

Engineering strategies for the enhancement of *Nannochloropsis gaditana* outdoor production: Influence of the CO₂ flow rate on the culture performance in tubular photobioreactors



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ABSTRACT

The aim of this study was to evaluate the influence of the CO₂-specific flow rate on pH control, CO₂ use efficiency and biomass and lipid productivity of *Nannochloropsis gaditana* cultivated in outdoor tubular photobioreactors of 3.0 m³. In that study was evaluated the injection of CO₂-specific flow rates ranging from 0.4 to 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹. The use of low CO₂-specific flow rates, from 0.4 to 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹, has enabled pH control. The highest biomass productivities and CO₂ use efficiency were obtained using the flow rates of 1.2 and 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹. The highest lipid productivities were verified in experiments with flow rates of 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹, followed by 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹. A high CO₂-specific flow rate increases CO₂ loss and reduces the performance of the cells due to inadequate pH control. By contrast, a low CO₂-specific flow rate prevents adequate pH control and the performance of the cultures. Considering the results provided, an optimal CO₂-specific flow rate (1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹) was obtained for outdoor photobioreactor operations to produce *N. gaditana*, thus contributing to cost reduction.

1. Introduction

Microalgae are microorganisms that, through photosynthesis, produce biomass that can be used in food, feed and cosmetics [1] for the extraction of high added-value biocompounds [2] and in biofuel production [3,4]. Additionally, these microorganisms can be applied to wastewater treatment [5] and flue gas cleaning because they use nutrients from the effluents as a source of nitrogen, phosphorus and carbon for their growth [5–7].

Microalgae can be produced in both open and closed reactors. Open reactors (ordinarily the raceway type) are commonly used in the production of robust and not easily contaminated microalgae, mainly strains such as *Spirulina* and *Dunaliella* [8]. The most widespread use of this type of system occurs mainly due to its simplicity, relative low cost of construction and operation, with the possibility of scale up [9,10]. Generally, open reactors have minimal control of the operating conditions because they are more susceptible to contamination [11]. Closed reactors (photobioreactors) are the most recommended system to

produce biomass of non-robust strains usually rich in high added-value compounds [12]. Among them, tubular photobioreactors are the most extended ones. Although photobioreactors have a higher cost of construction than open systems, the bioreactor design is considered the most promising, because it has greater control of cultivation parameters, resulting in higher biomass productivities and lower contamination risks [13].

The increase in microalgae biomass productivity can be achieved by the supply of mainly carbon, in addition to nitrogen and phosphorus, among others, regardless of the photobioreactor configuration. Carbon dioxide (CO₂), which can be injected pure or from flue gases, is used as a carbon source and to control pH in photobioreactors. When pure CO₂ is supplied, the carbon source can represent up to 30% of biomass production costs [14]. Owing to the high cost of pure CO₂, its utilization efficiency is an important aspect that must be considered when producing microalgae at an industrial scale. Studies have shown that losses can reach up to 50%, but improved design parameters and operating conditions can minimize these losses, reducing it below 20%, especially

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when using advanced control strategies [15,16].

Nannochloropsis gaditana is a marine strain characterized by its fatty acid profile and high lipid content. Thus, this microalga strain is proposed to produce biodiesel and have applications in aquafeed and as a source of polyunsaturated fatty acids [17]. Previous studies have reported that *N. gaditana* can be cultivated under outdoor conditions in raceway and tubular photobioreactors [18,19]. However, the productivity and quality of the produced biomass are better when using tubular photobioreactors [19]. In addition to the photobioreactor design, certain factors, such as the CO₂ supply for pH control and carbon source, need to be evaluated for microalgae growth. Thus, it is important to evaluate the effects of the operational conditions (such as the CO₂ flow rate and concomitant pH variation) in outdoor microalgae production.

As described above, the aim of the study was to evaluate the influence of different CO₂-specific flow rates on pH control, CO₂ use efficiency and biomass and lipid productivity by *N. gaditana* cultivated in outdoor tubular photobioreactors. Additionally, we sought to identify the optimal conditions to maximize the performance of the system to enlarge the potential for large-scale production.

2. Material and methods

2.1. Microorganisms and culture medium

The marine microalga used was *Nannochloropsis gaditana*, which belonged to the collection of cultures of the research group “Marine Microalgae Biotechnology Group of University of Almería (Spain)”. The culture medium used was prepared using artificial seawater (30 g L⁻¹ of NaCl), agricultural fertilizers (g L⁻¹) [Ca(NO₃)₂ (0.8), KH₂PO₄ (0.25), MgSO₄ (0.3)] and micronutrients (mg L⁻¹) [B (0.23), Cu (0.09), Fe (2.10), Mn (1.17), Mo (0.07) and Zn (0.35)] [20]. The components of the culture medium were dissolved in water and were filtered through an ultrafiltration system comprising a 0.02 μm pore membrane (SFP 2860; DOW™ Ultrafiltration Modules, DOW®, China), followed by sterilization with ozone. The inoculum of *N. gaditana* was propagated in the laboratory and then on a pilot scale (outdoor conditions) in bubble column photobioreactors (total volume: 0.1 m³) located inside a greenhouse, with constant aeration and CO₂ supply on-demand for pH control (7.8–8.0).

2.2. Tubular photobioreactors

The experiments were carried out in pilot-scale tubular photobioreactors of 3.0 m³ (working volume of 2.6 m³), located inside a greenhouse at the “Estación Experimental Las Palmerillas” property of Fundación CAJAMAR (Almería, Spain). The pilot plant comprises ten tubular photobioreactors, these ones consisting of solar light receivers and a bubble column, as previously described by Fernández et al. [21]. The light receivers comprising tubes of 19 m in length and 0.09 m in diameter, united in a loop configuration, with a total horizontal length of 400 m. The bubble column was 3.5 m high and 0.5 m in diameter, with a work volume of 0.45 m³. This column was used to eliminate gases accumulated in the system (mainly oxygen) to allow the entrance of the culture medium and temperature control. The temperature of the culture was maintained below 30 °C by passing water at room temperature, with a flow rate of 1.5 m³ h⁻¹ in stainless steel heat exchangers located inside the bubble column. The continuous recirculation of the culture (with a velocity of 0.9 m s⁻¹), between the column and light receivers, was performed using a centrifugal pump installed in the bottom of the column. Each photobioreactor is equipped with a dissolved oxygen probe (OD5120; Crison, Barcelona, Spain), pH and temperature probe (pH5083 T; Crison, Barcelona, Spain), which are located at the end of the loop and are connected to a multimeter (MM44; Crison, Barcelona, Spain). The solar radiation received was measured using a thermoelectric pyranometer connected to an AC-420

adapter (LP-02; Geónica S.A., Spain). The probes were connected to a transmitter (Labjack U12) and data acquisition software (Daqfactory, Azeotech, Arizona, USA) [22,23].

2.3. Operation conditions

The experiments performed consisted of varying the CO₂-specific flow rates at 0.4, 1.2, 1.9, 3.1 and 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ to determine the effect on the pH control and carbon supply during the production of *N. gaditana* in tubular photobioreactors. Independent of the CO₂-specific flow rate, the pH of the culture was controlled at 8.0 by the on-demand CO₂ supply. That control was realized by on-off algorithm, in which opens the CO₂ valve at a pH higher than 8.1 and closes it at a pH below 7.9. The pH sampling time was 10 s, which was lower than the cycling of the reactor that was 4 min. The CO₂-specific flow rates were monitored by flow meters (FR4500; Key Instruments, USA), and the gas was injected into the cultures at a working pressure of 0.2 kgf cm⁻² using on/off solenoid valves. The CO₂ was injected on-demand by the control system using an on/off controller, only during the light period (when solar radiation > 30 W m⁻²), to control the pH at the setpoint (8.0). Air was supplied to the bubble column at a flow rate of 200 L min⁻¹ for the desorption of gases, mainly oxygen, accumulated in the culture. The cultures were carried out in June (spring-summer) in tubular photobioreactors as described above. The cultures were assessed in the semicontinuous mode at a dilution rate of 0.16 d⁻¹. The supernatant was recovered after centrifugation, followed by separation of the biomass and recirculation after nutrient supplementation and cleaning (ultrafiltration + ozone).

2.4. Analytical determinations

The biomass concentration (C_b) was determined by vacuum filtration of 50 mL of culture in a 0.7 μm pore-size glass microfiber filter (Whatman™). The biomass was washed with 50 mL of ammonium formate solution 1.25% w v⁻¹ and was dried in an oven at 80 °C for 24 h.

The fluorescence of chlorophyll or the maximum quantum yield (F_v/F_m) was measured to determine the physiological status of the cells. For this analysis, a 3.5 mL aliquot of culture was kept in the dark for 15 min; later, the value of the F_v/F_m ratio was measured in a fluorimeter (AquaPen AP 100; Photon Systems Instruments, Czech Republic) [23].

The cultures were evaluated daily by microscopic observation (Leica, ICC50 HD, Germany) to verify that the strain used was the dominant microorganism throughout the experimental period.

The total inorganic carbon concentration (TIC; g L⁻¹) in the inlet and outlet culture medium was determined using an analytical kit (Hach-Lange LKC 381, Germany) and photometer (Dr Lange LASA 50, Germany) [7].

2.5. Harvesting and biomass composition

The biomass of each experiment was recovered from the liquid medium by centrifugation (GEA, Germany) and then was dried by freeze drying (FD80; Cuddon Freeze Dry, New Zealand) and was stored at -20 °C. The lipids were extracted with a chloroform: methanol mixture (1: 2) and were quantified according to the colorimetric method proposed by Marsh and Weinstein [24], which uses the standard curve of tripalmitin [25]. The moisture content in the biomass was determined by official methodology [26], and the lipid concentration was expressed on the dry basis.

2.6. Independent and response variables

The independent variable was the imposed CO₂-specific flow rate, whereas environmental variables that could not be controlled such as solar radiation and temperature were measured and registered online.

Direct response variables included the dissolved oxygen and pH of the culture, in addition to the number and duration of CO₂ injections and the overall mass flow of CO₂ supplied. These variables were also measured and registered online using data acquisition software (Daqfactory; Azeotech, Arizona, USA).

The biomass productivity (P_b , g L⁻¹ d⁻¹) was calculated according to the following equation: $P_b = C_b D$, where C_b is the biomass concentration (g L⁻¹) and D is the dilution rate (d⁻¹) [12]. The CO₂ demand by the cultures (D_{CO_2} , g d⁻¹) was calculated according to Eq. (1), where P_b (g L⁻¹ d⁻¹) is the biomass productivity, V_{work} (L) is the working volume of the photobioreactor, x_{cbm} is the mass fraction of carbon in the biomass (considered 0.5 g_{carbon} g_{biomass}⁻¹), and MM_{CO_2} (g mol⁻¹) and MM_C (g mol⁻¹) are the molecular weights of carbon dioxide and carbon, respectively.

$$D_{CO_2} = P_b V_{work} x_{cbm} \frac{MM_{CO_2}}{MM_C} \quad (1)$$

The CO₂ use efficiency (E_{CO_2} , % w w⁻¹) was calculated according to Eq. (2) from the ratio of the CO₂ demand (D_{CO_2} , g d⁻¹) and CO₂ mass rate (m_{CO_2} , g d⁻¹) injected into the cultures.

$$E_{CO_2} = \frac{D_{CO_2}}{m_{CO_2}} \quad (2)$$

The ratio between the CO₂ supply and biomass production ($R_{CO_2/biomass}$, g_{CO₂} g_{biomass}⁻¹) was calculated according to Eq. (3), where m_{CO_2} (g d⁻¹) is the CO₂ mass rate injected into the cultures, P_b (g L⁻¹ d⁻¹) is the biomass productivity and V_{work} (L) is the photobioreactor working volume.

$$R_{CO_2/biomass} = \frac{m_{CO_2}}{P_b V_{work}} \quad (3)$$

The lipid concentration was evaluated in the lyophilized biomass of each experiment, while the lipid productivity (P_{lipid} , mg L⁻¹ d⁻¹) was determined according to Eq. (4), where P_b is the biomass productivity and f_{lipid} is the lipid fraction present in the biomass.

$$P_{lipid} = P_b f_{lipid} \quad (4)$$

2.7. Statistical analysis

The results of the experiments were assessed by analysis of variance (ANOVA) followed by Tukey's test for the comparison of means at a 90% confidence level.

3. Results and discussion

This study evaluated the effect of CO₂-specific flow rates on pH control, biomass productivity, and CO₂ use efficiency of *N. gaditana* cultures carried out in tubular photobioreactors under outdoor conditions. All experiments were carried out in June (spring-summer) to ensure that the cultures were exposed to similar environmental conditions such as solar radiation and temperature. Fig. 1 shows the daily variation of dissolved oxygen, solar radiation, temperature and pH in a typical solar cycle. The maximum value of solar radiation in the daylight period was 564.8 W m⁻², the temperature ranging from 24 °C to 34 °C as extreme values during the experimentation time. The average temperature in the daylight period was 31 °C, while that during the night was 27 °C. The available cooling system prevents the temperature from increasing above 35 °C, which could strongly reduce microalgae growth. The increase in the culture temperature during the solar cycle occurs by the absorption of solar radiation as heat. Therefore, this increase may cause overheating of the culture, resulting in the inhibition of growth and even cell death [23]. Zitteli et al. [27] verified that the highest biomass productivity (0.8 g L⁻¹ d⁻¹) of *Nannochloropsis* sp. in tubular photobioreactors under outdoor conditions was achieved in the spring when the temperatures ranged from 28 °C to 29 °C. Similarly, San

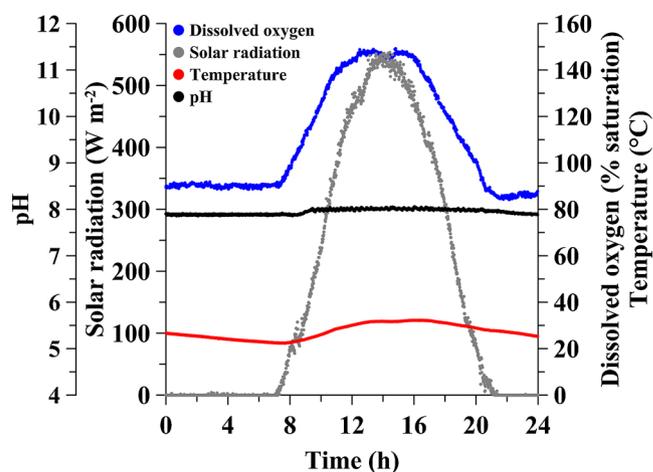


Fig. 1. Variation in dissolved oxygen (D.O.), solar radiation, temperature and pH of the culture during the production of *Nannochloropsis gaditana* in tubular photobioreactors at a 0.16 d⁻¹ dilution rate, when operating at a CO₂-specific flow rate of 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹.

Pedro et al. [18] observed the highest biomass productivity (0.19 g L⁻¹ d⁻¹) in *N. gaditana* cultures using raceway reactors under outdoor conditions, at temperatures of 29 °C.

The data in Fig. 1 show that, due to photosynthesis, the concentration of oxygen dissolved in the culture follows the sunlight intensity outline. During the night, the dissolved oxygen concentration was 89% Sat., lower than complete saturation with air due to cell respiration. The dissolved oxygen increases concomitantly with solar radiation, reaching a maximum value of 149% Sat. between 12 and 16 h. After 18 h, with the decrease in solar radiation, the dissolved oxygen concentration also declines gradually. According to these results, there was no high accumulation of dissolved oxygen in the culture that can damage the performance of the cells. Thus, the mass transfer capacity on the bubble column was higher than the oxygen production rate by the microalgae under the conditions analyzed. According to Ippoliti et al. [23], dissolved oxygen concentrations above 300% Sat. can be achieved when producing *Isochrysis* T-ISO in tubular photobioreactors at noon (12 h-highest solar radiation), indicating that the photobioreactor has insufficient mass transfer capacity to remove the oxygen produced. Under these conditions of a high dissolved oxygen concentration, photorespiration is enhanced, reducing the biomass productivity. The value of dissolved oxygen achieved in tubular reactors is highly dependent on the strain and operational conditions. According to Molina et al. [28], the dissolved oxygen concentration is increased up to 200% Sat., when producing *Phaeodactylum tricorutum* in tubular photobioreactors but is reduced at noon when the solar radiation exceeds 1500 μE m⁻² s⁻¹, due to the occurrence of photoinhibition. Experiments performed in the springtime do not show a decline in the dissolved oxygen at noon, and the cause was attributed to lower photoinhibition during the springtime when solar radiation is generally less than that in the summer.

In addition to temperature and dissolved oxygen, the carbon supply and pH control are major factors determining the performance of microalgae cultures [12]. According to Acien et al. [29], the on-demand injection of CO₂ was demonstrated to be an adequate method for pH control and the carbon supply in microalgae production processes. The required carbon can be supplied by gaseous streams containing high CO₂ concentrations at a low flow rate or, alternatively, using low CO₂ concentration streams at high flow rates. Additionally, the reliability of the system may also vary depending on the CO₂ utilization capacity related to biomass production and minimizing carbon losses to the atmosphere [7].

The results observed (Fig. 2) for all the CO₂-specific flow rates (0.4 to 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹) evaluated that the pH was controlled

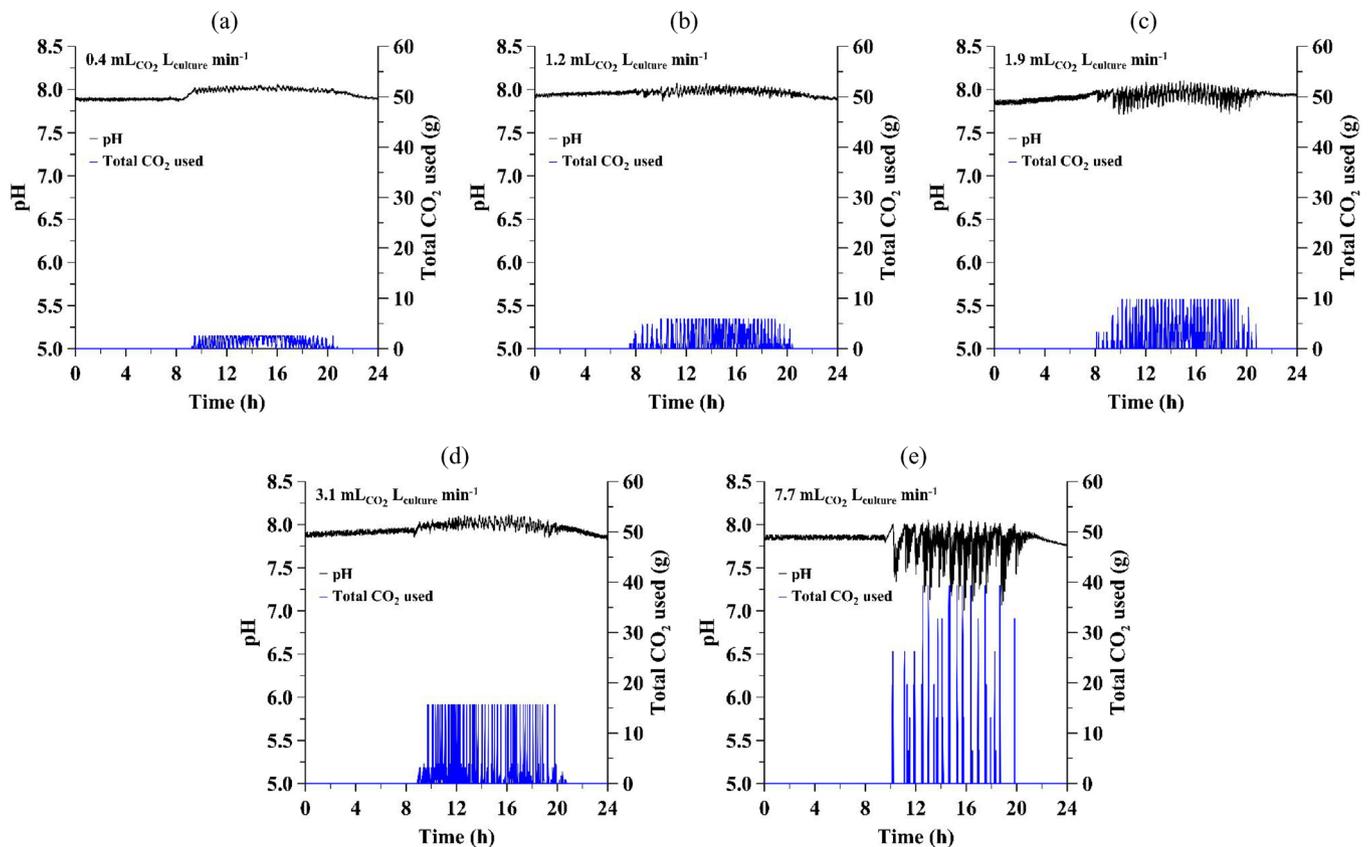


Fig. 2. Daily variation in the pH and total CO₂ used for pH control in the *Nannochloropsis gaditana* cultures, operated in the semicontinuous mode and carried out with CO₂-specific flow rates of 0.4 (a), 1.2 (b), 1.9 (c), 3.1 (d) and 7.7 mL_{CO₂} L_{culture} min⁻¹ (e).

Table 1

Experimental values of pH, chlorophyll fluorescence (Fv/Fm), biomass productivity (P_b), CO₂ mass rate (m_{CO₂}), CO₂ demand (D_{CO₂}), CO₂ use efficiency (E_{CO₂}), ratio between the CO₂ supply and biomass production (R_{CO₂/biomass}), CO₂ valve activation, and lipid concentration in biomass and lipid productivity (P_{Lipids}) as a function of the CO₂-specific flow rate in *Nannochloropsis gaditana* cultures.

Parameter	CO ₂ -specific flow rate (mL _{CO₂} L _{culture} ⁻¹ min ⁻¹)				
	0.4	1.2	1.9	3.1	7.7
pH Min	7.85 ^a ± 0.01	7.85 ^a ± 0.03	7.69 ^b ± 0.03	7.69 ^b ± 0.04	7.03 ^c ± 0.03
pH Max	8.07 ^b ± 0.01	8.08 ^{a, b} ± 0.01	8.11 ^a ± 0.01	8.12 ^a ± 0.01	8.04 ^b ± 0.01
pH Mean	7.99 ^a ± 0.03	7.98 ^{a, b} ± < 0.01	7.93 ^b ± 0.01	7.98 ^{a, b} ± < 0.01	7.78 ^c ± 0.03
Fv/Fm	0.70 ^a ± 0.01	0.70 ^a ± < 0.01	0.70 ^a ± 0.01	0.68 ^a ± < 0.01	0.69 ^a ± 0.03
P _b (g L ⁻¹ d ⁻¹)	0.21 ^b ± 0.01	0.22 ^{a, b} ± 0.01	0.24 ^a ± < 0.01	0.16 ^c ± < 0.01	0.12 ^d ± < 0.01
m _{CO₂} (g d ⁻¹)	966.3 ^c ± 14.1	1389.8 ^{a, b} ± 180.7	1665.6 ^a ± 10.6	1496.3 ^{a, b} ± 1.56	1356.7 ^b ± 42.6
D _{CO₂} (g d ⁻¹)	996.9 ^b ± 44.0	1032.5 ^{a, b} ± 37.7	1100.3 ^a ± 1.57	754.4 ^c ± < 0.01	549.8 ^d ± 15.7
E _{CO₂} (% w w ⁻¹)	103.0 ^a ± 6.1	74.0 ^b ± 5.7	67.0 ^b ± 0.5	50.4 ^c ± 0.1	41.0 ^c ± 2.4
R _{CO₂/biomass} (g g ⁻¹)	1.75 ^a ± 0.10	2.42 ^b ± 0.23	2.71 ^b ± 0.02	3.57 ^c ± < 0.01	4.45 ^d ± 0.27
CO ₂ valve activation	526 ^a ± 7.07	380 ^b ± 14.9	285 ^c ± 18.4	274.5 ^c ± 6.36	71 ^d ± 11.3
Lipids (% w w ⁻¹)	18.8 ^c ± 0.3	14.6 ^d ± 0.4	16.3 ^d ± 1.4	21.1 ^b ± 0.6	23.1 ^a ± 0.6
P _{Lipids} (mg L ⁻¹ d ⁻¹)	39.5 ^a ± 0.9	32.1 ^b ± 0.1	39.1 ^a ± 2.0	33.8 ^b ± 0.3	27.7 ^c ± 0.8

Identical superscript letters in the same line indicate that the means were not significantly different at a 90% confidence level ($p > 0.10$).

below the setpoint value of 8.0, including at noon with the highest photosynthetic activity. When using low CO₂-specific flow rates, from 0.4 to 1.2 mL_{CO₂} L_{culture}⁻¹ min⁻¹, the lowest variations of pH are observed because the supplied CO₂ is approximately consumed at the same rate that it is supplied (Fig. 2a-b). When increasing the CO₂-specific flow rate to 1.9 and 3.1 mL_{CO₂} L_{culture}⁻¹ min⁻¹, higher variations of pH are observed, with decreases up to 7.7 in the morning because the excess of CO₂ supplied reduces the pH of the culture after injections (Fig. 2c-d). The variations of pH are highly relevant when providing 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ of CO₂; in this case, the minimum pH reached was 7.03 (Fig. 2e). The number of activations of the on/off valve is

reduced proportionally from more than 500 when providing 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹ of CO₂ to less than 100 when providing 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ of CO₂, presenting a significant difference between the experiments ($p < 0.10$) (Table 1). This finding is important because the lifetime of the valves is limited; thus, many actuations imply a lower useful life of the equipment. The reduction of valve activations is interesting because it contributes to energy savings, increased equipment life and reduction of the CO₂ injection time [30,31]. However, increasing the CO₂-specific flow rate can result in pH variations (pH gradients), which influence the CO₂ use efficiency and biomass productivity. This phenomenon was observed by Santos et al. [7]: when a

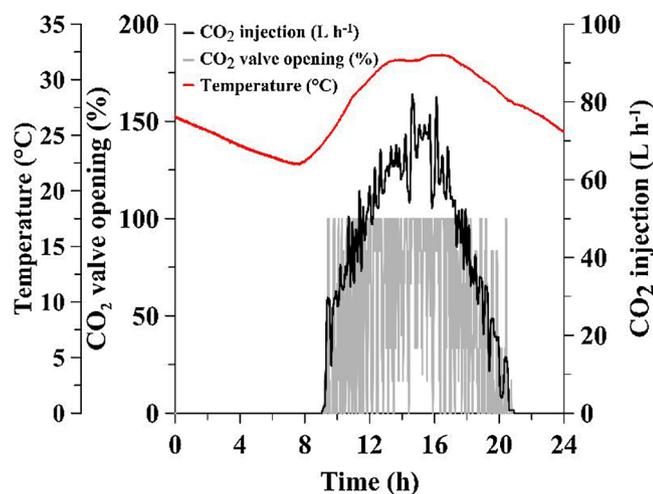


Fig. 3. Daily percentage variation in the position of the valve for CO₂ addition, CO₂ injection, and temperature of the *Nannochloropsis gaditana* cultures carried out in the semicontinuous mode, using a CO₂-specific flow rate of 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹.

higher CO₂ mass is injected, either by a high molar fraction or injection flow rate, there may be a delay between the pH measurement in the reactor and valve opening for the CO₂ supply. In that sense, more carbon would be supplied than required by the system, possibly affecting cultivation performance.

The frequency of which the CO₂ valve is open can be used to determine the total CO₂ injected that, in an ideal setting, must be the same as the net CO₂ demand by the cultures. Fig. 3 shows that, when a CO₂-specific flow rate of 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹ was used, the valve to CO₂ injection was open almost all the time. At any flow rate, the frequency of opening was higher at noon than in the morning and afternoon. Thus, the CO₂ injection rate shows the typical variation of sunlight, achieving its maximum value at noon with up to 80 L h⁻¹ of CO₂ injected. Arbib et al. [32] reported that it is possible to correlate the intervals of the flue gas injections in the culture with the photosynthetic activity of the microalgae cells. Thus, solar radiation was demonstrated to be the environmental parameter that directly influences the pH of the cultures. The results reported by Godos et al. [6] indicated that the highest frequency of flue gas additions (10% CO₂) in *Scenedesmus* sp. cultures was verified with the increase in solar radiation.

The experimental maximum, minimum and average values of the pH for each of the analyzed conditions are summarized in Table 1. The experimental data showed that the minimum pH remains higher than 7.69 at CO₂-specific flow rates lower than 3.1 mL_{CO₂} L_{culture}⁻¹ min⁻¹ but is reduced to 7.03 when the CO₂-specific flow rate is higher than this value, presenting a significant difference from that in the other experiments ($p < 0.10$). The maximum pH values ranged from 8.04 to 8.12. Although the data presented a significant difference between the treatments ($p < 0.10$), this variation was small. The mean pH values were similar, ranging from 7.99 to 7.78 ($p > 0.10$), for CO₂-specific flow rates of 0.4 to 3.1 mL_{CO₂} L_{culture}⁻¹ min⁻¹. In the experiment using a CO₂-specific flow rate of 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹, the average pH of the cultures was 7.78, significantly different from that in the other treatments ($p < 0.10$). These pH values are recommended for microalgae production, especially for *N. gaditana*. However, the pH gradients of the cultures negatively affect the performance of the cells. As reported by Rubio et al. [15], Berenguel et al. [33] and Santos et al. [7], when CO₂ is injected at high concentrations, pH gradients may occur within the reactor that may affect the culture. The increase in pH during the light period is due to photosynthesis; thus, according to Grobbelaar [34], the CO₂ consumption by microalgae results in the accumulation of OH⁻ ions in the medium and, consequently, in the increase in pH. The

on-demand injection of CO₂ allows the replacement of CO₂ consumed and maintenance of the pH; however, if excessive CO₂ is injected, the pH is reduced in excess, thus damaging the cells. According to Pawlowski et al. [30], the pH of cultures is influenced mainly by two phenomena, CO₂ supply and CO₂ uptake, which are functions of light availability. The CO₂ supplied contributes to the formation of carbonic acid (H₂CO₃), causing a reduction in the pH of the culture. On the other hand, microalgae in the presence of light perform photosynthesis, consuming CO₂ and producing O₂, increasing the pH of the cultures.

The experimental values of F_v/F_m do not show damage of the photosynthetic apparatus because these values were higher than 0.65, even with the pH variations in culture (Table 1), with no significant difference ($p > 0.10$) for this response among the experimental conditions. However, the biomass productivity shows a clear reduction when operating at CO₂-specific flow rates higher than 3.1 mL_{CO₂} L_{culture}⁻¹ min⁻¹, when the higher pH gradients were verified. Thus, the biomass productivity slightly increases from 0.21 to 0.24 g L⁻¹ d⁻¹ when the CO₂-specific flow rate is increased from 0.4 to 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ and is reduced to 0.16 and 0.12 g L⁻¹ d⁻¹ when the CO₂-specific flow rate is increased to 3.1 and 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ (Table 1). Among the three lowest utilized CO₂-specific flow rates, the lowest ($p < 0.10$) biomass productivity was observed when using a CO₂-specific flow rate of 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹, likely due to a carbon limitation effect. The highest values of biomass productivity were verified in the experiments with CO₂-specific flow rates of 1.2 to 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹, with a significant difference ($p < 0.10$) from that of the other treatments.

Independent of the CO₂-specific flow rate used, the total inorganic carbon concentration (TIC) in the inlet (0.119 g L⁻¹) was lower than that in the outlet because the CO₂ supplied oversaturated the culture medium with respect to air saturation. However, when using low CO₂-specific flow rates (0.4–1.2 mL_{CO₂} L_{culture}⁻¹ min⁻¹), the TIC concentration in the outlet fraction was lower (0.147–0.151 g L⁻¹) than when using the optimal CO₂-specific flow rate of 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ (0.157 g L⁻¹). At higher CO₂-specific flow rates (3.1–7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹), the TIC concentration in the outlet fraction was higher (0.167–0.169 g L⁻¹) because less carbon was fixed by the microalgae; thus, it persisted in the culture medium. Acien et al. [14] reported that, in microalgae cultures, the TIC concentration in the culture medium outlet is higher than that in the inlet, likely due to the added CO₂ for the control of pH remaining in the liquid phase but being lost with the culture medium when dilution is performed.

To determine the total amount of CO₂ effectively fixed into the biomass, an overall mass balance must be performed. The CO₂ mass rate (m_{CO_2}) increased from 966.3 to 1665.6 g d⁻¹ when the CO₂-specific flow rate was increased from 0.4 to 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ ($p < 0.10$). However, the increase in the CO₂-specific flow rate (3.1–7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹) caused an m_{CO_2} decrease from 1496.3 to 1356.7 g d⁻¹ (Table 1). Thus, the flow rate of the CO₂ mass injected is a direct function of biomass productivity. The estimated CO₂ demand shows a similar tendency, increasing from 996.9 to 1100.3 g d⁻¹ when the CO₂-specific flow rate increases from 0.4 to 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ ($p < 0.10$), but it decreases to 754.4 and 549.8 g d⁻¹ when the CO₂-specific flow rate increases up to 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ ($p < 0.10$) (Table 1). The CO₂ use efficiency decreases from 100% to 42% when the CO₂-specific flow rate increases from 0.4 to 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ ($p < 0.10$) (Table 1). The experiments using the CO₂-specific flow rates of 1.2 and 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ showed statistically similar CO₂ use efficiencies ($p > 0.10$). The efficiency does not only consider the CO₂ lost to the air by stripping but also considers the carbon accumulated in the culture medium; thus, it is a global efficiency value. The only approximation performed to calculate this efficiency is to consider a constant carbon content into biomass; because it is produced in the continuous mode, this approximation is quite reasonable. When considering the injection of CO₂ into microalgae cultures, it is assumed that microalgae comprise 50% of

carbon on average as biomass [34]. Therefore, it is necessary to provide a minimum of 1.8 kg of CO₂ to produce 1 kg of biomass [14]. The data obtained from experimental measurements show that the real values ranged from 1.75 to 4.45 g_{CO₂} g_{biomass}⁻¹ (Table 1), due to different phenomena occurring such as CO₂ absorption in the culture medium, CO₂ losses and reduction of biomass production by inadequate pH value/gradients. Under the optimal conditions found in the CO₂-specific flow rates of 1.2 and 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹, the optimal values were 2.42 and 2.71 g_{CO₂} g_{biomass}⁻¹, respectively. These results agree with those previously reported by Acien et al. [14] using *Scenedesmus almeriensis*. These authors verified the results with a CO₂ use efficiency of 74.5% and 2.34 g_{CO₂} g_{biomass}⁻¹. Chai and Zhao [35] verified a reduction of the CO₂ removal efficiency with the flow rate increase, from 2.5 to 15 mL_{mixture} L_{culture} min⁻¹ (10% CO₂), in cultivations of *Chlorococum* sp. The authors attributed this result to the reduction of the interfacial area between gas and liquid, suggesting that it can be improved by minimizing the bubble size at a lower CO₂-specific flow rate. In that sense, Ryu et al. [36] evaluated the effect of the injection of one CO₂ concentration (5%) on the different aeration flow rates and verified that, when the aeration rate was increased, there was a decrease in the CO₂ use efficiency by microalgae. The authors also evaluated the bubble size and concluded that, when the bubble size is larger, the mass transfer efficiency of the gas was decreased due to the reduction of the gas-liquid interface. Santos et al. [7] and Godos et al. [6] evaluated CO₂ transfer and carbon balance in raceway reactors and observed that an increased CO₂-specific flow rate resulted in the supply of more carbon than required by the microalgae cultures. According to the authors, this may contribute to the greater accumulation of this gas in the liquid medium and, consequently, higher losses to the atmosphere. Likewise, Moraes et al. [37] evaluated the effect of the gas mixture flow rate containing air and CO₂ (CO₂-specific flow rate fixed at 6 mL_{CO₂} L_{medium} min⁻¹) in *Spirulina* sp. LEB 18 cultivation and observed a reduction in the biomass productivity and E_{CO₂} with an increase in the flow rate from 50 to 300 mL_{mixture} L_{culture} min⁻¹.

In the present work, the lipid concentration in *N. gaditana* biomass varied between 14.6% and 23.1% (Table 1). The highest lipid concentration (p < 0.10) was verified in the experiment with injection of 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹. Thus, it was possible to observe that the use of a high CO₂ flow rate and occurrence of pH gradients may have caused a stress condition for the cultures, contributing to the increased lipid content of *N. gaditana*. However, when analyzing lipid productivity, it was possible to verify that the highest results, with significant difference among other results (p < 0.10), were found in the experiments with 0.4 mL_{CO₂} L_{culture}⁻¹ min⁻¹ (39.5 mg L⁻¹ d⁻¹) and 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ (39.1 mg L⁻¹ d⁻¹). The increase in the CO₂ flow rate from 0.4 to 7.7 mL_{CO₂} L_{culture}⁻¹ min⁻¹ resulted in a reduction of almost one-third in lipid productivity in *N. gaditana* cultures. According to Acien et al. [29], when microalgae are submitted to stress conditions, they can trigger the accumulation of lipids in the biomass. However, these conditions do not contribute to cell growth. In that sense, in addition to assessing the lipid content produced, one should consider lipid productivity. According to Chen et al. [38], lipid productivity is an important factor that needs to be evaluated because it allows verification of the potential of microalgal biomass application as raw material for biodiesel production.

In summary, the experimental values obtained demonstrate that CO₂ must be provided to *N. gaditana* cultures to avoid carbon limitation and inadequate pH. However, the CO₂ gas flow rate employed, independent of the photobioreactor, is not an arbitrary parameter. To enhance the CO₂ use efficiency, the CO₂-specific flow rate utilized in cultures must be minimized, but excessive reduction may have a negative effect on the biomass productivity. Thus, it is necessary to perform global analysis of the culture parameters (biomass productivity, photosynthetic efficiency, CO₂ use efficiency, CO₂ demand and biomass composition) and process (pH control, CO₂ valve activations, carbon mass supplied) to identify the most suitable experimental condition for the development of the production system.

4. Conclusion

The CO₂-specific flow rate used for carbon supply and pH control in *Nannochloropsis gaditana* cultures in tubular photobioreactors under outdoor conditions influenced the performance of the entire process. In that sense, in addition to defining the CO₂-specific flow rate to control the pH, it is necessary to analyze the existence of pH gradients, carbon use efficiency and biomass productivity in depth as a function of the cultivation operational conditions. Therefore, the use of a CO₂-specific flow rate of 1.9 mL_{CO₂} L_{culture}⁻¹ min⁻¹ was considered optimal to produce *N. gaditana* in tubular photobioreactors under outdoor conditions.

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