21.7	N9	21.2
		CE4	11.1

algal strains, and up to 20 in 5 % diluted centrate in *Parachlorella hussii* N9. Both *Cyanothece* sp. CE4 and *Parachlorella hussii* N9 showed an increase in the normalized carbohydrate-to-pigment ratio (Fig. 6) at the highest centrate dilutions (10 % and 5 %). At the lowest dilutions (50 % and 20 %), the ratios for both strains remained relatively low and similar. *Parachlorella hussii* N9 exhibited a more evident increase in this ratio compared to *Cyanothece* sp. CE4 at the 5 % centrate.

4. Discussion

This study demonstrated the potential of *Parachlorella hussii* N9 and *Cyanothece* sp. CE4 to support a sustainable waste management through biogas upgrading, nutrient recovery from wastewater (i.e., centrate) and metabolite production under varying biogas-to-centrate ratios. Both strains effectively contributed to CO_2 consumption, nitrogen removal, and valuable metabolite generation, although they exhibited different growth patterns and metabolic responses to centrate dilution.

4.1. Algal growth

Based on the patterns of CO_2 consumption, O_2 production, and optical density, *Parachlorella hussii* N9 exhibited a rapid exponential growth within the first 24 h, reaching the stationary phase in less than 3 days (Fig. 2, a–c–e). The control and the assays conducted with 10 % diluted centrate exhibited the highest OD_{680} values, while the medium composed of 50 % diluted centrate negatively impacted the growth, showing a reduction in OD_{680} of 15–40 % compared to the other cultivation conditions. The reduced growth observed with 50 % diluted

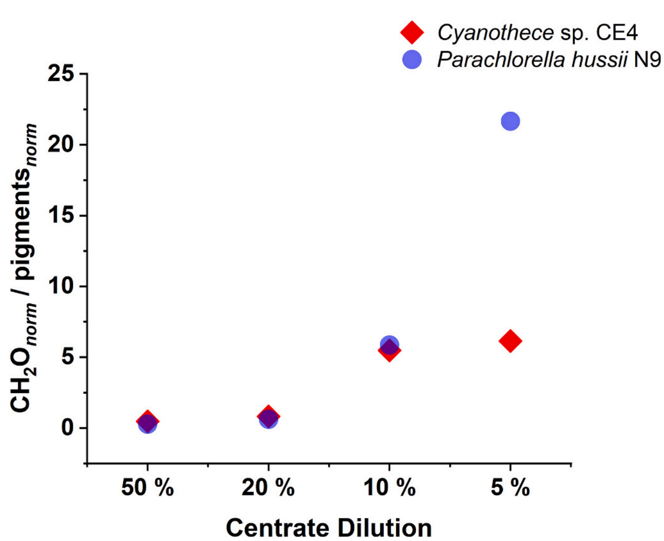

Fig. 6. Normalized carbohydrate-to-pigment ratio in the freeze-dried biomasses of *Parachlorella hussii* N9 (blue dot) and *Cyanothece* sp. CE4 (red rhombus) cultivated at different biogas–to–centrate ratios. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

centrate can be attributed to the higher ammonium concentration, resulting from lower centrate dilution, but also to other factors such as the presence of toxicants or medium turbidity, which may hinder light penetration and diffusion and impact biomass productivity [39–41]. Indeed, it has been observed that increasing the concentration of anaerobic digestion effluents microalgal growth is affected due to the strong absorbance of light at wavelengths below 650 nm in the effluents, reducing light transmittance [42]. On the other hand, nutrient limitation, likely associated with the lower centrate concentrations, may also reduce algal growth, as growth rates are directly influenced by the intracellular nutrient concentration [26]. Different trends within treatments in OD values compared to CO_2 concentration and O_2 production may be explained by the production of extracellular material (including EPS) that can affect the turbidity of the media [43], as also confirmed by the different sCH_2O and cCH_2O values (Fig. 3). On the contrary, *Cyanothece* sp. CE4 exhibited a lower growth, reaching the stationary phase in 3 days, while 5 days were needed for the assays with the highest N concentrations (control and 50 % centrate, Fig. 1, b-d-f). Despite the slight differences observed between 20, 10, and 5 % diluted centrate assays, the test with 50 % diluted centrate showed a reduction of 50–66 % in OD_{680} compared to the other cultivation conditions. Bohutskyi et al. [44] compared the growth of different strains of *C. sorokiniana*, *C. vulgaris*, *S. acutus*, and *S dimorphus* cultivated in primary or secondary domestic wastewaters characterized by a low nutrient load (TN $<$ 23 mg L^{-1}) supplemented with different ratios of centrate (TN $\sim$ 800 mg L^{-1}). All strains reached the stationary phase in 8–12 days. The authors observed an improvement in the growth of *C. sorokiniana* strains using 20 % diluted centrate (TN $\sim$160 mg L^{-1}). However, 20 % diluted centrate negatively influenced the growth of other microalgae, in particular *S. acutus*, for which the optimal centrate dilution ranged from 10 to 5 %. These results were in agreement with the optimal centrate dilution in terms of growth herein observed (10 %), but the growth of *Parachlorella hussii* N9 and *Cyanothece* sp. CE4 was faster, indeed, 2–5 days were enough to reach the stationary phase, which confirmed the potential of these strains to support a simultaneous biogas upgrading and nutrient recovery from digestate.

Despite the similar ammonia concentration, the OD_{680} of *Cyanothece* sp. CE4 cultivated in 50 % diluted centrate was 2.5 times lower than the OD_{680} recorded in the control. This suggests that other factors can be involved in the inhibition of *Cyanothece* sp. CE4 growth, such as the presence of other contaminants in the centrate and the lower salinity, as confirmed by the 37 % reduction in electrical conductivity observed in the control (100 % seawater, Supplementary Table S2). Thus, Hotos et al. [45] observed a 4-fold reduction in biomass concentration when cultivating *Cyanothece* sp. in a medium with a 50 % reduction in salinity. On the contrary, the light intensity exhibited a positive effect on the growth in these experiments, although the maximum light intensity tested was 184 μmol photons m^{-2} s^{-1}, that is much lower than in the present experiments. Similarly, Zhao et al. [46] observed a decrease in the growth rate of *Nannocholoropsis* sp. (a marine microalga) cultivated in a medium composed of 60 % biogas slurry and 40 % seawater compared to growth of *Nannocholoropsis* sp. in 40 % biogas slurry and 60 % seawater.

The increase in the pH of *Parachlorella hussii* N9 cultures up to 9.5 $\pm$ 1.5 (Fig. S1) was consistent with the higher photosynthetic activity and CO_2 uptake, which raised medium alkalinity [47]. In contrast, *Cyanothece* sp. CE4 maintained a pH of 7.5 $\pm$ 0.5 under all cultivation conditions (**Fig. S2**), likely due to the addition of TRIS buffer to increase the pH of the medium before *Cyanothece* sp. CE4 inoculation [48]. In this context, it is well-established that high pH values enhance CO_2 mass transfer and solubilization, an operational strategy typically implemented to improve photobiological biogas upgrading [20] without affecting C availability.

4.2. Biogas upgrading and pollutant removal

Both strains achieved an almost complete CO_2 consumption under all cultivation conditions (Fig. 2, a-d) except in 50 % diluted centrate, where CO_2 removals of 94 % and 84 % were observed for *Parachlorella hussii* N9 and *Cyanothece* sp. CE4, respectively. These results indicate that, in these conditions, up to 530 mg CO_2 can be abated per L of culture. Although the lowest centrate dilution reduced CO_2 absorption, the residual CO_2 concentration remained below 2 % in all cultivation conditions (except in *Cyanothece* sp. CE4 incubated under 50 % diluted centrate), which complied with the European biomethane standard EN 16723:2018 ($CH_4 \geq 90$ %, $CO_2 \leq 2$ %, $O_2 \leq 1$ % and negligible amounts of H_2S) [49]. This suggests that both strains can be employed for biogas upgrading. In this context, CO_2 consumption during photosynthetic biogas upgrading is influenced by several factors such as pH, temperature, photobioreactor configuration, symbiosis with bacteria, and the presence of other contaminants in the biogas, with maximum CO_2 removal efficiencies ranging from 62 to 99 %, where the highest values were observed in monoalgal cultures [15,48,50,51]. The CH_4 content in the headspace remained at 70–78 % across all tests, largely due to the replacement of CO_2 by O_2 during microalgal growth. However, an exception was observed with *Cyanothece* sp. CE4 cultivated at the highest centrate concentration. In this case, the growth was reduced compared to the other treatments, while the lower O_2 production coupled with increased CO_2 solubilization facilitated by the addition of TRIS buffer likely supported the increase in CH_4 content to 87 %. It should be mentioned that most standards for biomethane use require O_2 content below 1 %, but O_2 is commonly accumulated during microalgal growth [52]. To address this issue, different strategies have been studied to remove O_2 from biomethane, such as the use of gas-liquid membranes, or separated biogas absorption columns coupled with photobioreactors [52]. As instance, the use of algal-bacterial cultures, where O_2 is utilized by sulfur-oxidizing bacteria, implemented in two-stage continuous systems coupling biogas absorption columns and photobioreactors, can achieve CH_4 concentrations of up to 95 % following biological biogas upgrading [52,53]. The estimation of the molar ratio between the O_2 produced and CO_2 consumed, considering the headspace gas composition (CO_2 and O_2), the inorganic carbon concentrations and the estimated dissolved oxygen in the growth media (estimated using the Henry's law constant), revealed ratios close to the stoichiometric values for the two strains. In the case of the microalga, the average O_2/CO_2 ratio was 1.08 ± 0.12. In the case of the cyanobacterium, the value decreased to 0.99 ± 0.12. Conversely, the ratio estimated for *Cyanothece* sp. CE4 grown in 50 % diluted centrate was lower than 0.5, suggesting a lower photosynthetic efficiency and/or a decoupling between oxygen evolution and inorganic carbon uptake in the most stressful conditions. However, it's important to note that several factors can introduce uncertainty into the estimation of the O_2/CO_2 molar ratio. These include i) the buffering capacity of the medium, which can affect pH stability and, consequently, carbon speciation; ii) the presence of carbon concentrating mechanisms in certain organisms, which can alter the balance between CO_2 and bicarbonate utilization; and iii) measurement limitations related to gas exchange and inorganic carbon quantification [54,55]. For instance, the use of buffers like TRIS can influence the carbonate equilibrium, potentially leading to inaccuracies in estimating the actual inorganic carbon available for photosynthesis.

N removal was nearly complete in the assays conducted with 10 % and 5 % diluted centrate for both strains and for *Parachlorella hussii* N9 grown in 20 % diluted centrate (Fig. 4). Higher centrate concentrations exhibited reduced N removal efficiency as a result of the higher initial concentrations and the presence of inhibitory compounds in the centrate [56], suggesting an optimal nitrogen concentration of 150 mg L^{-1} in this experimental set-up. Nevertheless, it should be mentioned that, considering the volume of centrate adopted in the different tests, up to 250 mg TN can be removed per L of culture adopting 50 % centrate. This value gradually decreases reducing the centrate concentration, resulting in average values of 113, 66, and 34 mg TN removed per L of culture adopting 20 %, 10 %, and 5 % centrate, respectively. Higher centrate concentrations can be used to abate N pollution but lower centrate concentrations are needed to obtained cleaner water. Kusmayadi et al. [57] observed nitrogen removal of 95 % by *Chlorella sorokiniana* in a raw dairy wastewater characterized by 150 mg TN L^{-1}. The TN removal efficiency decreased by ~25 % when dairy wastewater was diluted 25–75 % using BG11 medium. Lower nitrogen removal efficiencies (58 %) were reported by Romero-Villegas et al. [24] using raw centrate characterized by an initial TN content of 470 mg L^{-1}. Different C/N ratios not only affect biomass growth and composition, but, as a consequence, also nutrient removal. For instance, Dang et al. [58] observed increased biomass productivity, C and N removal when co-cultivating a *Chlorella* sp. strain with activated sludge at a C/N ratio of 5 compared to 1 or 10 in a 10 L photobioreactor supplied with synthetic wastewater. The limited growth, CO_2 consumption and TN removal of *Cyanothece* sp. CE4 grown in 50 % diluted centrate suggested the occurrence of stressing conditions for the growth of this cyanobacterium compared to *Parachlorella hussii* N9. It is known that microalgae often exhibit faster growth rates and higher adaptability to different environmental stresses compared to cyanobacteria [59]. In this study, the high ammonia concentration (representing ~90 % of the TN concentration, ~ 318 mg L^{-1}), high light intensity (400 μmol photons m^{-2} s^{-1}), and the lower salinity compared to the other cultivation conditions represented stressing factors for the growth of the cyanobacterium.

On the other hand, Cu and Ni concentration in the centrate was similar or even lower than in other centrates reported in the literature [60,61], and was below the current European standards (Directive 2008/105/CE) in all the culture media at the beginning of the assays.

The absence of Ni removal compared to Cu can be attributed to the distinct chemical behaviors of these metals and their interactions with photosynthetic microorganisms. Indeed, Ni tends to form stable complexes with water and other ligands in solution, which limits its bioavailability and subsequent uptake or adsorption by microalgal cells [29,62]. In contrast, Cu plays an essential role in the photosynthesis, respiration and defence of microalgae, acting as a cofactor in various enzymatic processes [63]. This functional feature likely enhances Cu assimilation or biosorption mechanisms, as observed with *Parachlorella hussii* N9, which removed 40–60 % of the Cu present in the culture media (Supplementary Fig. S3). On the contrary, *Cyanothece* sp. CE4 was unable to remove Cu, potentially due to differences in cellular metabolism or metal tolerance mechanisms between the two strains. It should be mentioned that the upscale of the proposed process can pose further challenges due to the fluctuations in centrate composition, the necessity to remove the O_2 produced by microalgal metabolism, the presence of other contaminants in the biogas, and the interaction with the microbial community in non-sterile conditions. Despite microfiltration of culture medium and utilization of closed photobioreactors should be adopted to avoid contamination of the two tested strains, the possibility of creating specific microalgal-cyanobacterial consortia, ensuring their stability over time, and using mixed microalgal-bacterial cultures may be evaluated. Indeed, different studies have explored the efficacy of microalgal-bacterial consortia in wastewater treatment and biogas upgrading on a pilot scale [53,64,65]. In this case, even if the presence of H_2S in the biogas can inhibit microalgal growth, the concurrent presence of some sulfate oxidizing bacterial species and spontaneous oxidation of H_2S into sulfate by dissolved oxygen (DO) reduce H_2S inhibition [66].

4.3. Carbohydrate and pigment production

The highest cellular and soluble carbohydrate concentrations were observed in the assays carried out at the highest centrate dilutions (10–5 % centrate), with *Parachlorella hussii* N9 achieving up to 960 mg L^{-1} of cellular carbohydrates and 200 mg L^{-1} of soluble carbohydrates. Similarly, *Cyanothece* sp. CE4 achieved 745 mg L^{-1} of cellular carbohydrates and 143 mg L^{-1} of soluble carbohydrates (Fig. 3, a-d). Centrate dilutions of 10 and 5 % also promoted the highest cellular and soluble carbohydrate productivities, respectively (Table 2). In this context, Qu et al. [67] evaluated the growth, nitrogen removal and carbohydrate production of different microalgal strains cultivated in artificial low-N-content swine wastewater. The best performing strain was *Parachlorella kessleri,* which exhibited a cellular carbohydrate productivity of 381 mg L^{-1} d^{-1}. When a real wastewater containing higher N concentrations was used under the same cultivation conditions, the carbohydrate productivity of *P. kessleri* decreased. Despite the higher N concentrations, Qu et al. [67] reported that increasing light intensities and temperatures (600 μmol photons m^{-2} s^{-1}, 30 °C) supported carbohydrate productivities of up to 644 mg L^{-1} d^{-1}, which were even higher than the values herein recorded for *Parachlorella hussii* (max 547 mg L^{-1} d^{-1}). Ángeles et al. [68] found that cyclic N-deprivation increased the intracellular carbohydrate storage by two folds in a cyanobacteria/microalgae consortium cultivated using biogas as a carbon source. Nevertheless, the intracellular carbohydrate content never exceeded 29 % dw. On the other hand, the carbohydrate content in biomasses grown in centrate in previous works ranged between 10 and 52 %, depending on the cultivation conditions [12,69]. In this work, the carbohydrate content quantified on freeze-dried biomass reached 41–44 % dw (Fig. 5, a-b). Our empirical findings suggest an enhanced carbohydrate productivity when increasing centrate dilutions, which is in agreement with previous research showing that abiotic stresses, including high light intensities and N limitation, can promote carbohydrate accumulation [70].

Similarly, the synthesis of pigments varied with centrate dilution, with *Parachlorella hussii* N9 exhibiting the highest Chl *a* and *b* content at the highest centrate concentrations (up to 3.7 % Chl $a + b$ dw, Fig. 5c), while *Cyanothece* sp. CE4 was able to produce C-PC and APC at the highest centrate concentrations (0.6–0.9 % dw, respectively, Fig. 5d). The latter pigment accumulations were lower compared to those observed in other cyanobacteria, where C-PC can constitute up to 25 % dw [71]. Chl *a* concentration in *Cyanothece* sp. CE4 was lower than expected (< 0.3 % dw). In this context, a long-term exposure to high irradiances can result in photooxidation, characterized by a reduction in the number of active PSII centers and the photo-destruction of photosynthetic pigments, including chlorophyll and phycobiliproteins [72]. No statistical difference was observed in the carotenoid content of *Cyanothece* sp. CE4 (Fig. 5d, 0.3 % ± 0.1 dw), suggesting that the production of these pigments can be related to other factors different from centrate concentrations and thus nitrogen content. Overall, a reduction in chlorophyll content and chlorophyll-to-carotenoid ratio in cyanobacteria is typically observed when increasing light intensity [73,74]. Hotos and Antoniadis [75] reported an increase in carotenoid production coupled with a reduction in Chl*a* in a *Cyanothece* sp. culture when increasing light intensity from 40 μmol photons m^{-2} s^{-1} (0.07 and 1.2 % dw, carotenoid and Chl*a*, respectively) to 160 μmol photons m^{-2} s^{-1} (0.11 and 0.5 % dw, carotenoid and Chl*a*, respectively). In our study, the light intensity was 400 μmol photons m^{-2} s^{-1}, suggesting that the production of carotenoids in the cyanobacterium and the lower chlorophyll-to-carotenoid ratios compared to *Parachlorella hussii* N9 were likely due to the high light intensity.

C and N are the two most important elements in microalgal biomass, typically accounting for 50 % and 5–10 % (dry weight) of the biomass content [26]. It is known that microalgae and cyanobacteria adjust their nutrient uptake and composition based on their availability, storing excess nutrients or altering biomass composition with shifts in carbohydrates, lipids, proteins, or pigments when one or more of them become limiting [26].

The differences in biomass C/N and carbohydrate-to-pigment ratios observed at the varying centrate dilutions revealed a significant change in the balance of macronutrients, with a shift towards carbohydrate production under more diluted conditions. In particular, N limitation, which can be observed under the highest centrate dilutions, is typically associated with an interruption in amino acid synthesis that has likely caused also the interruption of pigment production and degradation of photosynthetic apparatus in *P. hussi* N9 grown with 5 % centrate (Figs. 5c and 6), while the photosynthetically fixed C in the Calvin cycle is converted to carbohydrates or other storage products (e.g. lipids). Conversely, the excess of N in combination with optimal C concentrations can foster protein synthesis [76,77]. These results indicate that a specific centrate dilution can be selected with the aim of not only maximizing biomass production or CO_2 and nitrogen consumption, but also targeting specific metabolite production. Therefore, centrate dilution and thus, biogas-to-centrate ratio can generate a biomass suitable as a feedstock for the synthesis of biofuel, biopolymers, biofertilizers, dietary feed ingredients, bioindicators or dyeing solutions for the textile industry, according to the pigment and carbohydrate production [5,78]. This will support the creation of a circular economy and enhancing resource efficiency goals [39]. Nevertheless, it should be mentioned that depending on the type of wastewater, and thus the contaminants present, and the desired application of the generated biomass, a proper risk assessment should be carried out to ensure quality and safety of the bioproducts [78]. Indeed, in pilot and industrial cultivation, the control of microbial contaminants can pose a serious challenge for microalgal growth but also for metabolite production. Different strategies have been proposed to control biological pollutants, including the selection of the most favourable conditions for microalgal growth, filtration systems, and chemical and biological drug additives [79]. On the other hand, it has been demonstrated that bacteria present in real anaerobic digestion effluents can positively interact with selected microalgae [80]. In the particular case of methanotrophic bacteria, methane consumption by methanotrophs present in AD effluent would be limited by the poor aqueous solubility of methane. Nevertheless, the elucidation of the positive interactions between selected microalgae and the indigenous microbial community for biogas upgrading, microalgal growth, and metabolite production requires further studies.

In addition to the environmental outcome, the use of wastewater and biogas as inputs for microalgal biomass production offers economic advantages. By replacing synthetic fertilizers and commercial-grade CO_2, production costs can be reduced up to 40 % [13,81]. These savings are further amplified when including the service of wastewater treatment cost and biogas upgrading as input for biomass production [17,81]. Moreover, the resulting biomass can be valorized in multiple markets, with estimated values ranging from €0.5–3 per kg for biofuels, chemicals and biopolymers, and from €3 up to €2650 per kg for pigments, depending on the compound and application [82,83].

5. Conclusion

This study demonstrated that new strains, compared to those commonly adopted, can be used for biogas upgrading and nutrient removal from digestate. Extending these processes to marine microorganisms will reduces the water footprint of the process. *Parachlorella hussii* N9 and *Cyanothece* sp. CE4 can effectively couple biogas upgrading with nutrient recovery from wastewater, offering a promising approach for a sustainable waste valorization. Indeed, complete CO_2 consumption was achieved under most conditions, with residual CO_2 levels compliant with European biomethane standards, while total nitrogen consumption was almost complete in all the media.

The results also indicated that a proper selection of biogas-to-digestate ratio should be carried out to address specific metabolite production. Indeed, under high biogas-to-digestate ratio, which caused a lower nitrogen availability, the intracellular accumulation of carbohydrates was enhanced (up to 44 % dry weight), indicating a viable route for producing biomass feedstock suitable for bio-based chemicals or biofuels. On the opposite, low biogas-to-digestate ratio positively influenced pigment production.

Despite promising lab-scale results, several factors require further investigation for practical application, including managing variable wastewater compositions, ensuring stable cultivation in non-sterile conditions, and designing systems for efficient O_2 removal. Future studies should explore optimized algal-bacterial consortia or co-cultivation systems that can improve resilience and enhance overall process efficiency at full scale.

CRediT authorship contribution statement

Matilde Ciani: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Laura Vargas-Estrada:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Alessandra Adessi:** Writing – review & editing, Supervision, Conceptualization. **Raúl Muñoz:** Writing – review & editing, Supervision, Resources, Methodology, Conceptualization.

Declaration of competing interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2025.104290.

Data availability

Data will be made available on request.

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