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The Microalga *Chlorella vulgaris* as a Natural Bioenergetic System for Effective CO₂ Mitigation—New Perspectives against Global Warming

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Abstract: In the present contribution, the differentiation in the molecular structure and function of the photosynthetic apparatus of the unicellular green alga *Chlorella vulgaris* was studied at several light intensities (0–400 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)) and various CO₂ concentrations (0.04–60% CO₂), in completely autotrophic conditions. Asymmetries that occur by different light intensities and CO₂ concentrations induce metabolic and functional changes. Using chlorophyll fluorescence induction techniques (OJIP test), we showed that *Chlorella vulgaris* tolerates extremely high CO₂ levels and converts them photosynthetically into valuable products, including O₂ and biomass rich in carbohydrates and lipids. Interestingly, the microalga *Chlorella vulgaris* under extremely high CO₂ concentrations induces a new metabolic state intensifying its photosynthetic activity. This leads to a new functional symmetry. The results highlight a potent CO₂ bio-fixation mechanism of *Chlorella vulgaris* that captures up to 288 L CO₂ L PCV \(^{-1} \) day \(^{-1} \) under optimal conditions, therefore, this microalga can be used for direct biological CO₂-reducing strategies and other green biotechnological applications. All of the above suggest that *Chlorella vulgaris* is one of the most prominent competitors for a closed algae-powered bioreactor that is able to consume huge amounts of CO₂. Thus, it is a sustainable and natural bioenergetic system with perspectives in dealing with major environmental issues such as global warming. In addition, *Chlorella vulgaris* cultures could also be used as bioregeneration systems in extraterrestrial missions for continuous atmospheric recycling of the human settlements, paving the way for astrobiological applications.

Keywords: *Chlorella vulgaris*; CO₂ mitigation; global warming; biofuels; photosynthesis; environmental biotechnology; astrobiology

1. Introduction

Over the last couple of centuries, industrialization along with urbanization have brought an immense increase in greenhouse gases (GHGs), mostly carbon dioxide (CO₂), which is considered one of the main causes of global warming [1] and other major environmental problems, such as ocean acidification [2]. Atmospheric CO₂ concentration increased rapidly from 280 ppm in 1850 [3] to more than 417 ppm in 2021, with more than half of this increase happening in the last 30 years [4]. Notably, CO₂ levels today are higher than at any other point in at least the past 800,000 years [5]. Human activities release about 35 gigatons of CO₂ every year, as opposed to almost 10 megatons 2 centuries ago [6]. There is already clear evidence that anthropogenic emissions of GHGs, such as CO₂, alter the natural carbon cycle, which leads to an accelerated warming of our planet [7]. Based on Representative Concentration Pathways (RCPs), adopted by the International Panel on Climate Change (IPCC), and other modelling scenarios, scientists predict that the amount of CO₂ in the atmosphere might double or even triple at the end of the 21st century [8,9]. This will result in a planet that is hotter by more than 4 °C compared to preindustrial ages [8,9], well above the 2 °C danger mark agreed in COP21 Paris Agreement [10].
Several techniques have been examined worldwide for reducing CO\(_2\) emission levels based on chemical, physical and biological methods [11–13]; among these, CO\(_2\) bio-fixation via the photosynthetic procedure is considered one of the most effective approaches for CO\(_2\) capture [14,15]. Photosynthesis is the process used by phototrophic organisms to convert light into chemical energy that is then invested in carbon fixation, the conversion of inorganic carbon compounds (usually CO\(_2\)) into organic carbon compounds (usually a carbohydrate such as glucose) [16]. Oxygenic photosynthesis, the mechanism that uses H\(_2\)O as a reducing agent and releases O\(_2\), evolved 3.8 × 10\(^9\) years ago and changed Earth’s atmosphere [17]. It slowly converted a strongly reducing, CO\(_2\) rich, atmosphere into the oxidizing one that we know today [18]. Undoubtedly, photosynthetic organisms have a fundamental regulatory role in the natural carbon cycle equilibrium [19], an attribute in which we should invest while tackling the spiraling problem of CO\(_2\) increase.

Regulations and controls on reducing our “carbon footprint” cannot help us much without serious socioeconomic impacts on existing infrastructure [1]. However, an efficient photosynthetic system, such as microalgae, could simultaneously be profitable through numerous applications, while providing a green solution for CO\(_2\) mitigation. Microalgae are the fastest growing photosynthetic organisms on earth, up to 50 times faster than their terrestrial plants [20]. They do not require cultivable land, while their use of fresh water is dramatically reduced [21]. Various microalgae tolerate a wide range of cultivation conditions [22], including extremely high CO\(_2\) concentrations [23,24]. In particular, *Chlorella vulgaris* photoautotrophic cultures cultivated in bubble column photobioreactors showed a high rate of CO\(_2\) fixation up to 10% [25,26]. Comparatively, photosynthetic activity in most terrestrial plants increases when CO\(_2\) levels rise, until some saturating concentration, which is typically around 0.1%. Higher CO\(_2\) concentrations lead to adverse effects [27].

The microalga *Chlorella vulgaris* is an extremely promising candidate also for large-scale cultivation that has already attracted a lot of interest for numerous applications [28]. A rapid growth rate and a resistance to harsh environmental conditions are some of the features that make this microalga a prominent candidate for efficient production of high-value biomass yields [29]. Due to its high nutritional value, it is one of the few microalgae that have been used successfully as alternative aquacultures, and a source of animal and even human food [30–32], as it exhibits a large number of therapeutic properties widely used in pharmaceutical and cosmetic industry [29]. Furthermore, agrochemical applications of *Chlorella vulgaris* can benefit common crops by both biofertilization [33] and growth acceleration [34]. Another interesting feature of this particular species is that it responds to different growth parameters by modifying its rich biochemical composition [28], which is ideal for third-generation and even fourth-generation biofuels, that are not involved in the “food vs. fuel dilemma” [35]. Both bioethanol and biodiesel production can be optimized by aiming at carbohydrate and lipid accumulation, respectively [36,37].

In the present contribution, the metabolic and functional differentiation of the unicellular green alga *Chlorella vulgaris* was studied at various light intensities and CO\(_2\) concentrations, aiming at effective CO\(_2\) mitigation, conversion of CO\(_2\) to O\(_2\) and the production of biomass rich in carbohydrates and lipids. Such a microalgal system could be the key for environmental and astrobiological applications.

2. Materials and Methods

2.1. Organism and Cultivation Conditions

Cultures of the wild type strain of the unicellular green alga *Chlorella vulgaris* (211-11b, SAG Culture Collection of Algae) [38] were used for this study. According to Wong et al. (2017), Bold’s Basal Medium (pH = 6.8 ± 0.1) was found to be the best liquid medium for biomass production in *Chlorella vulgaris* [39,40]. The cultures were grown in elongated glass tubes (Ø 5 cm × 50 cm), and incubated at controlled temperature (25–26 °C) [41] with continuous sterile air bubbling (50–60 L·h\(^{-1}\)), for about a week. Light was provided with warm white LED lamps with the intensity of 40–50 µmol m\(^{-2}\) s\(^{-1}\) and a photoperiod of 16 h light:8 h dark [42]. After reaching a sufficient amount of biomass with high photosynthetic
efficiency, the cultured cells were centrifuged at 1500 × g for 5 min. Subcultures with an initial concentration of 1 µL packed cell volume (PCV) per mL culture were initiated with new medium. The initial cell density of 1 µL PCV mL⁻¹ culture was the best starting point so that all the photosynthetically active cells absorb about the same amount of light, while avoiding fast nutrient depletion. All the experiments were carried out in 120 mL hermetically closed bottles (Ø 5 cm, height 9.5 cm) with rubber septa and in the absence of any organic carbon source. The final culture volume in each bottle was 50 mL autotrophic culture medium (liquid phase) and 70 mL gas phase with different CO₂ concentrations (0.04–60%). Increased CO₂ levels decreased the pH of the medium from 6.8 (without CO₂ addition) down to 5.2 (in treatments with 60% CO₂), which was then increased proportionately to the photosynthetic activity of the microalgae [43]. The bottles were kept in a temperature-controlled chamber (25–26 °C) at various light intensities (0–400 µmol m⁻² s⁻¹). Sampling took place at the same time (at the middle of the light period), using sterile gas tight needles, without opening the bottles.

2.2. Microalgae Growth Determination

The culture’s growth rate was estimated by measuring the packed cell volume (PCV) of the culture according to the method of Navakoudis et al. [44]. Briefly, a 1 mL sample of a homogenized cell suspension was centrifuged at 1500 × g for 5 min using graduated capillary hematocrit tubes (TPP, Sigma-Aldrich, St. Louis, MO, USA) and the cell volume was expressed as µL PCV mL⁻¹.

2.3. GC-TCD Measurements

Oxygen and nitrogen measurements were made utilizing gas chromatography, using a thermal conductivity detector (GC-TCD) (Shimadzu GC 2010 Plus, Kyoto, Japan). To separate O₂ and N₂, argon was used as the carrier gas under the pressure of 5 bars, and at an oven temperature of 120 °C. The column used was a capillary Vici Metronics MC (Poulsbo, WA, USA) with length of 30 m (diameter 0.53 mm) and film thickness of 20 µm. The temperature of TCD was set at 200 °C for the detector and 180 °C for the injector. A gas-tight syringe (250 mL) was used for sampling from the hermetically closed bottles. The quantification of all gases was carried out by injecting known quantities of O₂ and N₂ in the GC-TCD. For the CO₂ measurements, helium was used as the carrier gas under pressure of five bars and at oven temperature of 250 °C. The column used was the same as explained above. The temperature of TCD was set at 300 °C for the detector and 280 °C for the injector. The quantification of CO₂ was carried out by injecting known quantities in the GC-TCD. Preliminary experiments with microalgal cultures in high CO₂ concentrations and in closed cultivation systems confirmed that the decrease in CO₂ volume (photosynthetic CO₂ fixation) was about the same with the increase in O₂ volume (photosynthetic O₂ production) (data not shown).

2.4. Photosynthetic and Respiratory Activity Measurements

For the measurements of photosynthetic and respiratory activity of the cultivated microalgae, we used GC-TCD measurements (for details, see above). The changes in O₂ levels in *Chlorella vulgaris* closed cultivation systems (initial cell concentration 1 µL PCV mL⁻¹) was measured at known time intervals under light conditions and in absolute darkness (for the first 16 h of incubation in light and in continuous darkness respectively) for the determination of the photosynthetic and respiratory activity respectively. The photosynthetic activity represents the actual net microalgal photosynthetic rate in the corresponding light intensity and CO₂ concentration used in each experimental treatment. Each net photosynthetic value was calculated by the difference between gross photosynthesis (total O₂ production) and the O₂ consumption due to respiration. The photosynthetic and respiratory activity was expressed as µL O₂ µL PCV⁻¹ h⁻¹.
2.5. Fluorescence Induction Measurements: OJIP-Test

The Handy Plant Efficiency Analyser, PEA (Hansatech Instruments, Kings’s Lynn, Norfolk, UK) was used for the fluorescence induction measurements [45]. This protocol is based on the measurement of a fast fluorescence transient with a 10 µs resolution in a time span of 40 µs to 1 s. Fluorescence was measured at a 12-bit resolution and excited by three red light-emitting diodes providing a saturating light intensity of 3000 µmol m$^{-2}$ s$^{-1}$. For the fluorescence induction measurements, we put the flat bottom of the small culture bottle (Ø 5 cm) directly on the PEA sensor using a specific adaptor that ensures absolute darkness for at least 10 min (in order to open the PSII reaction centers) before the exposure to saturated light [43]. Under these conditions, and without opening the culture bottles, we measured the photosynthetic efficiency (Fv/Fm) of the culture in the ambient conditions, according to the OJIP method [45,46]. Out of all the other photosynthetic OJIP variables measured, we used the density of active photosynthetic reaction centers (RC/CS$_0$), the functional antenna size (ABS/RC), the primary photochemistry (PSI$_0$), the dissipated energy flux per active reaction center (DI$_0$/RC), the performance index on absorption basis (PI$_{abs}$) and the maximum photosynthetic efficiency (Fv/Fm) [47].

2.6. Lipid Extraction and Quantification

The protocol of Folch and Stanley [48] was used for the extraction of total lipids, as discussed by Sati et al. [49], with some slight modifications. Briefly, 3 mL of chloroform:methanol in a ratio 2:1 (v/v) were added in 2.5 µL PCV pellet of centrifuged microalgal cells (1500 × g for 5 min) and incubated for 15–20 min with frequent vortexing. The samples were mixed with 1 mL of 0.74% NaCl solution for two phase separation and centrifuged for 5 min at 1500 × g. The lower phase (chloroform with dissolved lipids) was isolated and incubated at 95 °C for complete chloroform evaporation [48,49].

For the total lipids quantification, the method of Park and Jeong [50] was used with slight modifications. Briefly, the lipids were dissolved in 200 µL of H$_2$SO$_4$ (99.99%), by vortexing briefly. The samples were then incubated at 95 °C for 10 min and immediately cooled at room temperature. 4 mL of phosphor-vanillin reagent (500 mL H$_3$PO$_4$ 85%, 125 mL ddH$_2$O and 0.75 g Vanillin) was added into the samples and incubated for an additional 10 min at room temperature, before being measured spectrophotometrically at 530 nm. The quantification of total lipids was estimated using a calibration curve with canola oil.

2.7. Carbohydrate Extraction and Quantification

For the extraction and quantification of total carbohydrates, the method of Schulze et al. [51] was used. Briefly, 1 mL of HCl 37% was added to 2.5 µL PCV pellet of centrifuged microalgal cells (1500 × g for 5 min) and incubated for 15 min. Samples were then diluted with 5 mL of ddH$_2$O. At 400 µL of the diluted samples, we added 900 µL of thymol-sulfuric acid reagent (0.1 g thymol into 100 mL of H$_2$SO$_4$ 99.99%), and vortexed briefly. The samples were then incubated for 30 min at 95 °C. The absorbance was measured at 505 nm. The quantification of total carbohydrates was estimated using a calibration curve with glucose [51].

2.8. Data Analysis

Each experiment was repeated at least three times, thus, each treatment included three independent samples. Standard errors of the average values are presented on all diagrams. ANOVA One Way and Tukey HSD paired tests were used for testing the significance of the values. The normality of samples was tested using the Shapiro–Wilk test and the homoscedacity using the Bartlett’s test.
3. Results

3.1. Effect of Different Light Intensities on the Microalgal Photosynthetic Mechanism

The effects of various light intensities (0–400 μmol m\(^{-2}\) s\(^{-1}\)) on the photosynthetic apparatus of *Chlorella vulgaris* under natural atmospheric conditions in closed cultivation systems were evaluated. The kinetics of the maximal photosynthetic efficiency (Fv/Fm) in Figure 1A showed that increased light intensity acts as a major stress factor for the photosynthetic mechanism under ambient CO\(_2\) (0.04%) concentration. In complete darkness (0 μmol m\(^{-2}\) s\(^{-1}\)), Fv/Fm remained constantly high. After a couple of days, under extremely low light conditions (10 μmol m\(^{-2}\) s\(^{-1}\)), Fv/Fm values showed a slight decrease. As light intensity increased to 50 μmol m\(^{-2}\) s\(^{-1}\), the photosynthetic efficiency drop came earlier and sharper, leading to a decrease in photosynthetic capacity. Stepping up to 100 μmol m\(^{-2}\) s\(^{-1}\) and 200 μmol m\(^{-2}\) s\(^{-1}\), Fv/Fm decreased down to 0.4 (Figure 1A). Specifically, at 400 μmol m\(^{-2}\) s\(^{-1}\) under ambient CO\(_2\), there was a total breakdown of the photosynthetic apparatus after the fourth incubation day (no reliable measurements with OJIP-test; no observed bullets in Figure 1A). These first results showed an extremely light sensitive photosynthetic mechanism under ambient carbon dioxide with limited biomass increase (Figure 1B).

![Figure 1](image)

**Figure 1.** (A): Kinetics of the microalgal photosynthetic efficiency, expressed in Fv/Fm, over the incubation time under different light intensities (0–400 μmol m\(^{-2}\) s\(^{-1}\)) in an air atmosphere with 0.04% CO\(_2\) (p < 0.05 for each day). (B): Microalgal biomass concentration expressed in μL PCV/mL on the sixth incubation day, over different light intensities (0–400 μmol m\(^{-2}\) s\(^{-1}\)). Significantly different are the biomass concentrations of the following treatments: 0 μmol m\(^{-2}\) s\(^{-1}\) versus 50, 100 and 400 μmol m\(^{-2}\) s\(^{-1}\); 10 μmol m\(^{-2}\) s\(^{-1}\) versus 50 μmol m\(^{-2}\) s\(^{-1}\) and 50 μmol m\(^{-2}\) s\(^{-1}\) versus 200 μmol m\(^{-2}\) s\(^{-1}\) (p < 0.05).

The effect of light on the molecular structure and function of the photosynthetic apparatus of the microalga *Chlorella vulgaris* is presented in more detail in Figure 2, which depicts a series of more specific OJIP parameters. The results reveal that stress was induced by increasing the light intensity under ambient CO\(_2\) concentration in closed cultivation systems. Concisely, the density of active photosynthetic reaction centers (RC/CS\(_0\)) decreased
while the antenna size per active reaction center (ABS/RC) increased, leading to decreased primary photochemistry (PSI₀) and increased dissipation energy per active reaction center (DⅠ₀/RC). As a result, the performance index (PI(abs)) decreased (Figure 2). The parameters mentioned above are the most representative indicators of the sensitivity/tolerance of the photosynthetic apparatus under abiotic stress and are in agreement with the observations made in abiotic stress conditions, such as ozone elevation [52], high UVB radiation [53,54] and low temperature [55].

Figure 2. Changes in RC/CS₀, ABS/RC, PSI₀, DⅠ₀/RC and PI(abs) (OJIP test) over the 6 days of incubation time compared to the corresponding values of day 0, under different light intensities (0–400 μmol m⁻² s⁻¹) in an air atmosphere (0.04% CO₂). (A): 0 μmol m⁻² s⁻¹, (B): 10 μmol m⁻² s⁻¹, (C): 50 μmol m⁻² s⁻¹, (D): 100 μmol m⁻² s⁻¹, (E): 200 μmol m⁻² s⁻¹ and (F): 400 μmol m⁻² s⁻¹.

3.2. Effect of Various Extreme CO₂ Concentrations at the Photosynthetic Mechanism under Several Light Intensities

A series of various extreme CO₂ concentrations (0.04, 10, 20, 30, 40 and 60%) under different light intensities (0, 10, 50, 100, 200 and 400 μmol m⁻² s⁻¹) were combined in order to test the effects of elevated CO₂ concentrations in the Chlorella vulgaris cultures (Figure 3). The results showed that under continuous darkness (0 μmol m⁻² s⁻¹), by increasing the CO₂ levels, the photosynthetic efficiency (Fv/Fm) decreased proportionally. However, in the presence of light, changes induced in the Fv/Fm were completely different. Rising CO₂ levels (up to 40%; 1000 times higher than the atmospheric concentration) had a protective role to the photosynthetic apparatus, expressed with higher Fv/Fm values (Figure 3), in contrast to the light-induced stress effect that appeared in treatments without any additional
CO₂ (Figure 3). The above-mentioned protective mechanism induced by elevated CO₂ allowed microalgae to grow even at high light intensities (400 μmol m⁻² s⁻¹), inducing a new functional symmetry. That was impossible under ambient CO₂ concentration. CO₂ concentrations at 60% and higher were not ideal for the microalgae, since they accounted for an additional stress factor in each tested light intensity, except the 100 μmol m⁻² s⁻¹. This light intensity seems to be the only one suitable for microalgal adaptation, even at 60% CO₂, since the Fv/Fm values were maintained to the levels of 40% CO₂ and were higher than the corresponding values in the treatment with ambient CO₂ concentration. Lower intensities (<100 μmol m⁻² s⁻¹) were not enough for efficient use of the extremely high CO₂ concentration (60%), while higher intensities (>100 μmol m⁻² s⁻¹), in combination with the excess of CO₂, caused further stress effects.

For the analytical parameters of the fluorescence induction measurements (OJIP-test) presented in Figure 4, three different states were chosen in order to study the combined effect of light and CO₂ concentration in closed cultivation systems of Chlorella vulgaris cultures: (a) the ambient CO₂ state at 0.04% CO₂; (b) the concentration of 30% CO₂, with the most protective effect against high light intensities (Figure 3); and (c) the extremely high concentration of 60% CO₂, where the CO₂ stress on the photosynthetic apparatus was detected. In the absence of light, obvious signs of stress were observed in the presence of high CO₂ concentrations. In contrast, there was an improvement of the functionality of the photosynthetic apparatus under light conditions combined with high CO₂ levels (30%). Briefly, the density of the reaction centers (RC/CS½) increased and the size of the
antenna per active reaction center (ABS/RC) decreased in the treatments with 30% CO\textsubscript{2} compared to the corresponding treatments with ambient (0.04%) and extremely high (60%) CO\textsubscript{2} concentrations. These changes improved the primary photochemistry (PSI\textsubscript{0}) and decreased the non-photochemical energy dissipation per active reaction center (DI\textsubscript{0}/RC), which led to an increased photosynthetic performance (PI\textsubscript{(abs)}) (Figure 4).

Figure 4. Changes in RC/CS\textsubscript{0}, ABS/RC, PSI\textsubscript{0}, DI\textsubscript{0}/RC and PI\textsubscript{(abs)} (OJIP test) over the 6 days of incubation time compared to the corresponding values of day 0, under different light intensities (0–400 μmol m\textsuperscript{-2} s\textsuperscript{-1}) in an air atmosphere with 0.04% CO\textsubscript{2}, 30% CO\textsubscript{2} and 60% CO\textsubscript{2}.

Comparing and combining all the above-mentioned results, the high concentration of 30% CO\textsubscript{2} was perfectly managed by the microalgal photosynthetic mechanism up to the light intensity of 100 μmol m\textsuperscript{-2} s\textsuperscript{-1}. Higher light intensities were not so beneficial...
for the microalga, since the non-photochemical energy dissipation was increased, mainly in the first incubation day. After that incubation time interval, there was some adaptation signs, which could permit the microalgal survival in high light intensities (up to 400 μmol m\(^{-2}\) s\(^{-1}\)), and high CO\(_2\) concentrations (up to 60%) (Figure 4). It is worth mentioning that the stress effect that appeared in the photosynthetic apparatus in 60% CO\(_2\) was quenched in the light intensity of 100 μmol m\(^{-2}\) s\(^{-1}\), compared to lower and higher light intensities, meaning that the green microalga *Chlorella vulgaris* could also survive in extremely high CO\(_2\) concentrations, changing its bioenergetic strategy.

3.3. Photosynthetic and Respiratory Activities under Different Light Intensities and CO\(_2\) Concentrations

The photosynthetic and respiratory activities of the microalga *Chlorella vulgaris*, grown in closed systems under a combination of six different light intensities and six different CO\(_2\) concentrations, were calculated (Figure 5). It was proven that the photosynthetic rate, expressed as μL O\(_2\) (μL PCV)\(^{-1}\) h\(^{-1}\), was almost six times higher at extreme CO\(_2\) levels (from 20% to 60%) compared to the ambient levels (0.04%) (Figure 5). In the case that the photosynthetic activity was expressed per chlorophyll content, the corresponding values at extreme CO\(_2\) levels also increased almost eight times with the same trend (data not shown). Furthermore, taking into consideration the overall results for each individual light intensity, it was clearly shown that the maximal photosynthetic rate was reached at light intensities of about 100–200 μmol m\(^{-2}\) s\(^{-1}\). Higher light intensities induced photoinhibition (Figure 4) and decreased the photosynthetic activity (Figure 5). It is remarkable that extreme CO\(_2\) concentrations strongly induced the photosynthetic activity (Figure 5) at low light intensities of 10–50 μmol m\(^{-2}\) s\(^{-1}\), while the measurements were definitely better at the high light intensities (100–200 μmol m\(^{-2}\) s\(^{-1}\)), highlighting the extremely high photosynthetic sensitivity/efficiency of this microalga and its increased demands for CO\(_2\) consumption. *Chlorella vulgaris* is supposed to be a very adaptable green microalga that survives in adverse conditions, changing its bioenergetic strategy, depending on the best combination of CO\(_2\) concentration and light intensity.

![Figure 5](image-url)  
*Figure 5*. Photosynthetic and respiratory activities of microalgae expressed in μL O\(_2\) μL PCV\(^{-1}\) h\(^{-1}\) cultivated in different light intensities (0–400 μmol m\(^{-2}\) s\(^{-1}\)) in an air atmosphere with different CO\(_2\) concentrations (0.04–60%) (p < 0.05 for each light intensity, each CO\(_2\) concentration and each day).

Kinetics of total O\(_2\) quantities per culture during the entire incubation time are presented in Figure 6. The trend of the measurements confirmed the above-mentioned photosynthetic activity results. Even though significant amounts of O\(_2\) were produced at very low light intensities (10 μmol m\(^{-2}\) s\(^{-1}\)) over the incubation period of six days, the maximum O\(_2\) production was reached at a light intensity of about 100–200 μmol m\(^{-2}\) s\(^{-1}\). Increasing
CO₂ concentrations improved photosynthetic O₂ production, even at extreme CO₂ values. Treatments with 30–40% CO₂ yielded the optimal results in our closed cultivation systems (Figure 6). Notably, in higher light intensities, 20% CO₂ was consumed and converted to O₂ in almost 2–3 days, while by the end of the 4–6 days of incubation, concentrations as high as 40% CO₂ were almost completely converted to O₂.

![Figure 6](image-url)

**Figure 6.** Kinetics of total O₂ production per microalgal culture over the incubation time under different light intensities (0–400 μmol m⁻² s⁻¹) in an air atmosphere with different CO₂ concentrations (0.04–60%). (A): 0 μmol m⁻² s⁻¹, (B): 10 μmol m⁻² s⁻¹, (C): 50 μmol m⁻² s⁻¹, (D): 100 μmol m⁻² s⁻¹, (E): 200 μmol m⁻² s⁻¹ and (F): 400 μmol m⁻² s⁻¹ (p < 0.05 for each light intensity and each day).

3.4. Microalgal Biomass Production under Different Light Intensities and CO₂ Concentrations

The microalgal biomass concentration under several light intensities and CO₂ concentrations is presented in Figure 7. The results clearly show that the optimal biomass increase (about four times higher than the initial cell biomass) was recorded at *Chlorella vulgaris* cultures, which were incubated in closed cultivation systems, when exposed to light intensities higher than 50 μmol m⁻² s⁻¹ and CO₂ concentrations of about 30–40% CO₂. Increasing the light intensity to 100 μmol m⁻² s⁻¹, 200 μmol m⁻² s⁻¹ and 400 μmol m⁻² s⁻¹ led to a slight improvement of biomass productivity, whereas increasing the CO₂ to 60% CO₂ limited the microalgal biomass production (Figure 7). This extreme CO₂ concentration (60%) changed the bioenergetic management of *Chlorella vulgaris* cultures. The microalgae limited the energy yields for growth (Figure 7), in order to face the extra stress (Figures 3 and 4). Oppositely, 30–40% CO₂ concentrations were ideal for the closed cultivation systems used...
in this contribution, leading to the best growth (Figure 7) and photochemistry (Figures 3–6), mainly to high light intensities (100–200 μmol m⁻² s⁻¹). Lower CO₂ concentrations did not seem to satisfy Chlorella vulgaris demands, leading to a limited functionality of the photosynthetic apparatus and limited growth.

To evaluate the quality of the biomass produced, we examined the levels of carbohydrates and lipids. The microalgae content in carbohydrates and lipids was measured in the three different “carbon states” mentioned above (0.04% CO₂–30% CO₂–60% CO₂) under different light conditions (Tables 1 and 2). In the absence of light, microalgae did not have any exploitable external energy source, thus they supplied their demand by breaking down their cellular stocks in carbohydrates and lipids, and therefore lower values were recorded. The same tendency was observed in all CO₂-deprived cultures at all tested light intensities. On the contrary, optimizing the CO₂ concentration at 30%, led to an accumulation of carbohydrates at approximately 30–37% of dry weight in the first incubation day (Table 1). No significant changes in the cellular lipid level between the treatments were observed, even under high light conditions (Table 2). Interestingly, pushing CO₂ levels up to 60% led to an accumulation of lipids increase of up to 19% of dry weight in the first incubation day under high light intensities (Table 2). This observation could be attributed to the microalgal attempt to face the excess stress caused by the extremely high CO₂ concentration of 60%. Biomass increase was restricted, due to the lipid accumulation for saving energy in a more valuable form.
### Table 1. Percentage (% dry weight) of total carbohydrate content over 0, 1, 3 and 6 days of incubation under 0.04% CO₂, 30% CO₂ and 60% CO₂ under different light intensities (0–400 µmol m⁻² s⁻¹).

| [CO₂]       | Light Intensity | Carbohydrates (% DW) ± SE | Day 0 | Day 1 | Day 3 | Day 6 | Carbohydrates (mg/L) ± SE | Day 0 | Day 1 | Day 3 | Day 6 |
|-------------|-----------------|---------------------------|-------|-------|-------|-------|---------------------------|-------|-------|-------|-------|
| 0%          | 0 µmol m⁻² s⁻¹   | 24.0 ± 1.0                | 17.9 ± 0.5 | 15.2 ± 1.2 | 13.5 ± 0.7 | 42.2 ± 1.8 | 31.5 ± 0.9 | 26.7 ± 2.0 | 237 ± 1.1 |
| 10 µmol m⁻² s⁻¹ | 24.0 ± 1.0       | 17.9 ± 0.5                | 8.6 ± 0.4  | 10.2 ± 0.2 | 42.2 ± 1.8 | 31.5 ± 1.8 | 23.1 ± 0.7 | 30.5 ± 0.2 |
| 50 µmol m⁻² s⁻¹ | 24.0 ± 1.0       | 14.6 ± 0.9                | 11.9 ± 1.0 | 11.3 ± 1.1 | 42.2 ± 1.8 | 26.1 ± 1.5 | 28.8 ± 2.5 | 30.5 ± 3.0 |
| 100 µmol m⁻² s⁻¹ | 24.0 ± 1.0      | 17.1 ± 1.2                | 12.5 ± 2.2 | 12.1 ± 1.0 | 42.2 ± 1.8 | 30.0 ± 2.2 | 26.4 ± 4.6 | 27.7 ± 2.2 |
| 200 µmol m⁻² s⁻¹ | 24.0 ± 1.0      | 20.1 ± 2.2                | 18.5 ± 0.4 | 18.4 ± 1.8 | 42.2 ± 1.8 | 35.3 ± 3.9 | 35.9 ± 10 | 38.8 ± 3.7 |
| 400 µmol m⁻² s⁻¹ | 24.0 ± 1.0      | 11.9 ± 0.4                | 8.6 ± 0.1  | 10.0 ± 0.1 | 42.2 ± 1.8 | 20.9 ± 0.8 | 16.8 ± 0.3 | 21.0 ± 0.4 |

### Table 2. Percentage (% dry weight) of total lipid content over 0, 1, 3 and 6 days of incubation under 0.04% CO₂, 30% CO₂ and 60% CO₂ under different light intensities (0–400 µmol m⁻² s⁻¹).

| [CO₂]       | Light Intensity | Lipids (% DW) ± SE | Day 0 | Day 1 | Day 3 | Day 6 | Lipids (mg/L) ± SE | Day 0 | Day 1 | Day 3 | Day 6 |
|-------------|-----------------|-------------------|-------|-------|-------|-------|-------------------|-------|-------|-------|-------|
| 0%          | 0 µmol m⁻² s⁻¹   | 8.90 ± 0.4        | 13.1 ± 0.2 | 11.1 ± 0.1 | 23.2 ± 0.8 | 15.6 ± 0.7 | 23.0 ± 0.4 | 19.5 ± 0.1 |
| 10 µmol m⁻² s⁻¹ | 13.2 ± 0.6      | 14.2 ± 0.2        | 9.60 ± 1.0 | 9.70 ± 0.2 | 23.2 ± 0.8 | 25.1 ± 0.3 | 16.9 ± 1.8 | 19.2 ± 0.4 |
| 50 µmol m⁻² s⁻¹ | 13.2 ± 0.6      | 12.4 ± 0.5        | 10.3 ± 0.2 | 8.50 ± 0.3 | 23.2 ± 0.8 | 20.3 ± 1.2 | 21.8 ± 0.7 | 25.1 ± 1.3 |
| 100 µmol m⁻² s⁻¹ | 13.2 ± 0.6     | 13.5 ± 0.6        | 13.5 ± 0.7 | 10.6 ± 0.7 | 23.2 ± 0.8 | 23.7 ± 1.0 | 28.5 ± 1.5 | 24.2 ± 1.6 |
| 200 µmol m⁻² s⁻¹ | 13.2 ± 0.6     | 13.3 ± 0.6        | 11.0 ± 0.1 | 9.30 ± 0.1 | 23.2 ± 0.8 | 23.4 ± 1.0 | 21.3 ± 0.3 | 19.7 ± 0.1 |
| 400 µmol m⁻² s⁻¹ | 13.2 ± 0.6     | 13.7 ± 1.0        | 10.5 ± 0.4 | 8.20 ± 0.7 | 23.2 ± 0.8 | 24.1 ± 1.7 | 20.4 ± 0.8 | 17.2 ± 1.5 |

### 4. Discussion

The present study examined the metabolic and functional differentiation of the green alga *Chlorella vulgaris* under various light intensities and extreme CO₂ concentrations. The results verified the ability of this species to modify hostile CO₂ atmospheres by converting CO₂ to O₂ through the photosynthetic management of solar radiation, for the benefit of the organism, producing a microalgal biomass rich in carbohydrates and lipids.

In the current study, we found that high CO₂ concentrations, even at extreme levels up to 40%, not only increased biomass yield of *Chlorella vulgaris*, but also protected the photosynthetic mechanism against photoinhibition and photodamage. Consequently, the microalga *Chlorella vulgaris* is a species that does not only tolerate extremely high CO₂ levels, but also, extremely high CO₂ levels (up to 1500 times higher concentration than the ambient atmospheric CO₂ concentration) strongly increase its photosynthetic activity.
Additionally, the microalgal culture quadrupled its biomass in just 5–6 days without any accumulation per culture (Tables 1 and 2). The importance of the present study lies in the fact that the microalga Chlorella vulgaris is a photosynthetic microorganism [60]. Most importantly, these systems aim for constant O₂ Systems (ECLSS) for water recovery, air revitalization and oxygen generation [59].

The potentiality of this photosynthetic system for CO₂ mitigation showed clearly that today’s Earth atmospheric CO₂ levels are not the optimal habitat for Chlorella vulgaris. In this work, we report optimal growth at extreme CO₂ concentrations (30–40%) with inhibitory effects appearing first at 60% CO₂, where a completely different bioenergetic strategy was used by the microalga in order to face the extra stress effect caused by the extreme CO₂ concentration. In a hermetically sealed cultivation system similar to ours, Papazi et al. [23] found that Chlorella minutissima grew best at around 25% CO₂, verifying the CO₂ tolerance of Chlorella sp. [23]. However, Chlorella minutissima was evidently stressed at 40% CO₂, while Chlorella vulgaris did not show any stress effects at that level [23]. Since Chlorella vulgaris demands extremely high CO₂ concentrations in order to yield the best biomass production and mitigates up to 288 L CO₂ L PCV⁻¹ day⁻¹, it is ideal for direct biological CO₂ capture from industrial fuel gases, such as in coal plants that usually generate 10–20% CO₂ fumes, and other similar applications [56].

In our experiments, in light intensities above 50 µmol m⁻² s⁻¹, CO₂ concentrations up to 30% and 40% were almost completely converted to O₂ in 2–3 and 4–6 days, respectively. Additionally, the microalgal culture quadrupled its biomass in just 5–6 days without any additional energy input. The microalgal biomass consists of relatively high amounts of carbohydrates and lipids, ideal for biofuel production, without significant differences in the cellular concentrations (20–37% carbohydrates and 13–19% lipids), considering light intensity and CO₂ concentration (Tables 1 and 2). However, extreme CO₂ concentrations led to a strong increase in carbohydrate (about 3.5 times higher amount in 3 days) and lipid (about 2.5 times higher amount in 3 days) accumulation per culture (Tables 1 and 2).

Large scale cultivation of Chlorella vulgaris could bring valuable biomass yield, while also solving environmental problems, mainly CO₂-driven climate change. The aforementioned is in agreement with recent techno-economic analyses and life-cycle assessments of microalgae-based production systems, suggesting the necessity of using the microalgal biomass in an integrated biorefinery system from which all the valuable components are extracted and utilized [57].

It is known that the annual global CO₂ emissions since 2000 consist of 34.07 × 10⁹ t CO₂ from fossil fuel and industry emissions, and 5.86 × 10⁹ t CO₂ from land-use change emissions. As a result, the total CO₂ emissions are 39.93 × 10⁹ t/year or 0.11 × 10⁹ t/day [58]. The importance of the present study lies in the fact that the microalga Chlorella vulgaris is not only resistant to extremely high CO₂ concentrations, but actually requires them for intense photosynthetic activity without any stress induction. These data highlight that this particular microalga is a unique tool for solving global warming. If we consider its excellent photosynthetic activity (12 L O₂ L PCV⁻¹ h⁻¹ or 288 L O₂ L PCV⁻¹ day⁻¹) when exposed to 20 or 30% CO₂, and the ratio of 1 L CO₂ gas = 0.001812 kg CO₂, a theoretical huge Chlorella vulgaris culture of 210 Km³ (210 × 10⁹ m³) consisting of a cell density of 1 µL PCV/mL culture could mitigate the total global CO₂ emissions, converting them to O₂ and valuable biomass.

In recent decades, space missions have seen revolutionary progress, and sending humans to other planets, such as Mars, is now a realistic and most anticipated goal. NASA is already equipped with, and constantly updates, Environmental Control and Life Support Systems (ECLSS) for water recovery, air revitalization and oxygen generation [59]. In addition to that, bioregenerative life-support systems (BLSS) are developed with the aim of continuously recycling the above resources, while generating food via oxygenic photosynthetic microorganisms [60]. Most importantly, these systems aim for constant O₂ regeneration from CO₂-rich atmospheres [61], in which the results of the present contribution may seem helpful. Chlorella vulgaris is a photosynthetic microalga that demands extreme CO₂ concentrations for its metabolism, having the ability to convert a CO₂-rich, hostile-for-humans atmosphere to a hospitable O₂-rich atmosphere. As illustrated in Figure 5, the maximal rate of O₂ production happened in the treatments with CO₂ concentrations of 20% and more, under a light intensity of 100–200 µmol m⁻² s⁻¹, and reached a
value of about 288 L O₂ L PCV⁻¹ day⁻¹. These results could be extrapolated and compared with a regular human’s oxygen requirements (about 590 L O₂ day⁻¹) [62], which could be completely covered from a Chlorella vulgaris culture consisting from approximately 2 L PCV microalgae. The continuous recycling of a human settlement’s atmosphere in another planet, such as Mars, in which O₂ is consumed and CO₂ is produced continuously, could be provided by a closed cultivation system of Chlorella vulgaris, creating an efficient and sustainable O₂ regeneration system for cosmonauts and even settlers on other planets.

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