Biofixation of Air Emissions and Biomass Valorization—Evaluation of Microalgal Biotechnology

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Abstract
This research appraised the simultaneous biofixation, that is not quite common in scientific literature, of carbon dioxide (CO2) and nitric oxides (NOx) by microalgae species Chlorella vulgaris, Haematococcus pluvialis, and Scenedesmus subspicatus. The experimental design was established by five treatments with gas concentrations between control—0.04% of CO2, 5 to 15% of CO2, and 30 to 100 ppm of NOx. Parameters such as pH, growth, productivity, lipids, protein, carbon/ nitrogen ratio, and astaxanthin were evaluated. For all species, the maximal growth and productivity were achieved with 5% of CO2 and 30 ppm of NOx. Regarding protein content, for all the three species, better results were obtained at higher concentrations of CO2 and NOx. These results prove the microalgae capacity for CO2 and NOx biofixation and reuse of biomass as a source of high value-added products, such as lipids, proteins, and astaxanthin. These findings support the indication of these species for flue gas treatment process and use in biorefineries systems.

Keywords Greenhouse gases · Biomitigation · Chlorella vulgaris · Haematococcus pluvialis · Scenedesmus subspicatus

Introduction
The energy production to meet the demands of industries and the global population has increased, over the years, the emission of greenhouse gases (GHG). This is mainly due to combustion in thermoelectric plants, whose emissions are around 10 to 15% of CO2 and...
100 to 300 ppm of NO\textsubscript{x} and SO\textsubscript{x} [1]. Activities like that, which use non-renewable natural resources, are one of the major anthropogenic causes of GHG emissions [2].

As a result of increased emissions, global warming and climate changes are reported [3, 4]. Furthermore, there are other consequences such as impairment of environmental quality due to air, water, and soil pollution; changes in the incidence of heat waves, drought, soil disruption, rising sea levels, and acid rain; and consequently, damage to human health, such as respiratory diseases, cancer, and death [5, 6].

Faced with this perspective, strategies have been developed for the mitigation of GHG emissions [2]. Among these strategies, biofixation is highlighted, which is characterized by the use of biological activity to fix gases such as CO\textsubscript{2} and its conversion into biomass [2].

Along with biofixation, the use of microalgae stands out due to its characteristics such as nutrient assimilation from diversified sources, and its conversion into high-value biomass. In addition, the microalgae present a biofixation potential from 10 to 50 times highest than terrestrial plants species [7], may utilize wastewater for growth [8], and present potential to concomitant use in environmental treatment (biofixation) and production of high value-added products [9].

\textit{Chlorella, Haematococcus,} and \textit{Scenedesmus} are the genera of microalgae that stand out for their applicability in environmental treatments such as biofixation [10], wastewater treatment and biofuels production [8], biofertilizers [11], food and feed supplementation [12, 13], and medical applications [14]. Therefore, promoting microalgae growth by the disposal of specific nutrients sources of carbon and nitrogen, like CO\textsubscript{2} and NO\textsubscript{x} from potentially polluting activities, may constitute a biotechnological solution [15, 16] to GHG mitigation [17].

This research emerged from the need for the development and application of an alternative for GHG mitigation in a thermoelectric power plant. Thus, it aimed to appraise \textit{Chlorella vulgaris}, \textit{Haematococcus pluvialis}, and \textit{Scenedesmus subspicatus} for simultaneous biofixation of CO\textsubscript{2} and NO\textsubscript{x} at different concentrations.

\textbf{Material and Methods}

The microalgae species used in this research were \textit{Chlorella vulgaris} Beyerinck (Algal Biotechnology Laboratory-UFSCar, Brazil), \textit{Haematococcus pluvialis} Flotow (Botany Department-UBC, Canada), and \textit{Scenedesmus subspicatus} Chodat (Algal Biotechnology Laboratory-UFSCar, Brazil). They were maintained in Bold’s Basal Medium (BBM) [18].

\textbf{Cultivation Conditions}

To appraise microalgae tolerance, the cultures were carried out in glass photobioreactors (Schott flasks) with a liquid volume of 2 L (working volume of 2 L with headspace of 200 mL), conditioned at 22 °C and a photoperiod of 10 h light and 14 h dark that was supported by fluorescent lamps of 40 w [1, 10, 19] at an exposition of 150 µmol photons m\textsuperscript{-2}s\textsuperscript{-1}, approximately. The initial concentration of the inoculum was 0.1 g L\textsuperscript{-1} [20]. The aeration was carried out by compressed air and simulated gas, with mixing of CO\textsubscript{2} and NO\textsubscript{x} at different concentrations, arranged in industrial cylinders, according to the different treatments protocols.

It was established five treatments: CT: control treatment—air (0.04% of CO\textsubscript{2}); and injection treatments (IT) CO\textsubscript{2} and NO\textsubscript{x} (NO\textsubscript{2}+N\textsubscript{2} balance): 1–5% of CO\textsubscript{2} and 30 ppm of
NO$_x$, IT 2–7% of CO$_2$ and 40 ppm of NO$_x$, IT 3–10% of CO$_2$ and 60 ppm of NO$_x$, and IT 4–15% of CO$_2$ e 100 ppm of NO$_x$. Each treatment was performed in triplicate.

Cultures aeration was carried out by air injection utilizing a compressor (Schultz model 20/250). The flow inlet was 1 L min$^{-1}$. The simulated gas injections were performed with the same flow (1 L min$^{-1}$). This was controlled by solenoid valves during the light period (adapted from 10). The duration of gas injection was 1 min every 19 min. The tests lasted 16 days. In the end, the biomass was recovered by filtration and used to measure elementary contents, stoichiometry, lipids, protein, and astaxanthin (Fig. 1).

**Analytical Methods**

Culture pH was measured every 24 h with a digital pH meter (Hanna, HI 2221 model). The growth was measured by absorbance in a spectrophotometer (Kasuaki, IL 226 model) at 670 nm [10], which was used for biomass determination (g L$^{-1}$), through a correlation calculation.

Productivity was calculated with the biomass values obtained gravimetrically. Every 48 h, samples of 10 mL were filtered through pre-weighed glass microfiber filters (GF-1, 25 mm), which were dried and weighted [21]. The obtained values were applied on the formula:

\[
P = \left( \frac{X_t - X_0}{T - T_0} \right)
\]

$X_t$ is the final biomass concentration, $X_0$ is the initial biomass concentration, $T$ is the final day, and $T_0$ is the initial day of culture [10]. The analysis was performed in triplicate and it was obtained $n=9$. All samples were taken before the first gas injection of the day.

*Fig. 1* Experimental design of microalgae cultivation

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**Diagram**

- **Chlorella vulgaris**
- **Haematococcus pluvialis**
- **Scenedesmus subspicatus**

**Microalgae cultivation:**
- pH
- Growth
- Productivity

**Biomass recovery**
- Protein
- Lipids
- Astaxanthin
- CHN elemental
- Stoichiometry
Biomass Analysis and CO$_2$ Biofixation

For elementary analysis of carbon, hydrogen, and nitrogen (CHN) from microalgal biomass, samples were sent to the Instrumental Analytical Center of São Paulo University. Therefore, 5 mg was processed in an elementary analyzer (Perkin Elmer CHN 2400 series II), which performed biomass combustion in an environment of pure oxygen. The gases obtained were automatically measured. These analyses were performed in duplicate and the values were used to calculate the carbon/nitrogen (C/N) ratio.

Nitrogen values obtained were used to calculate the protein of the biomass, for which was adopted the conversion value of 4.44 proposed by López et al. [22].

With the values of carbon content, it calculated the CO$_2$ biofixation rate according to the formula proposed by Duarte and Costa [23]:

$$\text{Biofixation} = Px.Xcbm.(Mco_2/Mc)$$

$Px$ is the productivity in mg L$^{-1}$ day$^{-1}$, $Xcbm$ is the fraction of carbon contained in biomass, and $Mco_2$ and $Mc$ are the molecular weights of carbon dioxide and carbon.

The lipid content analysis was performed by the Bligh and Dyer [24]-based method. Samples of 50 mg of microalgal biomass were weighed and destined for the cold extraction of lipids. The biomass was treated with 3 mL of chloroform/ methanol (2:1, v/v) and 10 µL of butylhydroxytoluene (BHT) 1% in methanol. The samples were placed in Falcon tubes covered with aluminum for light protection. These tubes were destined for an ultrasonic bath (3 times $\times$ 15 min.). At the end of the cycles, the samples were conditioned at 4 °C for approximately 24 h. After this period, samples were again sonicated (3 times $\times$ 15 min.), centrifuged at 5000 rpm for 3 min. Then, the supernatant was recovered and reserved, and to the pellets in the Falcon tubes were added 1.5 mL of chloroform/methanol solution and centrifuged in the same conditions. The supernatant was recovered over again and added to the reserved ones. In the recovered liquid were added 2 mL of reverse osmosis water and 1 mL of chloroform. It was performed one more cycle of centrifugation in the same conditions previously established. The recovered liquids were conditioned in glass flasks and dried on the stove at 50 °C for solvents evaporation. Then, the flasks were cooled under vacuum until constant weight and the lipids content were determined gravimetrically.

Astaxanthin extraction from *Haematococcus pluvialis* biomass was performed by an acidic extraction (HCl and acetone). The extracts analysis was carried out by high-performance liquid chromatography (HPLC-LC 10AD, Shimadzu, Japan) equipped with a C18 column. For carotenoids separation, it used a gradient of eluent concentration (A, acetone, and B, methanol: water 9:1 v/v), under the following conditions: B 80 to 20% by 25 min; 20% by 10 min; and 20 to 80% by 5 min. The flow rate was 0.8 mL min$^{-1}$ and the temperature at 40 °C [25]. The detection was carried out at 476 nm. For astaxanthin identification and quantification, an astaxanthin standard was used (purity 96–98.2%, Carbosynth, USA). Astaxanthin was quantified by the formula:

$$y = 3.10^8x + 10124$$

$Y$ is the area of the astaxanthin curve obtained for each treatment. This formula was obtained by a linear correlation ($R^2 = 1$).
Statistical Analysis

Using Statistica 7 software, distribution analysis was performed using the Shapiro–Wilk method. The significant differences were identified by Tukey and Kruskal–Wallis tests, and the level of significance was set at \( p \leq 0.05 \).

Biomass, productivity, and biofixation were used to comparisons between time intervals (1, 4, 8, 12, and 16 days) and concentrations of simulated gas (treatments). For pH, protein, lipids, C/N ratio, and astaxanthin, comparisons were performed between treatments. Correlation analyses were carried out using Spearman’s test and data about gas concentration, biomass, protein, lipids, and astaxanthin. The correlation coefficient considered was null (0), weak (0 to 0.3), regular (0.3 to 0.6), strong (0.6 to 0.9), and very strong (0.9 to 1.0), based on Callegari-Jacques [26].

Results and Discussion

pH

The values of pH presented in CT and IT treatments by the three species of microalgae followed linearity, with a variance of 6.7 to 10.1 during the 16 days of cultivation. For S. subspicatus, the mean between treatments does not present statistical differences, while for C. vulgaris and H. pluvialis, the differences were observed between the treatments with the highest gas concentrations, which presented the lowest pH averages (Table 1).

It was identified that the lower pH was not a limiting factor for growth, since the values of biomass (Fig. 2) and productivity (Table 2) of the treatment IT 2 presented equivalency to the control CT. However, between the treatments with gas injection, those with lower pH (IT 3 and IT 2) were also the ones with the lowest growth.

The average pH range for C. vulgaris, H. pluvialis, and S. subspicatus was, respectively, 8.8, 8.4, and 8.7, and are associated with higher rates of biomass of the culture. These values were obtained from the treatment IT 1 (5% of \( \text{CO}_2 \) + 30 ppm of \( \text{NO}_x \)).

Radmann et al. [10] evaluated the tolerance of Spirulina sp., Scenedesmus obliquus, S. nidulans, and Chlorella vulgaris to the exposure of 12% of \( \text{CO}_2 \), 60 ppm of \( \text{SO}_2 \), and 100 ppm of \( \text{NO}_x \), concentrations similar to the treatment IT 4, reported a pH range between 6.0 and 10.0. These authors also concluded that the highest biomass was related to a pH

### Table 1: Average pH range presented by Chlorella vulgaris, Haematococcus pluvialis, and Scenedesmus subspicatus during cultivations under different concentrations of simulated gas

| Treatments    | Chlorella vulgaris | Haematococcus pluvialis | Scenedesmus subspicatus |
|---------------|--------------------|-------------------------|-------------------------|
| CT (AIR)      | 8.9 ± 0.6<sup>b</sup> | 8.7 ± 0.4<sup>c</sup>  | 9.1 ± 0.7<sup>a</sup>  |
| IT 1 (5% + 30 ppm) | 8.8 ± 0.7<sup>b</sup> | 8.4 ± 0.7<sup>c</sup>  | 8.7 ± 0.8<sup>a</sup>  |
| IT 2 (7% + 40 ppm) | 8.9 ± 1.0<sup>b</sup> | 7.8 ± 0.4<sup>b</sup>  | 8.6 ± 1.0<sup>a</sup>  |
| IT 3 (10% + 60 ppm) | 7.7 ± 0.6<sup>a</sup> | 7.7 ± 0.4<sup>a</sup>  | 8.2 ± 0.7<sup>a</sup>  |
| IT 4 (15% + 100 ppm) | 8.5 ± 0.9<sup>ab</sup> | 8.3 ± 0.5<sup>a</sup>  | 8.6 ± 0.9<sup>a</sup>  |

Lowercase letters on the same column represent differences between treatments for each species. Significance level of 95% (\( p \leq 0.05 \)) by Tukey’s test
Fig. 2  Microalgae biomass (g L\(^{-1}\)) increase throughout the experimental period. Biomass on a dry basis
range between 8.0 and 9.0, which corroborated with the results obtained for *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Scenedesmus subspicatus*.

Injections of gases such as CO$_2$ and NO$_x$ in the culture medium alters the pH, which interferes in the microalgae development by decreasing the bioavailability of some nutrients such as phosphorus and inorganic carbon [10, 27] and change the solubility of CO$_2$ in the medium [27, 28]. The data obtained for pH during cultivation proves that the gas injection did not interfere with the pH in order to prevent microalgae growth.

### Growth

The biomass measurement by absorbance (OD$_{670\text{nm}}$) demonstrated that *Chlorella vulgaris* has the biggest values for IT 1, followed by the treatments 2, 3, and 4, consecutively. To appraise these results, it was observed a strong and negative correlation ($-0.65$) between increased simulated gas concentration and decreased growth (Fig. 2).

These results demonstrated that the treatments with gas injection IT 1 (5% of CO$_2$ + 30 ppm of NO$_x$) and IT 2 (7% of CO$_2$ + 40 ppm of NO$_x$) presented higher growth than the control. For IT 1, the maximum biomass of the culture (g L$^{-1}$) obtained was...
32% higher than the control treatment. This corroborates with the hypothesis that *C. vulgaris* presents the capacity to grow in culture systems with different concentrations of CO$_2$ and NO$_x$.

*H. pluvialis* presented the highest biomass of the culture (g L$^{-1}$) in treatments IT 4 (15% of CO$_2$ + 100 ppm of NOx) and IT 1 (5% of CO$_2$ + 30 ppm of NOx) (Fig. 2). When comparing the maximum biomass obtained, the differences were, respectively, 12% and 11% higher than the control treatment. The lowest biomass of the culture was observed during the IT 2 (7% of CO$_2$ + 40 ppm of NOx), which was also observed with a low pH value (7.8) (Table 1). For *H. pluvialis*, it has not established a link between increased simulated gas concentration and decrease of biomass. Furthermore, this species demonstrated tolerance to different concentrations of CO$_2$ and NO$_x$.

Chekanov et al. [29] state that moderate concentrations of CO$_2$ (5%) are beneficial for cultures of *H. pluvialis*, which advantage the carbon accumulation and biomass production. However, CO$_2$ injections at level $\geq 10\%$ are harmful and result in decreased growth. This data corroborates with the results obtained in IT 1, which was the treatment with the highest growth. On the other hand, it is not consistent with the results of IT 4 (15% of CO$_2$ + 100 ppm of NOx), which presented a maximum growth of 12% higher than the control treatment (air—0.04% CO$_2$).

Cheng et al. [30] demonstrated the tolerance of *H. pluvialis* to exposure at 15% of CO$_2$ and reported that this concentration improved the culture yield when compared to air injection. According to these authors, this response is associated with the increase of photosynthetic activity and CO$_2$ competition.

*S. subspicatus* obtained approximate values of biomass between the treatments with gas injections (IT), which presented higher growth of biomass than the control treatment (Fig. 2). The biggest growth was registered in IT 1 (5% of CO$_2$ + 30 ppm of NOx) and IT 3 (10% of CO$_2$ + 60 ppm of NOx), which presented a maximum biomass production 23% higher than the control treatment. Thereafter, the treatment IT 4 increased 21% more than control. The lower biomass of the culture was observed in IT 2 (7% of CO$_2$ + 40 ppm of NOx). The data presented indicated a regular and positive correlation (0.51) between simulated gas concentration and biomass production.

These results prove that *S. subspicatus* is tolerant to exposure to different concentrations of CO$_2$ and NOx. However, contrary to what was observed for *C. vulgaris*, increased concentrations of simulated gas did not promote significant decreases in the growth of *S. subspicatus*. It should be noted that the treatments with gas injections presented growth similar to each other and higher than the control.

Nayak et al. [8], when appraised the growth of *Scenedesmus* sp. at different concentrations of CO$_2$, did not find a correlation between gas concentration and microalgae growth. Li et al. [21] also obtained similar results for *Scenedesmus raciborskii* at exposure to 7% of CO$_2$ and to simulated gas with 15% of CO$_2$, 200 ppm of SO$_2$, and 100 ppm of NO. There were no significant differences between these two treatments.

The results of biomass production confirm the tolerance of the three microalgae species at different concentrations of CO$_2$ and NO$_x$. In addition, the best scenario for growth was achieved during the concentration IT1 (5% CO$_2$ + 30 ppm NO$_x$).
Productivity

When the biomass productivity was evaluated, it was verified that each microalgae species presented a different behavior during the cultivation. However, for the three of them, the highest productivity was obtained in IT 1 (5% of CO$_2$ + 30 ppm of NO$_x$).

*C. vulgaris* presented the highest productivity on day 8 for the tests IT 1, IT 2, and IT 4, respectively (Table 2). For IT 3, the better result observed was 0.05 g L$^{-1}$ day$^{-1}$ at days 12 and 16 (Table 2). Radmann et al. [10] appraised *C. vulgaris* and reported maximum productivity of 0.05 g L$^{-1}$ day$^{-1}$ with an injection of 12% of CO$_2$, 60 ppm of SO$_2$, and 100 ppm of NO. This value is the same that was obtained in the treatment IT 4 (15% of CO$_2$ + 100 ppm of NOx).

For *H. pluvialis*, the maximal biomass productivity was 0.05 g L$^{-1}$ day$^{-1}$ obtained in IT 1 (5% of CO$_2$ + 30 ppm of NO$_x$) between time intervals 8–12 days, while the lower biomass productivity was registered in the treatment IT 2, which had a larger adaptation phase, as is shown in Table 2. These results indicate that a trend related to gas concentration and biomass productivity has not been established. Nevertheless, the treatments IT 1 and IT 4 promoted a productivity improvement.

Li et al. [31] reported that *H. pluvialis*, at an exposure of 15% of CO$_2$, obtained maximum productivity of 0.66 g L$^{-1}$ day$^{-1}$. According to these authors, aeration with 15% of CO$_2$ significantly increases photosynthesis and carbon assimilation.

When the productivity of *S. subspicatus* was evaluated, the maximum values were obtained at day 8 for the treatments IT 1 and IT 2, with a productivity of 0.09 g L$^{-1}$ day$^{-1}$ for both. For the treatment IT 4, the biggest productivity was 0.07 g L$^{-1}$ day$^{-1}$ registered on day 4 (Table 2). The IT 3 presented the lowest productivity between the gas injections treatments. However, all treatments with gas injection (IT 1, 2, 3, and 4) had an improvement in productivity in comparison with the control treatment (Table 2).

Radmann et al. [10] obtained the maximum productivity of 0.04 and 0.05 g L$^{-1}$ day$^{-1}$, for *S. obliquus* and *C. vulgaris*, respectively. The values presented by *C. vulgaris* were the same, while for *S. subspicatus* were higher (0.07 g L$^{-1}$) during IT 4 (15% of CO$_2$ + 100 ppm of NOx).

Radmann et al. [10] assume that the exposure at time zero to gases containing molecules such as nitrogen oxides (NO$_x$) can inhibit the growth of microalgae. However, as observed by Vaz et al. (2016), the microalgae appraised in this research were not inhibited for the exposure to gases. Contrarily, it presented higher growth in the treatments with gas injection than control treatment (air).

From obtained data, the three microalgae species presented maximal biomass productivity for the treatment IT 1 (5% of CO$_2$ + 30 ppm of NO$_x$). When comparing the biomass productivity between microalgae species, *S. subspicatus* stands out with its productivity, which values up to 0.09 g L$^{-1}$ day$^{-1}$, after 8 days from cultivation.

CO$_2$ Biofixation

*C. vulgaris* presented a CO$_2$ biofixation range between 40.3 ± 7.7 and 146.2 ± 68.3 (mg L$^{-1}$ day$^{-1}$). The highest ranges were observed for treatments IT 1 (146.2 ± 68.3) and IT 4 (124.7 ± 22.6), both at day 4. In this interval, these averages were significantly higher than treatments CT and IT 3. This indicates that, for these treatments, the biggest biofixation
has occurred in the initial days of cultivation. On days 8, 12, and 16 were observed higher values for IT 1 and IT 2 (Table 3).

Biofixation performance by *H. pluvialis* presented a range between 15.0 ± 6.9 and 101.6 ± 13.3 mg L⁻¹ day⁻¹. Better averages were observed on treatments IT 1 at day 12 (101.6 ± 13.3 mg L⁻¹ day⁻¹), and IT 4 at day 4 (53.5 ± 18.2 mg L⁻¹ day⁻¹) (Table 3). In treatments IT 2 and IT 3, maximum averages were obtained at day 12.

Regarding CO₂ biofixation by *S. subspicatus*, its values were between 68.8 ± 5.5 and 156.7 ± 19.6 mg L⁻¹ day⁻¹. The highest significant averages were registered during IT 1 and IT 2 on day 8 (Table 3). These averages were significantly higher than the other treatments. For IT 3, the maximum average was achieved at day 12 (88.2 ± 8.3 mg L⁻¹ day⁻¹) and for IT 4 at day 4 (116.7 ± 18.0 mg L⁻¹ day⁻¹). *S. subspicatus* presented the highest biofixation between days 4, 8, and 12.

Yadav et al. [20] evaluated *Chlorella* sp. cultivated with the flue gas (10% CO₂, 0.554% CO, 8.33% O, 61 ppm NOₓ, 0.3% SOₓ, and 9 ppm hydrocarbons) and they obtained a CO₂ fixation range of 175 ± 10 (mg CO₂ L⁻¹ day⁻¹). Duarte and Costa [23] cultivated *Synechococcus nidulans* at 10% of CO₂, 100 ppm of NO, and 60 ppm of SO₂, and registered a value of 155.8 ± 13.0 mg L⁻¹ day⁻¹. These results are close to those obtained for *C. vulgaris* and *S. subspicatus*. Chekanov et al. [29] reported a reduction in CO₂ fixation mediated by *H. pluvialis* when CO₂ concentration increased to 10% and

| CO₂ biofixation | Time intervals |
|-----------------|----------------|
|                 | 4              | 8              | 12 | 16 |
| *Chlorella vulgaris* | CT (0.04% CO₂) | 69.7 ± 9.3Bb | 90.9 ± 9.3Ab | 89.5 ± 9.6Ab | 89.1 ± 7.6Ab |
|                 | IT 1 (5% + 30 ppm) | 146.2 ± 68.3Bb | 132.3 ± 20.0Bc | 121.5 ± 14.8Ab | 95.5 ± 12.1Ac |
|                 | IT 2 (7% + 40 ppm) | 80.2 ± 5.1Ab | 95.9 ± 10.3Bbc | 104.4 ± 14.1Bb | 76.5 ± 12.5Ab |
|                 | IT 3 (10% + 60 ppm) | 40.3 ± 7.7Ab | 54.3 ± 5.4Ba | 74.6 ± 6.2Ca | 78.7 ± 6.2Cb |
|                 | IT 4 (15% + 100 ppm) | 124.7 ± 22.6Bb | 79.8 ± 11.2Ab | 68.4 ± 9.3Ab | 62.5 ± 10.5Ab |
| *Haematococcus pluvialis* | CT (0.04% CO₂) | 70.1 ± 16.3Bb | 42.8 ± 7.5AAb | 36.1 ± 3.9Ab | 40.4 ± 7.6Ab |
|                 | IT 1 (5% + 30 ppm) | 69.7 ± 20.8Ab | 91.5 ± 13.3Bc | 101.6 ± 13.3Bc | 88.0 ± 13.3Ab |
|                 | IT 2 (7% + 40 ppm) | 15.0 ± 6.9Ab | 24.3 ± 5.6Bc | 37.2 ± 2.0Cabc | 32.3 ± 5.9Ac |
|                 | IT 3 (10% + 60 ppm) | 53.3 ± 8.4Bc | 32.8 ± 4.5AAb | 47.4 ± 3.3Bc | 42.1 ± 3.1Ab |
|                 | IT 4 (15% + 100 ppm) | 53.3 ± 18.2Ab | 52.2 ± 6.9Bc | 41.9 ± 8.6Ab | 41.3 ± 3.7Ab |
| *Scenedesmus subspicatus* | CT (0.04% CO₂) | 95.5 ± 10.5Bb | 71.5 ± 6.6AAb | 78.0 ± 4.5Bc | 63.0 ± 4.8Ab |
|                 | IT 1 (5% + 30 ppm) | 148.1 ± 14.1Cd | 156.5 ± 5.0Ch | 130.1 ± 9.4Bb | 115.5 ± 6.2Ac |
|                 | IT 2 (7% + 40 ppm) | 141.1 ± 22.9Bd | 156.7 ± 19.6Bb | 135.7 ± 2.7Bb | 112.2 ± 3.5Abc |
|                 | IT 3 (10% + 60 ppm) | 72.0 ± 7.6Aa | 68.8 ± 5.5Aa | 88.2 ± 8.3Ab | 85.5 ± 6.7Ab |
|                 | IT 4 (15% + 100 ppm) | 116.7 ± 18.0Bc | 88.6 ± 12.4Aab | 95.9 ± 4.7Bb | 83.5 ± 5.3Ab |

Capital letters on the same line represent differences between time intervals averages. Lowercase letters in the same column represent differences between treatment averages. Significance level of 95% (p ≤ 0.05) Kruskal–Wallis (n = 9)
20%. According to these authors, this may be a negative effect on photosynthetic carbon fixation. This was not observed in this research, since *H. pluvialis* presented a different behavior, with a better biofixation on IT 4 (15% CO₂) than IT 3 (10% CO₂).

Between the microalgae species evaluated in this research, *S. subspicatus* presented the highest rates of CO₂ biofixation (up to 156.7 ± 19.6 mg L⁻¹ day⁻¹) and these rates were related with the highest growth and productivity (Fig. 2). This corroborates with Adamczyk et al. [32] which affirm that the highest biofixation capacity is associated with the highest productivity and growth.

**Biomass Analysis**

**Protein**

The total protein levels obtained at the end of cultures presented averages with differences between treatments for the three microalgae species. *C. vulgaris* treatments IT 3 and IT 4 presented the highest significant values of 15.1% and 15.7%, respectively. The lower value was observed for treatment IT 1 (5% CO₂ + 30 ppm NOₓ) (Table 4). Thus, there was registered a strong and positive correlation (0.68) between increased simulated gas concentration and protein production, and a strong and negative correlation (−0.82) between the decrease of growth and high level of protein.

Concerning *H. pluvialis* in treatments with gas injection, the highest level of total protein was obtained for IT 3 (25%) followed by IT 2 (22%). The treatment IT 1 was presented with the lowest protein content (Table 4). From this, a regular and negative correlation (−0.43) between growth and protein content was registered.

| Table 4 | Content of protein and lipids % w/w on a dry basis and stoichiometry from biomass of *Chlorella vulgaris*, *Haematococcus pluvialis*, and *Scenedesmus subspicatus* cultivated at different concentrations of CO₂ and NOx |
|-----------------|-----------------|-----------------|
| **Chlorella vulgaris** | **Protein (%)** | **Lipids (%)** | **C:N ratio** |
| CT (0.04% CO₂) | 13.2 ± 0.2b | 20.1 ± 2.0ab | 15.5 ± 0.1bc |
| IT 1 (5% + 30 ppm) | 9.9 ± 0.6a | 24.7 ± 1.9c | 21.2 ± 1.0d |
| IT 2 (7% + 40 ppm) | 12.0 ± 0.1b | 19.7 ± 1.9a | 16.8 ± 0.0f |
| IT 3 (10% + 60 ppm) | 15.1 ± 0.3c | 19.6 ± 0.7a | 13.3 ± 0.1a |
| IT 4 (15% + 100 ppm) | 15.7 ± 0.0e | 23.1 ± 1.1bc | 13.1 ± 0.0a |
| **Haematococcus pluvialis** | **Protein (%)** | **Lipids (%)** | **C:N ratio** |
| CT (0.04% CO₂) | 26.8 ± 1.6d | 4.4 ± 0.5a | 8.3 ± 0.4a |
| IT 1 (5% + 30 ppm) | 10.0 ± 0.4a | 7.7 ± 1.0ab | 24.0 ± 0.7b |
| IT 2 (7% + 40 ppm) | 22.4 ± 0.5bc | 22.6 ± 1.3c | 10.0 ± 0.0a |
| IT 3 (10% + 60 ppm) | 25.0 ± 0.0cd | 15.8 ± 4.1bc | 8.5 ± 0.0p |
| IT 4 (15% + 100 ppm) | 21.2 ± 0.2b | 13.8 ± 1.1bc | 10.2 ± 0.0b |
| **Scenedesmus subspicatus** | **Protein (%)** | **Lipids (%)** | **C:N ratio** |
| CT (0.04% CO₂) | 12.4 ± 0.3b | 29.0 ± 1.7a | 17.4 ± 0.6ab |
| IT 1 (5% + 30 ppm) | 8.2 ± 0.1a | 31.2 ± 3.8a | 26.3 ± 0.1f |
| IT 2 (7% + 40 ppm) | 11.7 ± 1.4b | 31.3 ± 4.0a | 18.5 ± 2.1ab |
| IT 3 (10% + 60 ppm) | 14.1 ± 0.7b | 27.8 ± 4.4a | 15.1 ± 0.5a |
| IT 4 (15% + 100 ppm) | 10.8 ± 0.0ab | 29.3 ± 5.0a | 19.6 ± 0.0bc |

Lowercase letters in the same column represent differences between treatment averages for each microalgae species. Significance level of 95% (p ≤ 0.05). *Tukey and #Kruskal–Wallis (n = 9)
When evaluating *S. subspicatus*, treatments IT 3 and IT 2 presented averages of the protein content of 14.1% and 11.7%, respectively. These values were significantly higher than treatment IT 1 (5% CO₂ + 30 ppm NOₓ), which presented the lowest value (8.2%) (Table 4).

The three microalgal species presented a similar response, the lowest protein content associated with IT 1 (5% of CO₂ + 30 ppm of NOₓ). In addition, it was found that for *C. vulgaris*, the protein content increased with lowest growth at higher concentrations of gas. For the other species, this behavior was not observed. *H. pluvialis* and *S. subspicatus* presented the same standard response for protein production, with the higher protein content presented by IT 3, IT 2, IT 4, and TI 1, consecutively (Table 4).

Moreover, when comparing the three microalgae species, *H. pluvialis* presented the highest value of protein content, which was between 10.0 and 25.0%, followed by *C. vulgaris* (9.9–15.7%), and *S. subspicatus* (8.2–14.1%). This finding suggests that the increase in protein content was improved with NOₓ injection and, between the three microalgae species, *H. pluvialis* presented better NOₓ fixation.

Yadav et al. [20] also observed a significant increase in protein content of *Chlorella* sp. cultivated at different CO₂ concentrations. According to Li et al. [21], simulated gas injection (15% CO₂ + 200 ppm SO₂ + 100 ppm NO) promotes protein biosynthesis in *Scenedesmus raciborskii*. For microalgal metabolism, NOₓ serves as an additional source of nitrogen [21, 33].

**Lipids**

Content of total lipids from biomass obtained at the end of cultivations was demonstrated that *C. vulgaris* registered the highest values at treatments IT 1 and IT 4, with lipid concentrations of 25% and 23%, respectively. Between the injection treatments, the lowest lipid content (19.6%) was observed for IT 3 (10% CO₂ + 60 ppm NOₓ) (Table 4). Therefore, these values were higher than presented by Yadav et al. [20] when they appraised *Chlorella* sp. and obtained 12.7 ± 1.7 and 4.5 ± 1.0% for concentrations of 5% of CO₂ and flue gas (10% CO₂, 0.554% CO, 8.33% O, 61 ppm NOₓ, 0.3% SOₓ, and 9 ppm of hydrocarbons).

On the other hand, a different response was observed for *H. pluvialis*. Treatment IT 2 (7% CO₂ + 40 ppm NOₓ) presented 22.6% of lipid content, a value significantly higher than IT 3, IT 4, and IT 1, which presented, respectively, 15.8%, 13.8%, and 7.7% (Table 3). From these results, a lipid content decrease of 7% from IT 2 to IT 3 was found. Cheng et al. [30] also obtained similar results for *H. pluvialis*, with a lipid decline of 6% due to an increase of CO₂ concentration. According to these authors, lipid production may be strongly affecting astaxanthin accumulation.

*S. subspicatus* did not present a significant difference between treatments, which presented a range between 27.8 and 31.3%. Therefore, the highest averages of lipid content were observed for treatments IT 1 and IT 2, with 31.2 and 31.3% (Table 4). In addition, *S. subspicatus* presented the highest lipid content, followed by *C. vulgaris* and *H. pluvialis*, consecutively.

The difference in lipid accumulation may be a biological response of microalgae to a stress factor, such as greenhouse gas injections in the culture medium. According to results obtained by Nayak et al. [8], the lipid accumulation may decrease due to an increase in CO₂ concentration. When these authors appraised *Scenedesmus* sp. at 10% of CO₂, the lipid content was 16.6%. This value is 40% lower than that obtained for *S. subspicatus* in treatment IT 3 (10% of CO₂ + 60 ppm of NOₓ) (Table 4).
The stoichiometry of main elements (C/N) was demonstrated that the treatment IT 1 (5% \(\text{CO}_2\) + 30 ppm \(\text{NO}_x\)) stands out with the highest C/N ratios for the three microalgae species (Table 4). Chekanov et al. [29] also observed this response for \emph{H. pluvialis}, with a rise of C/N ratio due to the 5% \(\text{CO}_2\) concentration in the medium.

Otherwise, results of lowest C/N ratios were related with high concentrations of gas injections, as 15% \(\text{CO}_2\) + 100 ppm \(\text{NO}_x\) for \emph{C. vulgaris} and 10% \(\text{CO}_2\) + 60 ppm \(\text{NO}_x\) for \emph{H. pluvialis} and \emph{S. subspicatus}. These treatments also presented the highest protein and astaxanthin content (Table 4). According to Chekanov et al. [29], the lowest C/N ratios were associated with microalgae physiological conditions such as cells with high content of nitrogen compounds like protein, nucleic acids, and chlorophyll.

### Table 5

| Treatment          | Astaxanthin µg mL |
|--------------------|-------------------|
| CT (0.04% \(\text{CO}_2\)) | 0.23 ± 0.14abc    |
| IT 1 (5% + 30 ppm) | 0.14 ± 0.04abc    |
| IT 2 (7% + 40 ppm) | 0.14 ± 0.08b      |
| IT 3 (10% + 60 ppm)| 0.27 ± 0.11c      |
| IT 4 (15% + 100 ppm)| 0.24 ± 0.12abc   |

Lowercase letters represent differences between treatment averages. Significance level of 95% \((p \leq 0.05)\). Kruskal–Wallis \((n = 12)\)

### Carbon:Nitrogen Ratio

Astaxanthin profiles were obtained by chromatography and the results indicate that, for \emph{H. pluvialis}, astaxanthin was identified in all treatments (0.04–15% of \(\text{CO}_2\) and 0–100 ppm de \(\text{NO}_x\)). Astaxanthin, as well protein and lipids content, may be useful to assess microalgae response to stress conditions.

When performing the astaxanthin quantification, it was observed that the highest astaxanthin concentration was in IT 3 (10% \(\text{CO}_2\) +60 ppm \(\text{NO}_x\)), which also had the highest protein content in injections treatment. A correlation was established weak and positive (0.26) between increase of gas concentration and astaxanthin content. These results demonstrated that the injection of simulated gas at concentrations between 10 and 15% of \(\text{CO}_2\) + 60 and 100 ppm of \(\text{NO}_x\) did not inhibit astaxanthin biosynthesis, once this can raise its production (Table 5).

Chekanov et al. [29] related that concentrations up to 20% of \(\text{CO}_2\) did not induce inhibition in astaxanthin biosynthesis of \emph{H. pluvialis}. Nevertheless, according to Cheng et al. [30], the accumulation of lipids is one of the essential factors for the astaxanthin synthesis.

### Conclusions

\emph{Chlorella vulgaris}, \emph{Haematococcus pluvialis}, and \emph{Scenedesmus subspicatus} presented tolerance to growth under simultaneous injections of \(\text{CO}_2\) and \(\text{NO}_x\). These species were capable of simultaneous GHG biofixation and production of proteins, lipids, and astaxanthin.
when cultivated under gas injections at concentrations near to emissions of coal and natural
gas thermoelectric plants. *S. subspicatus* presented the highest potential for CO₂ biofixa-
tion and *H. pluvialis* for NO₃ fixation. This raises its applicability for the flue gas treatment
process. Thus, *Chlorella vulgaris, Haematococcus pluvialis*, and *Scenedesmus subspica-
tus* are indicated for use in biorefinery systems with the production of high value-added
bioproducts.

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**Declarations**

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