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# Comparison of the Photoautotrophic Growth Regimens of *Chlorella sorokiniana* in a Photobioreactor for Enhanced Biomass Productivity

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**Abstract:** Microalgae have a wide industrial potential because of their high metabolic diversity and plasticity. Selection of optimal cultivation methods is important to optimize multi-purpose microalgal biotechnologies. In this research, *Chlorella sorokiniana* AM-02 that was isolated from a freshwater lake was cultured under various high photosynthetic photon flux density (PPFD) conditions and CO<sub>2</sub> gas levels in standard Bold's basal medium (BBM). Furthermore, a wide range of nitrate levels (180–1440 mg L<sup>-1</sup>) was tested on the growth of *C. sorokiniana*. Microalgae growth, pigment concentration, medium pH, exit gas composition, as well as nitrate, phosphate, and sulfate levels were measured during an experimental period. The preferred high PPFD and optimal CO<sub>2</sub> levels were found to be 1000–1400 μmol photons m<sup>-2</sup> s<sup>-1</sup> and 0.5–2.0% (v/v), respectively. The addition of nitrate ions (up to 1440 mg L<sup>-1</sup>) to the standard growth medium increased final optical density (OD<sub>750</sub>), cell count, pigment concentration, and total biomass yield but decreased the initial growth rate at high nitrate levels. Our findings can serve as the basis for a robust photoautotrophic cultivation system to maximize the productivity of large-scale microalgal cultures.

**Keywords:** *Chlorella sorokiniana*; PPFD; growth; nutrient removal; photobioreactor; productivity; characterization

## 1. Introduction

Microalgae-based biotechnologies have received much attention worldwide, and they are presented as complex processes for purification of air and wastewater as well as for the production of substantial amounts of lipids, polysaccharides, proteins, commercially important pigments (such as lutein and astaxanthin), and other useful products. Pharmaceuticals for various purposes, nutraceuticals, fertilizers, biodiesel, bioethanol, and biogas are obtained from different microalgae species [1–4].

Currently, individual microalgae that are capable of adsorbing or transforming certain pollutants have become excellent candidate species for simultaneously capturing carbon dioxide and removing nutrients, hydrocarbons, pesticides, and cyanide compounds [1,5] as well as for remediation of aquatic ecosystems polluted with heavy metals [6]. The practical application of microalgae metabolic potential can be limited by certain factors, such as photoinhibition and a slow response to different light irradiance, which lead to a low yield of biomass or target products. To increase the cell growth rate, the efficiency of biomass production, and the yield of target products, which means the effectiveness of various microalgae-based technologies, light intensity, photoperiod, temperature, pH, adequacy of macro- and micronutrients, and cultivation regimen (autotrophy, heterotrophy, and mixotrophy) must be thoroughly studied [4,7–9].

Multifarious microalgae species, including algae of the genus *Chlorella* (*Chlorophyta*; *Trebouxiophyceae*; *Chlorellales*; *Chlorellaceae*), have been studied in various experimental projects to assess their potential for nutrient bioextraction, bioremediation, and bioenergy processes [7,10]. Species in the genus *Chlorella* are thought to have high potential for industrial use because of their fast and stable growth characteristics [11–13]. *Chlorella sorokiniana* is considered to be one of the promising *Chlorella* species that is used for wastewater treatments as well as for producing valuable products, including lipids, polysaccharides, proteins, and pigments [10,14–16]. *Chlorella sorokiniana* UTEX 1230 was originally isolated from a stream in Texas in 1951 by Dr. Constantine Sorokin [17], and more detailed information about this species of *Chlorella* was presented in subsequent research studies [18–20]. *C. sorokiniana* can grow effectively in a wide temperature range (25–40 °C) and under different light intensities [11,18–20].

Representatives of freshwater microalgae have the potential for large-scale cultivation. Researchers have developed various methods of cultivation depending on further practical application goals for the object that is being studied. In photoautotrophic regimens, microalgae cultures assimilate inorganic carbon, and light intensity can significantly affect CO<sub>2</sub> assimilation and biomass production [3,10,21]. Among cultivation trends for various industrially attractive microalgae, attention to heterotrophic (substrate dependent) or mixotrophic cultivation (auto- and heterotrophic) is given, which explains the choice with a higher growth rate and lower operating costs of green microalgae [7,11,15]. Although microalgae cultivation under heterotrophic and mixotrophic conditions has some advantages, this process is at risk of contamination with heterotrophic microbes because organic compounds are used as carbon and energy sources [22], and this leads to the release of carbon dioxide as one of the major greenhouse gases. The use of gaseous CO<sub>2</sub> as a carbon source by photoautotrophic microalgae contributes to sequestration of carbon dioxide, which is one way to reduce the risks that are associated with global warming [23,24]. Furthermore, when synthesis of the target products is the cell's physiological response to high light stress, it is important to develop and apply effective strategies for photoautotrophic cultivation [10]. Therefore, additional studies are needed on the characteristics of algae metabolism at different light intensities and CO<sub>2</sub> concentrations for each microalgae culture. Moreover, the isolation of new strains with high rates of nitrogen, phosphorus, and sulfur removal is important for the development of new wastewater treatment technologies.

Despite recent success in this research area, it is important to develop novel methods and optimize previous methods of growing and harvesting of microalgae so that many attractive species for biotechnology can be grown in a short time and with less corresponding energy use. *C. sorokiniana* as one of the attractive species for wastewater treatment was selected for the current study. Thus, this study evaluated the optimal high photosynthetic photon flux density (PPFD) conditions and CO<sub>2</sub> levels on the growth rate and the ability to utilize important nutrients, such as nitrate, phosphate, and sulfate, by indigenous *C. sorokiniana* AM-02 that tolerates high irradiance. In addition, based on the optimal growth parameters for *C. sorokiniana*, the influence of additional nitrogen and sulfur sources on the biomass productivity was investigated. The results presented here offer a new strategy for improving productivity and decreasing resource costs.

## 2. Materials and Methods

### 2.1. Isolation and Identification of Microalgal Strain

The microalgae culture of *Chlorella sorokiniana* AM-02 was isolated from a freshwater lake located in Kazan (Russia) in 2018. The isolated strain was made axenic by continuous sub-culturing using Bold's basal medium (BBM) [25] on agar plates supplemented with ampicillin and kanamycin (10 and 50 µg mL<sup>-1</sup> in the medium, respectively). The axenic culture was identified using molecular analysis of the ribulose biphosphate carboxylase large subunit (*rbcL*) gene and translational elongation factor Tu (*tufA*) gene markers. For this, a FastDNA spin kit (MP Biomedicals, Solon, OH, USA) and a FastPrep-24 homogenizer (MP Biomedicals, Solon, OH, USA) were used to extract DNA from microalgae in

accordance with the manufacturer's protocol. DNA was then quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) and stored at  $-20\text{ }^{\circ}\text{C}$  until use. Primers for the *rbcL* and the *tufA* genes were selected from published studies and were used to identify microalgae (Table 1). Amplicons obtained with these primers were purified using a QIAquick PCR Purification kit (Qiagen, Hilden, Germany) and sequenced on a 3730 DNA Analyzer (Thermo Fisher Scientific, Wilmington, DE, USA) according to the manufacturers' protocols. Sequencing data was confirmed by BLAST searches in the GenBank's database and using the BOLD Identification System for the ribulose biphosphate carboxylase gene. The determined *rbcL* gene and *tufA* gene sequences were deposited in the GenBank database under accession numbers MT647139 and MT647140, respectively.

**Table 1.** List of primers used in this study to identify the strain AM-02.

| Molecular Marker   | Primers             | Sequence 5' → 3'        | Reference |
|--|---------------------|-------------------------|-----------|
| <i>rbcL</i> (ribulose biphosphate carboxylase large subunit) | <i>rbcL</i> -M379 F | GGTTTCAAAGCTYTWCGTGC    | [26]      |
|  | <i>rbcL</i> LFP R   | GTAAATACCACGGCTACGRTCTT |           |
|  | Fw_rbcL_192         | GGTACTTGGACAACWGTWTGGAC | [27]      |
|  | Rv_rbcL_657         | GAAACGGTCTCKCCARCGCAT   |           |
| <i>tufA</i> (translational elongation factor Tu)             | <i>tufA</i> F       | GGNGCNGCNCAAATGGAYGG    | [28]      |
|  | <i>tufA</i> R       | CCTTCNCGAATMGCRAAWCGC   |           |

## 2.2. Cultivation Conditions

Before starting the experiments in a photobioreactor, *C. sorokiniana* was placed in 250 mL Erlenmeyer flasks containing 30 mL of BBM under sterile conditions to eliminate any contamination. The inoculum was grown with shaking at 120 rpm at  $+26\text{ }^{\circ}\text{C}$  under continuous illumination ( $150\text{ }\mu\text{mol photons m}^{-2}\text{ s}^{-1}$ ). After 5 days, the biomass was concentrated by centrifugation at  $5000\times g$  for 5 min and then used as an inoculum for experiments in a photobioreactor.

## 2.3. Cultivation in a Photobioreactor

In order to study the influence of operational factors on photoautotrophic cultivation of *C. sorokiniana* for accelerated growth and biomass production, microalga with an initial  $\text{OD}_{750}$  of 0.01 (optical density at 750 nm) was grown in a sterilized 3.6 L Labfors 4 Lux photobioreactor (Infors HT, Bottmingen, Switzerland) with a 2.4 L working volume with controlled luminous flux levels. The experiments were divided into three main groups: (1) cultivation under different photosynthetic photon flux density (PPFD) conditions, (2) cultivation at various levels of carbon dioxide, and (3) cultivation in the presence of various levels of nitrate and sulfate ions.

The photobioreactor was illuminated with 1–16 Gro-Lux tubes with high blue and red radiation (per tube: 120 lumen (lm), maximum: 1920 lm) uniformly distributed around the culture vessel. Light intensity was additionally measured using a photosynthetically active radiation meter (PAR meter, Apogee Instruments, Logan, UT, USA). In experiments on the selection of optimal lighting, we tested the growth of culture at different values of illumination. Three treatments were carried out under different PPFD conditions ( $1000$ ,  $1200$ , and  $1400\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ ). For an optimized strategy for the growth and the accumulation of biomass, we analyzed the following conditions of the carbon dioxide regimen: cultures sparged with atmospheric air only ( $\sim 0.04\%$   $\text{CO}_2$ ) and cultures sparged simultaneously with atmospheric air and continuously added  $\text{CO}_2$  (finally  $0.5\%$ ,  $1.0\%$ , and  $2.0\%$   $\text{CO}_2$  ( $v/v$ )). Aeration ( $0.55$  volume of air per volume of liquid per minute) was provided by a compressor via a  $0.45\text{ }\mu\text{m}$  filter. Carbon dioxide addition was provided by thermal mass flow meter and controller (Vögtlin Instruments, Aesch, Switzerland). They were mixed and injected into the reactor. The temperature was controlled at  $+26$ – $26.5\text{ }^{\circ}\text{C}$ . A relatively low temperature was chosen in our experiments since high temperatures lead to higher control costs and higher energy consumption of the process. The reactor was continuously stirred at 120 rpm. In all experiments, light was maintained on a 16:8 light/dark cycle. When observing the foam, a sterile  $2\%$   $v/v$  solution of antifoam (Antifoam B, Sigma-Aldrich, St. Louis,

MO, USA) was added. The experiments continued in the range of 112–232 h. Light, temperature, medium pH, pressure inside the reactor, carbon dioxide flow, and the percentage of released oxygen and carbon dioxide were measured by the Infors devices and displayed online on a computer screen.

Samples for analysis of algal growth, cell number, pigment content, and ions concentration in the culture medium were collected every day. pH as well percentage of the released molecular oxygen and carbon dioxide were measured throughout the experiments. Two independent experiments were performed to test reproducibility, and the results are presented as the mean.

#### 2.4. Measurement of Algal Growth and Biomass Dry Weight

Optical density was measured using a Lambda 35 spectrophotometer (Perkin Elmer, Singapore) at 750 nm. Cell suspensions were diluted prior to measurements to obtain a final  $OD_{750}$  of less than 0.5 for measurements. Cell number was additionally determined by using a hemocytometer. The determination was conducted in triplicate, and the mean values are presented along with standard deviations. After 112–232 h (depending on the experiment), the centrifuged biomass (at  $5000\times g$  for 10 min) was washed twice with sterile deionized water to remove medium salts and centrifuged again. The remaining microalgal pellets were dried at  $+80\text{ }^{\circ}\text{C}$  for 48 h. The final biomass yield ( $\text{g L}^{-1}$ ) was calculated as the difference between the weight of a centrifuge tube with dried microalgal pellet and the weight of the biomass-free centrifuge tube.

#### 2.5. Measurement of Pigment Concentrations

Chlorophylls a and b, carotenoids, and total pigments ( $\text{mg L}^{-1}$ ) were determined using dimethyl sulfoxide extraction and optical absorption correlation, as previously described by Chai et al. [8] and Wellburn [29]. Optical densities at 480, 649.1, and 665.1 nm were measured with a Lambda 35 spectrophotometer (Perkin Elmer, Singapore). The concentration of pigments was measured in triplicate, and the mean values are presented along with standard deviations.

#### 2.6. Ion Chromatography

Nitrate, phosphate, and sulfate concentrations were measured using a Dionex ICS-900 Ion Chromatography System (Thermo Fisher Scientific, Wilmington, DE, USA) equipped with an IonPac AG22 ( $4\times 50\text{ mm}$ ) guard column, and an IonPac AS22 ( $4\times 250\text{ mm}$ ) analytical column. The mobile phase consisted of  $4.5\text{ mM Na}_2\text{CO}_3/1.4\text{ mM NaHCO}_3$  at a flow rate of  $1.5\text{ mL min}^{-1}$ . Data acquisition and instrument control were performed with the Dionex Chromeleon software (Sunnyvale, CA, USA). Analyses were conducted in triplicate, and the mean values are presented with standard deviations.

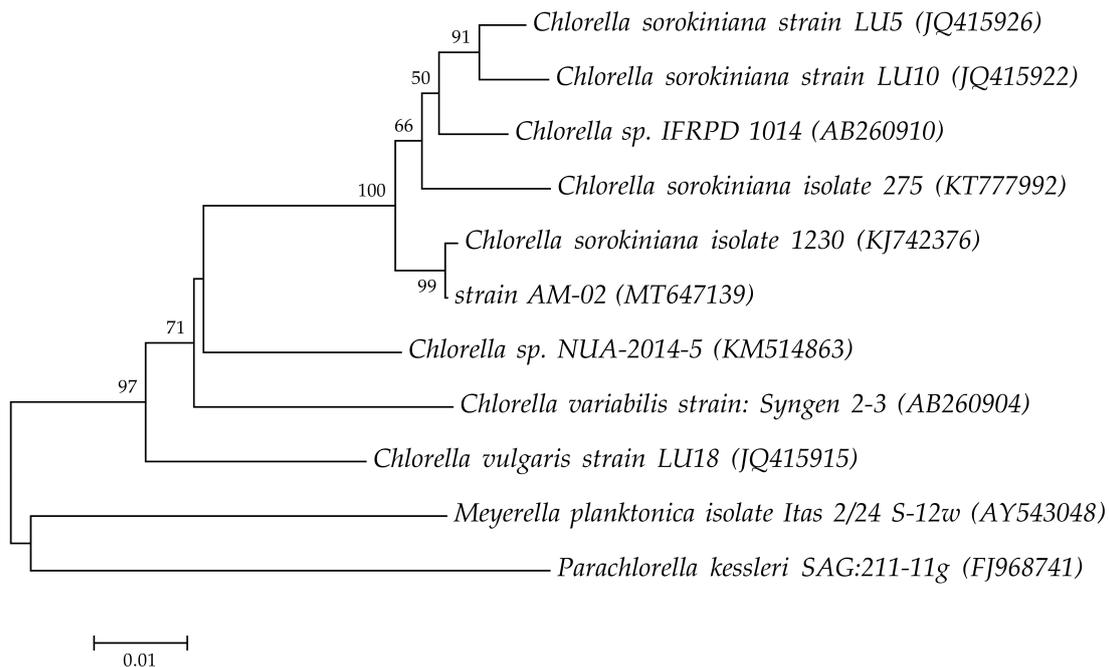
#### 2.7. Data Analysis

The effects of PPF,  $\text{CO}_2$ , and nitrate concentration values on the photoautotrophic growth of *C. sorokiniana* were evaluated. The influence of these parameters on microalgal biomass was significant when the  $p$  value was below 0.05 (based on a two-tailed  $t$  test).

### 3. Results and Discussion

#### 3.1. Effects of PPF and $\text{CO}_2$ Concentration Values on Growth of Algae

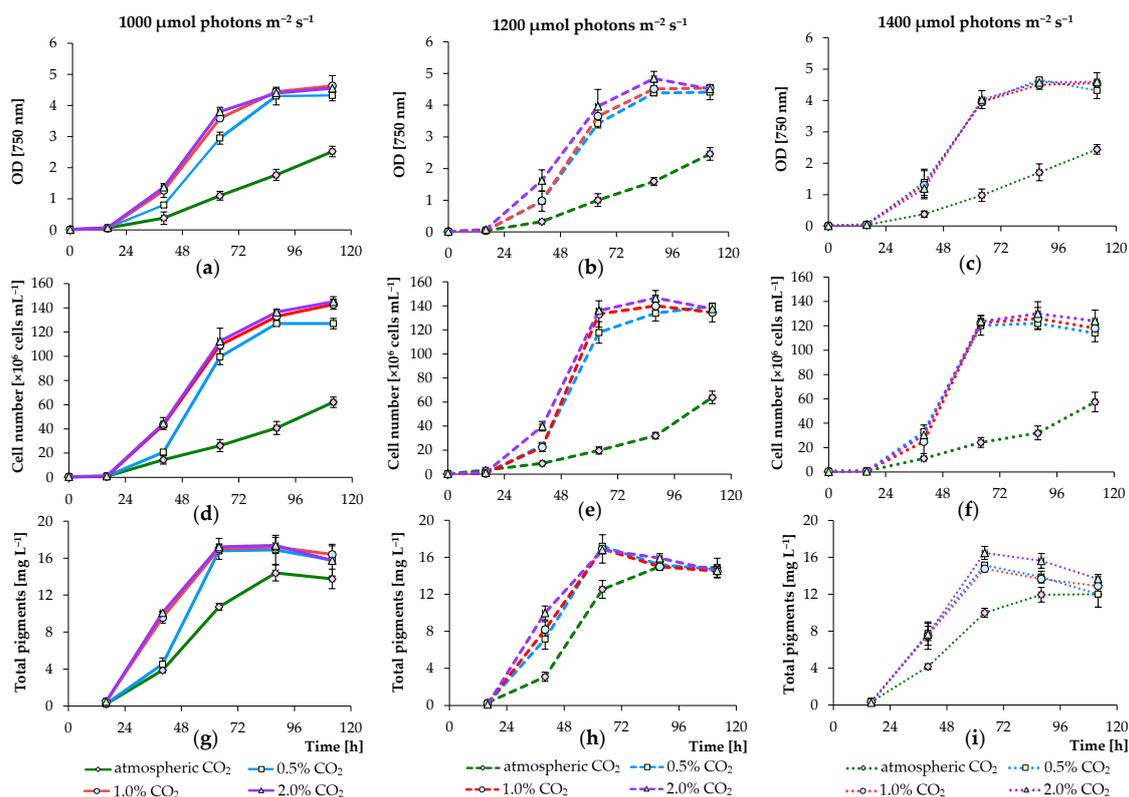
In this research study, various PPF and  $\text{CO}_2$  concentration values as well as nutrient requirements for maximum growth and biomass accumulation of *C. sorokiniana* AM-02 were determined. Phylogeny of the strain AM-02 based on *rbcL* gene is presented in Figure 1. Based on these data, this strain was assigned to *C. sorokiniana*.



**Figure 1.** Phylogenetic tree based on *rbcL* gene sequences. Analysis was conducted in MEGA7 [30] using the neighbor-joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method. *Meyerella planktonica* and *Parachlorella kessleri* were used as outgroup.

To control the algal growth,  $OD_{750}$  was measured and number of cells was counted (results are summarized in Figure 2). Three high PPFD conditions were chosen (1000, 1200, and 1400  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) for the experiments. We chose high PPFD conditions, because we plan to continue testing the growth of this strain in digested agricultural waste materials (such as pig manure and chicken manure), which are dark in color. In experiments, when cultures were bubbled with air supplemented with final 0.5%, 1.0%, and 2.0%  $\text{CO}_2$  (for all tested PPFD conditions), a 1.7–2.4-fold increase in growth in standard BBM was observed compared to that detected in treatments with non- $\text{CO}_2$  augmented cultures. The mean  $OD_{750}$  values of *C. sorokiniana* grown at 1000, 1200, and 1400  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and sparged with air after 112 h of cultivation were 2.52, 2.46, and 2.44, respectively, whereas in experiments with the addition of extra  $\text{CO}_2$ , the growth values were above 4.3 (Figure 2a–c).

The cultures in experiments supplied with different concentrations of carbon dioxide (under different PPFD conditions) increased in cell number until stationary growth was achieved at  $1.22\text{--}1.46 \times 10^8$  cells  $\text{mL}^{-1}$  (after 88 h), while in treatments supplied with atmospheric carbon dioxide, the cultures increased in cell number to  $5.7\text{--}6.4 \times 10^7$  cells  $\text{mL}^{-1}$  only (after 112 h) (Figure 2d–f). Overall,  $OD_{750}$  correlated with the calculated cell numbers (Figure 2). The concentration of total pigments (chlorophyll a, b, and total carotenoids) also increased to 14–17  $\text{mg mL}^{-1}$  with higher accumulation in treatments supplemented with 2% carbon dioxide but decreased thereafter (Figure 2g–i).



**Figure 2.** Growth of *C. sorokiniana* AM-02 (OD<sub>750</sub> (a–c) and cell number (d–f) and total pigment concentration in cells (g–i) cultured under various light and CO<sub>2</sub> regimens (1000, 1200, and 1400 μmol photons m<sup>-2</sup> s<sup>-1</sup> and atmospheric CO<sub>2</sub>, 0.5, 1.0, and 2.0% CO<sub>2</sub>) in standard Bold’s basal medium (BBM).

Table 2 presents the tested regimens of cultivation and the average yield of dry biomass of microalgae after photoautotrophic cultivation. As can be seen from the cell density, cell number, average biomass yield of dry microalgae, as well as pigment content, the growth had the similar trends under all lighting conditions with an additional CO<sub>2</sub> supply; however, the growth of *C. sorokiniana* was slightly higher in experiments in which PPF and CO<sub>2</sub> concentration values were 1200 μmol photons m<sup>-2</sup> s<sup>-1</sup> and 2.0%, respectively. In this regard, we used cultures grown at 1200 μmol photons m<sup>-2</sup> s<sup>-1</sup> and sparged with 2.0% carbon dioxide for further selection of optimal nutrient concentrations in modified BBM. At 1000 μmol photons m<sup>-2</sup> s<sup>-1</sup> and 0.5% CO<sub>2</sub>, the lower growth rate was additionally observed (in treatments with a changing level of CO<sub>2</sub>). However, at 1400 μmol photons m<sup>-2</sup> s<sup>-1</sup>, the growth was almost identical regardless of the level of CO<sub>2</sub> addition (0.5%, 1.0%, or 2%). It is believed that, from an industrial point of view, transportation, storage, and delivery of gaseous carbon dioxide are expensive for large-scale algae production [12]. Therefore, the cultivation of *C. sorokiniana* AM-02 on an industrial scale can also be achieved with lower levels of CO<sub>2</sub> supplementation.

In several previous studies, other PPF conditions (100–500 μmol photons m<sup>-2</sup> s<sup>-1</sup>) were tested for culturing other *C. sorokiniana* strains in flasks and bottles, and no significant differences were found between different conditions for photoautotrophic cultures [6,11,13,31]. Due to the shading of the cells one above the other, the design of photobioreactors is also important to ensure adequate light distribution, as reported by Leon-Vaz et al. [32]. An increase in light intensity is not a solution to overcome shading in many photoautotrophic cultures with a high cell density, since many microalgae cannot grow efficiently at extremely high light intensities. However, Cuaresma et al. [33] reported a high growth rate of *C. sorokiniana* CCAP 211/8K under continuous illumination of 2100 μmol photons m<sup>-2</sup> s<sup>-1</sup> using red light emitting diodes. In our research, we show that our strain has optimal cultivation conditions in standard BBM at a high surface irradiance between 1000–1400 μmol photons m<sup>-2</sup> s<sup>-1</sup> with Gro-Lux tubes in a 3.6 L photobioreactor with a working volume of 2.4 L.

**Table 2.** The average dry weight levels of algal biomass at the end of the experiments.

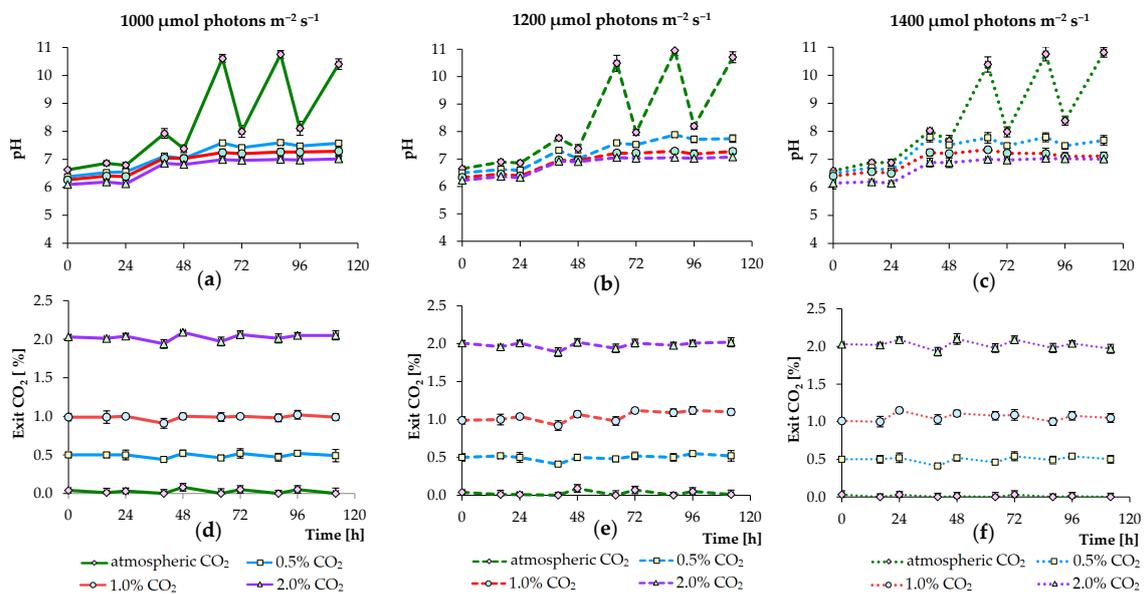
| PPFD ( $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) | Regimen  |                           | Dry Weight ( $\text{g L}^{-1}$ ) |
|---|--|---------------------------|----------------------------------|
|   | Initial $\text{NO}_3^-$ ( $\text{mg L}^{-1}$ ) | Initial $\text{CO}_2$ (%) |                                  |
| 1000  | 180  | ~0.04                     | $0.43 \pm 0.02$                  |
| 1000  | 180  | 0.5                       | $1.07 \pm 0.03$                  |
| 1000  | 180  | 1.0                       | $1.08 \pm 0.02$                  |
| 1000  | 180  | 2.0                       | $1.02 \pm 0.01$                  |
| 1200  | 180  | ~0.04                     | $0.45 \pm 0.02$                  |
| 1200  | 180  | 0.5                       | $1.02 \pm 0.02$                  |
| 1200  | 180  | 1.0                       | $1.08 \pm 0.04$                  |
| 1200  | 180  | 2.0                       | $1.10 \pm 0.03$                  |
| 1400  | 180  | ~0.04                     | $0.44 \pm 0.01$                  |
| 1400  | 180  | 0.5                       | $1.05 \pm 0.02$                  |
| 1400  | 180  | 1.0                       | $1.08 \pm 0.01$                  |
| 1400  | 180  | 2.0                       | $1.07 \pm 0.02$                  |
| 1200  | 180  | 2.0                       | $1.10 \pm 0.03$                  |
| 1200  | 360  | 2.0                       | $1.76 \pm 0.08$                  |
| 1200  | 720  | 2.0                       | $2.53 \pm 0.12$                  |
| 1200  | 1080   | 2.0                       | $3.11 \pm 0.21$                  |
| 1200  | 1440   | 2.0                       | $3.45 \pm 0.24$                  |

PPFD: photosynthetic photon flux density.

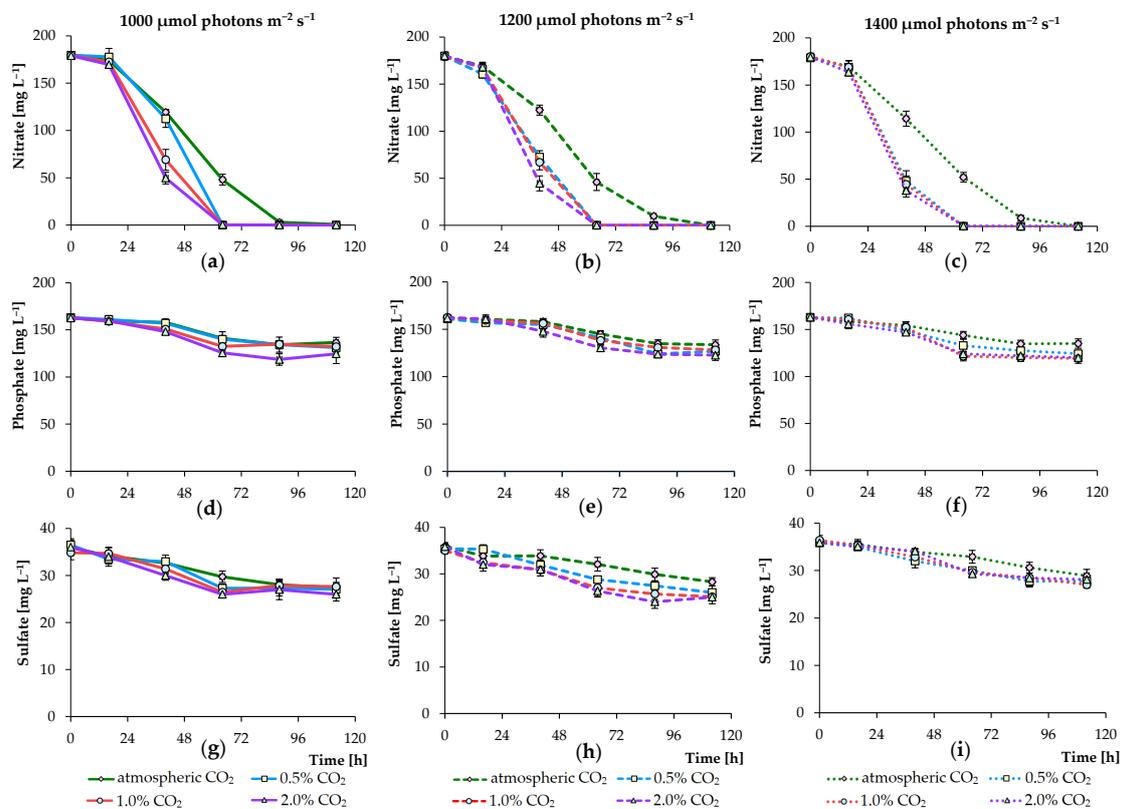
Figure 3 demonstrates the changes in pH and the percentage of released  $\text{CO}_2$  under selected conditions. Thus, the pH increased from an initial ~6.6 to 10.4–10.9 under all lighting conditions in the non- $\text{CO}_2$  augmented cultures (autotrophic cultivation leads to hydroxyl formation, which increases the pH), whereas the pH initially dropped to values 6.1–6.4 but ultimately did not increase above 7.8, 7.3, and 7.0 under all lighting conditions with the addition of  $\text{CO}_2$  up to 0.5%, 1.0%, and 2.0%, respectively (Figure 3a–c). During the light period, pH increased, while cultivation in the dark period led to a slight decrease in pH (in  $\text{CO}_2$  augmented cultures). In the non- $\text{CO}_2$  augmented cultures, pH decreased substantially during the dark period. In the case of  $\text{CO}_2$  release from the photobioreactor, a decrease and an increase in the  $\text{CO}_2$  level were observed during the light period and the dark period under all lighting conditions, respectively (Figure 3d–f). The increase in pH found in the culture medium can be explained by the growth of *C. sorokiniana* and the uptake of carbon from dissolved  $\text{HCO}_3^-$ , leaving  $\text{OH}^-$  ions. Similarly, higher pH levels in the non- $\text{CO}_2$  augmented cultures can be attributed to lower dissolved  $\text{CO}_2$  and less dissociated  $\text{H}^+$  ions [7,34]. In addition, sodium nitrate nutrition leads to an increase in pH of the medium [35].

Figure 4 shows the nutrient levels in the BBM with nitrate levels of about  $180 \text{ mg L}^{-1}$  at the beginning of cultivation. After 64 h and 88 h, nitrogen was completely utilized in standard BBM in the  $\text{CO}_2$  augmented and the non- $\text{CO}_2$  augmented cultures, respectively, and, therefore, decreases in the growth of the microalgae cultures and the pigment concentration were noted. It should also be reported that nitrate, orthophosphate, and sulfate were consumed faster by culture of *C. sorokiniana* AM-02 when air containing 2.0%  $\text{CO}_2$  was continuously bubbled into the reactor. Ion chromatography analysis demonstrated that the added amounts of phosphate (around  $160 \text{ mg L}^{-1}$ ) and sulfate (around  $35 \text{ mg L}^{-1}$ ) slightly decreased but were in excess during the entire experimental period, while the nitrate concentration sharply decreased, and  $\text{NO}_3^-$  completely was utilized after 64–88 h of cultivation (Figure 4). Thus, the concentration of  $\text{NO}_3^-$  was initially insufficient for the active growth and development of the culture in photoautotrophic treatments; therefore, the growth values were low, and the following experiments were performed to identify the effects of increasing  $\text{NO}_3^-$  content in the culture medium. These data confirm several reports in the literature on the rapid growth and rate of removal of nutrients but under other, different conditions [11]. In addition, some previous studies have demonstrated that various other *C. sorokiniana* strains can grow in wastewater with the effective

removal of nitrates and phosphates [34,36], providing high potential for their application in developing wastewater treatment technologies.



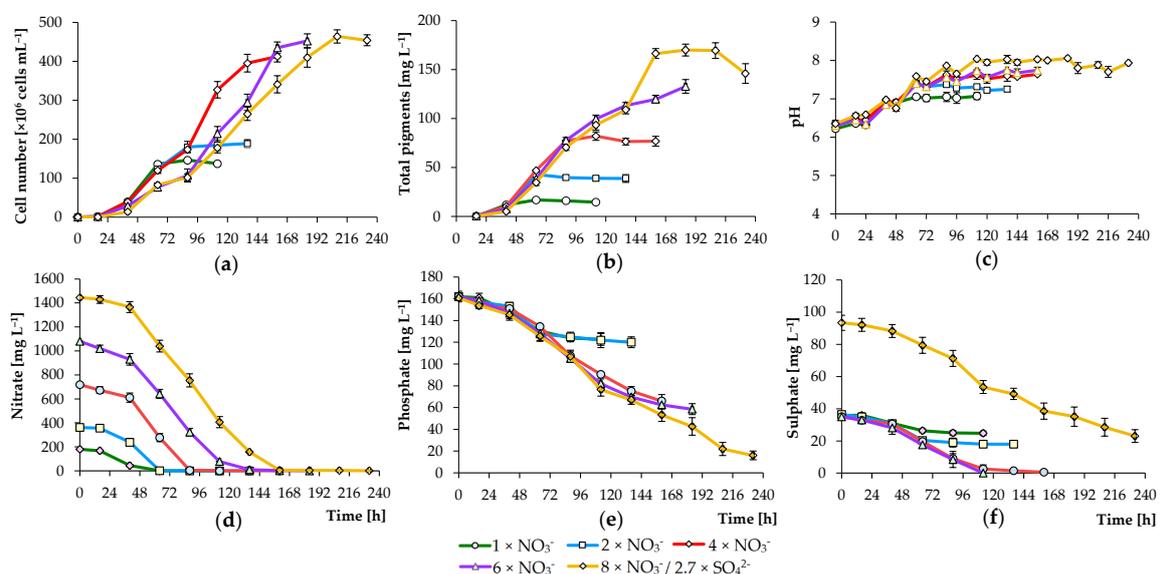
**Figure 3.** Changes in culture medium pH (a–c) and the released carbon dioxide level (d–f) observed during the growth of *C. sorokiniana* AM-02 cultured under various light and CO<sub>2</sub> regimens (1000, 1200, and 1400 μmol photons m<sup>-2</sup> s<sup>-1</sup> and atmospheric CO<sub>2</sub>, 0.5, 1.0, and 2.0% CO<sub>2</sub>) in standard BBM.



**Figure 4.** Changes in nitrate (a–c), phosphate (d–f), and sulfate (g–i) concentrations in culture medium during the growth of *C. sorokiniana* AM-02 cultured under various light and CO<sub>2</sub> regimens (1000, 1200, and 1400 μmol photons m<sup>-2</sup> s<sup>-1</sup> and atmospheric CO<sub>2</sub>, 0.5, 1.0, and 2.0% CO<sub>2</sub>) in standard BBM.

### 3.2. Effects of Nutrient Concentration on Algal Growth

In standard BBM (a medium that has been used to grow a variety of green algae cultures),  $\text{NaNO}_3$  is used as a source of nitrogen. The growth of strain AM-02 in treatments containing  $180 \text{ mg L}^{-1} \text{NO}_3^-$  (standard concentration) under the selected PPFD and  $\text{CO}_2$  concentration values ( $1200 \mu\text{mol m}^{-2} \text{s}^{-1}$  and 2%  $\text{CO}_2$ ) was low, as measured by low cells number ( $1.47 \times 10^8 \text{ cells mL}^{-1}$ ), pigment concentration ( $16.8 \text{ mg L}^{-1}$ ), and dry biomass weight ( $1.10 \text{ g L}^{-1}$ ) (Figure 5; Table 2). No  $\text{NO}_3^-$  was detected in standard BBM sparged with air containing 2.0%  $\text{CO}_2$  on 66 h and onwards. Figure 5 shows that all key parameters were improved by adding an additional level of  $\text{NO}_3^-$ . In the  $360 \text{ mg L}^{-1} \text{NO}_3^-$ -treatments (double  $\text{NO}_3^-$  concentration), number of cells, pigment concentration, and dry biomass weight increased significantly ( $p < 0.001$ ; up to  $1.9 \times 10^8 \text{ cells mL}^{-1}$ ,  $42.8 \text{ mg L}^{-1}$ , and  $1.76 \text{ g L}^{-1}$ , respectively). However, even at a double concentration of introduced  $\text{NO}_3^-$ , nitrogen began to be limited after 66 h, and the cell concentration did not exceed  $1.9 \times 10^8 \text{ cells mL}^{-1}$ . Under both conditions,  $\text{PO}_4^{3-}$  and  $\text{SO}_4^{2-}$  levels also decreased but remained abundant for culture growth (Figure 5).



**Figure 5.** Growth of *C. sorokiniana* AM-02 (a), total pigment concentration in cells (b), culture medium pH (c), as well as nitrate (d), phosphate (e), and sulfate (f) removal from the medium in different treatments with modified BBM (PPFD and  $\text{CO}_2$  concentration values:  $1200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  and 2.0%  $\text{CO}_2$ , respectively).

A further increase in the concentration of the nitrogen source to  $720 \text{ mg L}^{-1}$  of  $\text{NO}_3^-$  led to a significant increase in the number of cells and was reflected in the yield of final dry biomass ( $p < 0.001$ ; Figure 5a; Table 2). After 160 h, the culture increased in cell concentration until reaching stationary growth at  $4.13 \times 10^8 \text{ cells mL}^{-1}$ . After 112 h, the concentration of total pigments increased to  $82.1 \text{ mg mL}^{-1}$  and remained almost stable during cultivation. As shown in Figure 5b, the pigment concentrations in *C. sorokiniana* cultivated in the presence of high  $\text{NO}_3^-$  levels were generally higher than those observed in cells cultured at lower levels of nitrate ions. A clear positive correlation was observed between chlorophyll levels and the color of the microalgae culture. High concentrations of  $\text{NO}_3^-$  promoted the synthesis of pigments. Under these conditions, nitrate and sulfate were completely utilized by 88 h and 160 h, respectively (Figure 5d,f). Phosphate levels also decreased from an initial  $160$  to  $66 \text{ mg L}^{-1}$  by 160 h and remained at around these levels until the end of growth (Figure 5e). In  $6 \times \text{NO}_3^-$  and  $8 \times \text{NO}_3^-$ -treatments, the final biomass yield and pigment concentration were significantly higher than previously reported ( $p < 0.001$ ); however, the best growth rate was observed under  $4 \times \text{NO}_3^-$  conditions. Since  $\text{SO}_4^{2-}$  was completely utilized under  $4 \times \text{NO}_3^-$  and  $6 \times \text{NO}_3^-$  conditions, we increased its level to  $95 \text{ mg L}^{-1}$  in  $8 \times \text{NO}_3^-$  treatments. Under all conditions, nitrate, phosphate, and sulfate were

efficiently utilized by culture of *C. sorokiniana* (Figure 5). These results clearly demonstrate that *C. sorokiniana* AM-02 can grow at high nitrate concentrations and can be further tested for wastewater treatment. However, remarkably high  $\text{NO}_3^-$  concentrations added to the medium reduced the specific growth, which can be explained by increased osmotic stress and inhibition by the intermediate [37].

The pH increased to 7.1, 7.3, 7.6, 7.8, and 8.0 in  $1\times \text{NO}_3^-$ ,  $2\times \text{NO}_3^-$ ,  $4\times \text{NO}_3^-$ ,  $6\times \text{NO}_3^-$ , and  $8\times \text{NO}_3^-$  -treatments, respectively (Figure 5c). In the case of  $\text{CO}_2$  emission from the photobioreactor, a decrease and an increase in the  $\text{CO}_2$  level were observed during the light period and the dark period, respectively, with an increase in the  $\text{CO}_2$  level by the entire period in experiments with a high nitrogen content (from an initial 2.0% to 2.6%, data not shown). Therefore, based on our results, nitrogen and sulfur are the main nutrients that become insufficient in standard BBM, while the phosphorous level is sufficient to support the growth of microalgae. Our findings also demonstrate that high level of inorganic anions removal is possible (100% for  $\text{NO}_3^-$  and  $\text{SO}_4^{2-}$  and 91% for  $\text{PO}_4^{3-}$  (146 mg  $\text{L}^{-1}$ )), which indicates the potential use of the strain for wastewater treatment.

Nutrient availability affects the growth of photoautotrophic organisms. One of the important components affecting the growth of microalgae and physiological activity is nitrogen [14,38,39]. For numerous applications of promising photosynthetic microorganisms, an increase in biomass productivity is considered to be an important step. Various potential nitrogen sources and their concentrations, such as urea, nitrate, and ammonium ions, have been evaluated by several other researchers for the cultivation of other *C. sorokiniana* strains. Lizzul et al. [34] observed that *C. sorokiniana* UTEX1230 prefers ammonium ions rather than nitrate ions as a source of nitrogen when cultured in wastewater and BBM medium. Ramanna et al. [36] reported that urea provided the highest biomass yield during cultivation of *C. sorokiniana* strain NIES: 2173 in wastewater. Kim et al. [7] found that growth rate and nitrogen and phosphorus removal rates by *C. sorokiniana* UTEX 1670 were higher under heterotrophic conditions than under autotrophic conditions. The wastewater nitrogen and phosphate were also effectively utilized by several other *C. sorokiniana* strains [40]. Another species of the same genus, *C. vulgaris*, also effectively removed nitrogen and phosphorus from domestic wastewater [41]. In this work, *C. sorokiniana* AM-02 was isolated from a freshwater lake and therefore can be adapted to different environmental changes and thus potentially provides a competitive advantage when grown in open water.

#### 4. Conclusions

A study was conducted comparing the effect of various PPFD and  $\text{CO}_2$  concentration values on the growth of the new isolated strain *C. sorokiniana* strain AM-02. Thus, the photoautotrophic cultures of *C. sorokiniana* in modified BBM (720 mg  $\text{L}^{-1}$  of  $\text{NO}_3^-$ ) showed better growth performance. It was found that the preferred high PPFD and  $\text{CO}_2$  concentration values are 1000–1400  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  and 0.5–2.0%, respectively. In addition, the high growth of *C. sorokiniana* AM-02 makes this strain particularly suitable for the rapid production of biomass, attractive for bioremediation of aquatic environments contaminated with high levels of nitrate, phosphate, and sulfate. Moreover, our results show that adding an additional nitrogen source can significantly increase final biomass and pigment yield compared to standard BBM. Finally, our findings prove that the new isolated strain *C. sorokiniana* AM-02 is a fast-growing microalgae strain that can also be used in industry.

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