The Effects of Total Dissolved Carbon Dioxide on the Growth Rate, Biochemical Composition, and Biomass Productivity of Nonaxenic Microalgal Polyculture

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Abstract: The biosequestration of CO₂ using microalgae has emerged as a promising means of recycling CO₂ into biomass via photosynthesis, which could be used to produce biofuels as an attractive approach to CO₂ mitigation. We investigated the CO₂ fixation capability of the native nonaxenic microalgal culture using a 2 L photobioreactor operated in batch mode. The cultivation was carried out at varying concentrations of total dissolved CO₂ (Tco₂) in the bulk media ranging from 200 to 1000 mg L⁻¹ and the temperature and light intensities were kept constant. A maximum CO₂ fixation rate was observed at 400 mg L⁻¹ of Tco₂. Characteristic growth parameters such as biomass productivity, specific growth rate, maximum biomass yield, and biochemical parameters such as carbohydrate, protein, and lipids were determined and discussed. We observed that the effect of CO₂ concentration on growth and biochemical composition was quite significant. The maximum biomass productivity was 22.10 ± 2.85 mg L⁻¹ day⁻¹, and the rate of CO₂ fixation was 28.85 ± 3.00 mg L⁻¹ day⁻¹ at 400 mg L⁻¹ of Tco₂. The maximum carbohydrate (8.17 ± 0.49% dry cell weight) and protein (30.41 ± 0.65%) contents were observed at 400 mg L⁻¹, whereas the lipid content (56.00 ± 0.82% dry cell weight) was the maximum at 800 mg L⁻¹ of Tco₂ in the bulk medium.

Keywords: nonaxenic culture; biomass productivity; polyculture; carbon dioxide; CO₂ biosequestration; microalgae

1. Introduction

Anthropogenic activities have led to intensive greenhouse emissions, with carbon dioxide (CO₂) being the largest contributor to climate change [1,2]. One of the most crucial current challenges for scientific research is to reduce atmospheric CO₂ levels to pre-industrial levels. Innovative interdisciplinary solutions to rising levels of CO₂ are thus a need of the moment on all fronts. Geological sequestration and microalgal biofixation are two of the most frequently studied ways to capture atmospheric CO₂. Capturing CO₂ in the form of microalgal biomass recycles CO₂ into the biotic carbon pool. Algae convert atmospheric CO₂ into biomass by photoautotrophic mechanism and account for a large proportion (~50%) of the 111–117 billion metric tonnes of carbon per year of the global primary productivity. Therefore, microalgae have the potential to enable the sustainable production of energy, chemicals, and materials [3,4].

Generally, on a lab scale, monocultures of microalgae that are not associated with or contaminated by other species, i.e., axenic microalgal cultures are studied for the production of biofuels and other value-added compounds. Microalgae such as Botryococcus braunii,
Chlorella pyrenoidosa, Dunaliella salina, Scenedesmus sp., and Chlamydomonas reinhardtii are some of the organisms which are frequently studied in algal biofuel applications [5–7]. However, the outdoor mass cultivation of microalgae monocultures is often limited by low biomass yields, the susceptibility to crashes caused by contamination with or the natural introduction of invasive species, and low temporal stability to fluctuating conditions.

The challenges faced by large-scale monocultures of microalgae could be overcome by multifunctional polycultures as microalgae naturally function symbiotically with other living organisms present in aquatic ecosystems to utilise dissolved nutrients and organic carbon. In terms of multifunctionality, diverse polycultures also have the potential to better resist damage from grazers and invasions whilst maintaining a stable ecosystem over time [8]. However, the role of biodiversity in improving the multifunctionality of outdoor mass cultivation of algae is poorly understood. For example, Shurin et al. [9] and Stockenreiter et al. [10] have shown that polycultures of algae exhibit a higher lipid concentration and total cell biovolume compared to monocultures. Narwani et al. [8], on the other hand, have shown that diverse cultures do not lead to improved multifunctionality. Apart from being multifunctional in terms of increased culture productivity and stability, polycultures with algal species such as Ankistrodesmus falcatus, Chlorella vulgaris, Chlorella pyrenoidosa, and Scenedesmus sp., could also make the harvesting process more energy efficient by promoting the flocculation of the biomass [11].

Since cultures that are free from pest or predator species rarely develop in a full-scale application, it is crucial to study microalgal polycultures under nonaxenic conditions, i.e., in the presence of other organisms such as bacteria, amoebae, ciliates, and rotifers. Previous studies involving nonaxenic cultures of microalgae have shown that the presence of contaminating microorganism can either be beneficial or detrimental to the overall culture performance. For instance, Watanabe et al. [12] showed that in nonaxenic cultures of Chlorella vulgaris, the bacterial contaminants (i.e., in a strict sense, the organisms which make the culture nonaxenic) had both increasing and decreasing effects on the culture lifetime. Such interspecies interactions create complementary environmental niches and contribute to culture stability. Therefore, for our study, we have chosen a microalgal polyculture with microbial composition relevant to outdoor mass cultivation of microalgae [13–15]. As far as we know, studies involving polycultures have not been evaluated for the role of the availability of dissolved CO$_2$ in the bulk media on the performance of the polyculture in nonaxenic conditions. In this study, we also monitor the change in growth and biochemical composition of algae grown at various concentrations of dissolved CO$_2$ in the bulk media. Based on the results, we discuss the potential of using mixed microalgal cultures for sustainable CO$_2$ biosequestration in outdoor mass cultivation.

2. Materials and Methods

2.1. Microalgal Polyculture and Culture Medium

A nonaxenic polyculture of native microalgae reported by Sasongko et al. [13] was collected from the research-based Minamisoma pilot plant, Fukushima, Japan, and was grown at the Algae Biomass and Production facility in Kurihara, Tsukuba, Japan. The species diversity of this nonaxenic microalgal polyculture was previously studied by Demura et al. [16] who reported that it was composed of chlorophytes such as Ankistrodesmus sp., Chlamydomonas sp., Coelastrium sp., Desmodesmus sp., Dictosphaerium sp., Eudorina sp., Kirchneriella sp., Klebsormidium sp., Microactinium sp., Monoraphidium sp., Pediastrum sp., Scenedesmus sp., Selenastrum sp., Staurastrum sp., Tetraspora sp., and Uronema sp., Heterokontophytes, Ciliophora, Euglenozoa, Amoebozoa, and Cyanobacteria. The growth media used in this study contained MgSO$_4$ 7H$_2$O 30 mg L$^{-1}$, NaNO$_3$ 140 mg L$^{-1}$, CaCl$_2$·2H$_2$O 10 mg L$^{-1}$, NH$_4$NO$_3$ 22 mg L$^{-1}$, KH$_2$PO$_4$ 10 mg L$^{-1}$, K$_2$HPO$_4$ 5 mg L$^{-1}$, CaCO$_3$ 10 mg L$^{-1}$, citric acid 2 mg L$^{-1}$, Fe-citrate 2 mg L$^{-1}$, biotin 2 µg L$^{-1}$, thiamine HCl 10 µg L$^{-1}$, vitamin B6 1 µg L$^{-1}$, vitamin B12 1 µg L$^{-1}$, and Trace element mix A5 with Co (Merck KGaA, Darmstadt, Germany) 5 mL.
2.2. Experimental System

The photobioreactor (PBR) consisted of SIMAX borosilicate glass bottles with diameters of 136 mm and heights of 260 mm (Kavalierglass, Sazava, Czech Republic). The working volume of the PBR was two litres. The PBR was operated in batch mode owing to the simplistic design and flexibility of batch cultivation of microalgae [17]. The media were inoculated with the microalgae mixture collected by centrifugation form a starter culture. The initial dry weight content was set at 200 mg L\(^{-1}\). Pure industrial-grade CO\(_2\) was initially bubbled into the algae media at a flow rate of 0.5 L min\(^{-1}\) to reach 200, 400, 600, 800, and 1000 mg L\(^{-1}\) of total dissolved CO\(_2\) in the bulk media. The CO\(_2\) flow was cut off when the required concentration of total dissolved CO\(_2\) was reached, pH was adjusted to 8.5 using 5 M of NaOH, and the bottles were then sealed off to reduce CO\(_2\) escaping due to gassing out. The cultures were stirred at 100 rotations per minute by a magnetic stirrer and incubated at 28 ± 2 °C under continuous illumination of fluorescent lamps with a photon flux density of 50 \(\mu\)mol m\(^{-2}\) s\(^{-1}\).

2.3. Determination of Total Dissolved Carbon Dioxide

When CO\(_2\) dissolves in the bulk media, it exists as carbonic acid, bicarbonate, and carbonate ions. Thus, total dissolved CO\(_2\), Tco\(_2\) (mg L\(^{-1}\)) is the sum of [H\(_2\)CO\(_3\)], [HCO\(_3^-\)] and [CO\(_3^{2-}\)] and is given by the equation:

\[
T_{CO_2} = [H_2CO_3] + [HCO_3^-] + [CO_3^{2-}] \tag{1}
\]

Tco\(_2\) in the bulk media was experimentally determined by lowering the pH of the sampled culture solution to below four by using a citrate buffered ionic strength adjuster where virtually all these ions in the solution become CO\(_2\) gas. A carbon dioxide ion-selective electrode (Handheld carbon dioxide meter CGP-31, TOA DKK, Japan) was then immersed in the sample solution, which enabled CO\(_2\) in the sample solution to diffuse through a gas-permeable membrane until an equilibrium was reached between the partial pressures of CO\(_2\) in the sample solution and the CO\(_2\) in the high concentration sodium carbonate internal filling solution.

2.4. Microscopy and Morphological Analyses

The algae were morphologically observed using an optical microscope equipped with an advanced colour interpolation system (Leica DFC 7000T, Leica Microsystems, Tokyo, Japan). The images were acquired and processed with a LAS X software.

2.5. Measurement of Growth Parameters

Optical density (OD) does not serve as a reasonable estimate of biomass in mixed cultures [18]. Therefore, the biomass concentration was expressed as dry cell weight (DW). 10 mL of culture samples were filtered through dried and pre-weighed GF/C glass microfibre filter papers (Whatman, Chalfont, UK). Excess salts were removed from the filter paper containing biomass by passing 20 mL of 0.5 N NH\(_4\)HCO\(_3\) through the filter papers. The filters were dried at 48 °C overnight and weighed. The difference in mass was taken as the dry cell weight (DW).

The biomass specific growth rate, \(\mu\) (day\(^{-1}\)) was calculated using:

\[
\mu = (\ln N_2 - \ln N_1) / (t_2 - t_1) \tag{2}
\]

where \(N_1\) and \(N_2\) are the biomass at time \(t_1\) and \(t_2\), respectively.

Biomass productivity, \(P_x\) was obtained with Equation (3) where \(X_{max}\) and \(X_i\) are the maximum concentration of microalgae, and initial concentration of microalgae, respectively, and \(t_f\) and \(t_i\) are the times required to reach \(X_{max}\) and \(X_i\), respectively:

\[
P_x = (X_{max} - X_i) / (t_f - t_i) \tag{3}
\]
The CO₂ fixation rate, \( R_{\text{CO}_2} \) (mg CO₂ L\(^{-1}\) day\(^{-1}\)) is estimated using the formula as follows:

\[
R_{\text{CO}_2} = C_{\text{avg}} P_x \left( \frac{M_{\text{CO}_2}}{M_C} \right)
\]

where \( C_{\text{avg}} \) is the average carbon content of the microalgal cells (% w/w) measured by an elemental analyser (UNICUBE, Elementrar, Langenselbold, Germany), \( M_{\text{CO}_2} \) and \( M_C \) are the molar masses of CO₂ and elemental carbon, respectively.

### 2.6. Biochemical Characterisation of Microalgal Cells

For the biochemical characterisation of microalgal cells, dry biomass was harvested by centrifugation at 10,000 × g for 10 min (MX-307, Tomy Digital Biology Co., Ltd., Tokyo, Japan), washed with distilled with H₂O, and freeze-dried (Eyela FDH-2110, Tokyo Rikakikai Co., Ltd., Tokyo, Japan) to prevent the degradation of any heat sensitive metabolites and stored at −20 °C till further analysis. The carbohydrate content was estimated and analysed by the Dubois phenol-sulphuric acid method [19]. The total organic carbon and nitrogen were determined by the CHNS elemental analyser (UNICUBE, Elementrar, Langenselbold, Germany) calibrated using sulphanilamide as a reference standard. About 5 mg of lyophilised microalgal biomass were combusted in pre-weighed aluminium capsules with helium as a carrier gas. A total elemental nitrogen to protein conversion ratio of 4.44 was used [20]. The total lipid content was estimated by the Folch chloroform-based lipid extraction method [21].

### 2.7. Statistical Analysis

All experiments were performed in duplicates, and each measurement was made three times. Hence, all reported values are the mean of six data points. The effect of \( \text{Tco}_2 \) on biomass growth and biomass composition were statistically evaluated by the analysis of variance (ANOVA) test for equal variances using Minitab 19 (Minitab, LLC, Pennsylvania, PA, USA).

### 3. Results

#### 3.1. Biomass and Growth Analyses

We studied the effect of various concentrations of \( \text{Tco}_2 \) in the bulk media on the microalgal polyculture. During the microscopic examination of the polyculture, *Desmodesmus* sp., *Scenedesmus acuminatus*, *Dictyosphaerium* sp., and *Phormidium* sp. were observed as the primary dominant species (Figure 1). Comparing the various growth curves, although a net increase in biomass concentration was observed at all the defined \( \text{Tco}_2 \), higher levels of \( \text{Tco}_2 \) were found to be detrimental to the rate of biomass accumulation (Figure 2).

The effect of the different levels of \( \text{Tco}_2 \) in the bulk media on biomass accumulation was triphasic: an initial period of acclimation and little growth (days 0–3) is followed by a period of rapid biomass accumulation (days 3–7), after which the microalgal population entered a stationary phase from days 7–10. Comparing the specific growth rate at different phases (Figure 3), and the percentage increase in biomass accumulation from the start to the end of each phase (Table 1) elucidates the role of \( \text{Tco}_2 \) in culture growth. In the first growth phase (1–3 days), the specific growth rate of the culture was highest when the \( \text{Tco}_2 \) was 600 mg L\(^{-1}\) (\( \mu = 0.04 \) day\(^{-1}\), 15.75% increase in biomass) and was slightly higher than that observed at 400 mg L\(^{-1}\) of \( \text{Tco}_2 \). In the second growth phase (3–7 days), the specific growth rate was highest at 400 mg L\(^{-1}\) (74.50% increase in biomass) followed by that at 200 mg L\(^{-1}\) of \( \text{Tco}_2 \) (50.12% increase in biomass). In the third growth phase (7–10 days), while the growth rate vastly slowed down at all concentrations of \( \text{Tco}_2 \), it was still highest at 400 mg L\(^{-1}\) of \( \text{Tco}_2 \), indicating that CO₂ at this level was the most favourable for growth. At 1000 mg L\(^{-1}\) of \( \text{Tco}_2 \), the highest level tested in our study, the increase in biomass was the least. Thus, the maximum specific growth rate was observed in culture with a \( \text{Tco}_2 \) of 400 mg L\(^{-1}\) at the second phase after an initial acclimation during the first phase.
1000 mg L\(^{-1}\) of Tco\(_2\), the highest level tested in our study, the increase in biomass was the least. Thus, the maximum specific growth rate was observed in culture with a Tco\(_2\) of 400 mg L\(^{-1}\) at the second phase after an initial acclimation during the first phase.

**Figure 1.** Photomicrograph showing some of the representative species observed in the polyculture. A: *Desmodesmus* sp. (4 cells), B: *Scenedesmus acuminatus* (4 cells), C: *Dictyosphaerium* sp., D: *Phormidium* sp.

**Figure 2.** Mean ± standard deviation of the biomass, as measured by the dry cell weight (DW), at different concentrations of total dissolved carbon dioxide (Tco\(_2\)) in the bulk media.

**Figure 2.** Mean ± standard deviation of the biomass, as measured by the dry cell weight (DW), at different concentrations of total dissolved carbon dioxide (Tco\(_2\)) in the bulk media.
Figure 3. The specific growth rate of the microalgal polyculture within the three phases (0–3 days, 3–7 days, and 7–10 days) of culture under various levels of total dissolved CO\(_2\) (Tco\(_2\)).

Table 1. Increase in biomass of the nonaxenic microalgal culture at different concentrations of total dissolved CO\(_2\) at the end of different growth phases.

| Tco\(_2\) (mg L\(^{-1}\)) | Phase 1 | Phase 2 | Phase 3 | Overall |
|---------------------------|---------|---------|---------|---------|
| 200                       | 8.25    | 50.12   | 4.62    | 70.00   |
| 400                       | 12.75   | 74.50   | 6.99    | 110.50  *|
| 600                       | 15.75   | 27.43   | 0.85    | 48.75   |
| 800                       | 7.50    | 30.24   | 0.73    | 45.00   |
| 1000                      | 5.00    | 30.24   | 0.73    | 37.75   |

* maximum value in each column.

At a Tco\(_2\) level of 200 mg L\(^{-1}\), which showed a 70% overall increase in biomass, the overall productivity and maximum specific growth rate were 14.00 mg L\(^{-1}\) day\(^{-1}\) and 0.101 day\(^{-1}\), respectively (Figure 3). Whereas at Tco\(_2\) level of 400 mg L\(^{-1}\), which showed a 110.50% overall increase in biomass, the productivity and maximum specific growth rate were 22.10 mg L\(^{-1}\) day\(^{-1}\) and 0.13 day\(^{-1}\), respectively. The maximum CO\(_2\) fixation rate and biomass productivity were 28.85 ± 3.00 mg L\(^{-1}\) day\(^{-1}\) and 22.10 ± 0.7 mg L\(^{-1}\) day\(^{-1}\), respectively, at 400 mg L\(^{-1}\) of Tco\(_2\) whereas 1000 mg L\(^{-1}\) of Tco\(_2\) showed the least CO\(_2\) fixation rate of 6.92 ± 1.2 mg L\(^{-1}\) day\(^{-1}\) and lowest biomass productivity of 7.50 ± 0.4 mg L\(^{-1}\) day\(^{-1}\) (37.75% overall increase in biomass). The overall increase in biomass at Tco\(_2\) levels of 600 mg L\(^{-1}\) and 800 mg L\(^{-1}\) was 48.75% and 45%, respectively.

3.2. Effect of CO\(_2\) Concentration on Biochemical Composition

The content of carbohydrates, proteins, and lipids of the microalgae cultivated under different concentrations of Tco\(_2\) was determined and depicted in Figure 4. The carbohydrate content did not change significantly at any of the tested levels of Tco\(_2\).
Varying $T_{\text{CO}_2}$ concentration significantly affected the protein content of the microalgae. The percentage of accumulated protein at different levels of $T_{\text{CO}_2}$ ranged from 8.92 to 30.41%. The protein content was higher in the groups in which higher growth rates were also observed, i.e., at 200 and 400 mg L$^{-1}$ of $T_{\text{CO}_2}$. The maximum and minimum protein content obtained, 30.41 and 8.92%, were observed at 400 and 600 mg L$^{-1}$ of $T_{\text{CO}_2}$, respectively.

The total lipid content at different levels of $T_{\text{CO}_2}$ ranged from 26.39 (minimum observed at 200 mg L$^{-1}$ of $T_{\text{CO}_2}$) to 56.00% (maximum observed at 800 mg L$^{-1}$ of $T_{\text{CO}_2}$). A gradual increase in lipid content was observed with increasing levels of $T_{\text{CO}_2}$. The lipid content was higher at 600, 800, and 1000 mg L$^{-1}$ of $T_{\text{CO}_2}$ than that observed at 200 and 400 mg L$^{-1}$ of $T_{\text{CO}_2}$. The overall biomass growth was also lower at the levels where lipid content was high. The results indicate that the level of dissolved CO$_2$ can significantly affect lipid content.

**Figure 4.** Effect of total dissolved CO$_2$ ($T_{\text{CO}_2}$) in the bulk media on lipid, protein, and carbohydrate composition. ***, and * on top of the columns represent statistical significance at 5%, and 10%, respectively.**

4. Discussion

4.1. Biomass and Growth Analyses

Only a limited number of reported studies were conducted on nonaxenic cultures of microalgal polyculture. As far as we know, most of these studies correlate algal growth parameters to CO$_2$ concentration in the gaseous phase. CO$_2$ concentration in the gaseous phase does not reflect the actual level of CO$_2$ available in the bulk media available for uptake by algal cells, as it is determined by the culture pH and effective mass transfer [22–24]. Anjos et al. [24] also observed this likely lack of dependence of the high biomass accumulation rate on the level of CO$_2$ in the gaseous phase. Similarly, Yang et al. [25] reported that when operated under continuous mode, *Scenedesmus* sp., and *Desmodesmus* sp., did not show any increase in growth rate beyond 30 and 60 µM of CO$_2$, respectively.

At higher levels of $T_{\text{CO}_2}$, the microalgae continued to grow but at less than half the specific growth rate at a $T_{\text{CO}_2}$ concentration of 400 mg L$^{-1}$. Since $T_{\text{CO}_2}$ is the sum of $[\text{H}_2\text{CO}_3]$, $[\text{HCO}_3^-]$ and $[\text{CO}_3^{2-}]$, the stimulatory effect of CO$_2$ is due to the availability of carbon. Thus, for the consortium under study, 400 mg L$^{-1}$ of $T_{\text{CO}_2}$ was ideal for microalgal growth. Below the ideal concentration, algal growth becomes limited, whereas CO$_2$
abundance influences several key enzymes in carbon metabolism, such as ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and carbonic anhydrase [26,27]. The increased availability of CO$_2$ increases the carboxylating activity of RuBisCO, leading to increased photosynthesis, but can also adversely affect the activity of extracellular carbonic anhydrase and inhibit cell growth [28–30]. The low values observed for biomass growth parameters of the polyculture indicate that a higher level of Tco$_2$ is inhibitory. It also points to the presence of CO$_2$ tolerant species in the culture [31,32]. The high initial cell density and the diversity of cell morphology likely increased the tolerance against high levels of Tco$_2$ and reduced the long adaptation period [33]. In mixed cultures, a niche partitioning is generally observed in terms of CO$_2$ assimilation activity and nitrogen-fixing [8,34]. Thus, the growth parameters of the microalgae were significantly influenced by Tco$_2$, and the microalgae showed a wide range of tolerance to Tco$_2$. However, further studies are required to identify the optimum combination of traits of algal polyculture which would have the desired effect on biomass production.

4.2. Effect of CO$_2$ Concentration on Biochemical Composition

The biochemical composition is a crucial factor that determines the applicability of potential algal species for CO$_2$ biosequestration. The carbohydrate content of the culture did not show any significant change at any of the studied levels of total dissolved CO$_2$. Our result is contrary to what is generally observed in monocultures [35–37]. In the case of protein content, significant changes were observed with respect to Tco$_2$. The maximum protein content was at 400 mg L$^{-1}$ of Tco$_2$. At higher levels of Tco$_2$, the protein content decreased, likely due to a lack of availability of carbon and nitrogen species. There was a noticeable increase in lipid content at the Tco$_2$ level of 600 mg L$^{-1}$ and higher. Elevated CO$_2$ concentration in the bulk media creates a stressful environment for the microalgal cells, leading to the production of lipids [38]. The presence of oleaginous and high CO$_2$ tolerant Scenedesmus sp., and Desmodesmus sp., in the polyculture also likely contributed to increased lipid composition [25,26,39]. The biosynthesis of major biochemical constituents such as carbohydrates, proteins, and lipids involves many complex enzymatic reactions such as the light-driven generation of ATPs [40]. Thus, the accurate measurement of metabolic fluxes of CO$_2$ uptake in photosynthetic microorganisms leading to the formation of carbon backbones is complicated due to the presence of high levels of compartmentalisation [41]. The carbon backbones generated in the first few steps of CO$_2$ biofixation are utilised for the biosynthesis of carbohydrate during the dark period. The process can be driven towards the synthesis of proteins and lipids by limiting the dark period. Moreover, the enzymes involved in lipid biosynthesis require Mg$^{2+}$ at their active centres. Therefore, we suggest that, by implementing a two-stage cultivation process, comprising a first stage to promote cell growth with nutrient replete condition followed by a nutrient deplete stage whilst supplementing the media with Mg$^{2+}$ ions at pH between 8 and 10 could enhance the accumulation of lipids for the large-scale production of biofuels from microalgae [29,30,42].

5. Conclusions

Our results indicate that (i) the nonaxenic microalgal polyculture was tolerant to all tested total dissolved CO$_2$ concentrations up to 1000 mg L$^{-1}$; (ii) even though increased total dissolved CO$_2$ levels reduced the overall biomass accumulation, it was also found to be the most conducive to lipid accumulation. Our results also show that the growth of polyculture microalgae stabilise over time in nonaxenic conditions even at very high concentrations of total dissolved CO$_2$ and are suited for large scale biomass cultivation applications in CO$_2$ biosequestration, and accumulation of carbohydrate, proteins, and lipids. Further research, however, is required to understand the interaction between different species leading to niche partitioning and niche complementarity in nonaxenic microalgal polycultures. In order to develop regimens for the stable and sustainable production of biofuels from microalgae, future research efforts should also focus on identifying the agents responsible for the failure of large outdoor cultivation systems.
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References
1. United States Environmental Protection Agency. Sources of Greenhouse Gas Emissions | Greenhouse Gas (GHG) Emissions. US EPA. Available online: https://www.epa.gov/ghgemissions/sources-greenhouse-gas-emissions (accessed on 27 July 2020).
2. Nam, K.; Lee, S.; Jeon, H. Nonlinearity between CO₂ emission and economic development: Evidence from a functional coefficient panel approach. *Sustainability* 2020, 12, 258. [CrossRef]
3. Benemann, J.R.; Oswald, W.J. *Systems and Economic Analysis of Microalgae Ponds for Conversion of Carbon Dioxide to Biomass; Final Report*; U.S. Department of Energy Office of Scientific and Technical Information: Washington, DC, USA, 1996. [CrossRef]
4. Hillier, J.; Hawes, C.; Squire, G.; Hilton, A.; Wale, S.; Smith, P. The carbon footprints of food crop production. *Int. J. Agric. Sustain.* 2009, 7, 107–118. [CrossRef]
5. Kumar, K.; Mishra, S.K.; Shrivastav, A.; Park, M.S.; Yang, J.W. Recent trends in the mass cultivation of algae in raceway ponds. *Renew. Sustain. Energy Rev.* 2015, 51, 875–885. [CrossRef]
6. Chiu, S.Y.; Kao, C.Y.; Chen, T.Y.; Chang, Y.B.; Kuo, C.M.; Lin, C.S. Cultivation of microalgal Chlorella for biomass and lipid production using wastewater as nutrient resource. *Bioresour. Technol.* 2015, 184, 179–189. [CrossRef]
7. Wu, Y.H.; Hu, H.Y.; Yu, Y.; Zhang, T.Y.; Zhu, S.F.; Zhuang, L.L.; Zhang, X.; Lu, Y. Microalgal species for sustainable biomass/lipid production using wastewater as resource: A review. *Renew. Sustain. Energy Rev.* 2014, 33, 675–688. [CrossRef]
8. Narwani, A.; Lashaway, A.R.; Hietala, D.C.; Savage, P.E.; Cardinale, B.J. Power of Plankton: Effects of Algal Biodiversity on Sustainable Biofuel Systems. *J. Appl. Phycol.* 2015, 28, 51, 350–356. [CrossRef] [PubMed]
9. Shurin, J.B.; Abbott, R.L.; Deal, M.S.; Kwan, G.T.; Litchman, E.; McBride, R.C.; Mandal, S.; Smith, V.H. Industrial-strength ecology: Trade-offs and opportunities in algal biofuel production. *Ecol. Lett.* 2013, 16, 1393–1404. [CrossRef]
10. Steckeneiter, M.; Graber, A.K.; Haupt, F.; Stibor, H. The effect of species diversity on lipid production by micro-algal communities. *J. Appl. Phycol.* 2012, 24, 45–54. [CrossRef]
11. Wu, Y.H.; Hu, H.Y.; Yu, Y.; Zhang, T.Y.; Zhu, S.F.; Zhuang, L.L.; Zhang, X.; Lu, Y. Microalgal species for sustainable biomass/lipid production using wastewater as nutrient resource: A review. *Renew. Sustain. Energy Rev.* 2014, 33, 675–688. [CrossRef]
12. Watanabe, K.; Takihana, N.; Aoyagi, H.; Hanada, S.; Watanabe, Y.; Ohmura, N.; Saiki, H.; Tanaka, H. Symbiotic association in Chlorella culture. *FEMS Microbiol. Ecol.* 2005, 51, 187–196. [CrossRef] [PubMed]
13. Sasongko, N.A.; Noguchi, R.; Ito, J.; Demura, M.; Ichikawa, S.; Nakajima, M.; Watanabe, M.M. Engineering study of a pilot scale process plant for microalga-oil production utilizing municipal wastewater and flue gases: Fukushima microalgae pilot plant. *Energies* 2018, 11, 1693. [CrossRef]
14. Padmaerumana, G.; Kapoore, R.V.; Gilmour, D.J.; Vaidyanathan, S. Microbial consortia: A critical look at microalgal co-cultures for enhanced biominiuturing. *Crit. Rev. Biotechnol.* 2018, 38, 690–703. [CrossRef]
15. Godwin, C.M.; Lashaway, A.R.; Hietala, D.C.; Savage, P.E.; Cardinale, B.J. Biodiversity improves the ecological design of sustainable biofuel systems. *GCB Bioenergy* 2018, 10, 752–765. [CrossRef]
16. Demura, M.; Yoshida, M.; Yokoyama, A.; Ito, J.; Kobayashi, H.; Kayano, S.; Tamagawa, Y.; Watanobe, M.; Date, N.; Osaka, M.; et al. Biomass productivity of native algal communities in Minamisoma city, Fukushima Prefecture, Japan. *Algal Res.* 2018, 29, 22–35. [CrossRef]
17. Riebesell, U.; Wolf-Gladrow, D.A.; Smetacek, V. Carbon dioxide limitation of marine phytoplankton growth rates. *Nature* 1993, 361, 249–251. [CrossRef]
18. Shurin, J.B.; Mandal, S.; Abbott, R.L. Trait diversity enhances yield in algal biofuel assemblages. *J. Appl. Ecol.* 2014, 51, 603–611. [CrossRef]
19. DuBois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric Method for Determination of Sugars and Related Substances. *Anal. Chem.* 1956, 28, 350–356. [CrossRef]
20. López, C.V.G.; del Carmen Cerón García, M.; Fernández, F.G.A.; Bustos, C.S.; Chisti, Y.; Sevilla, J.M.F. Protein measurements of microalgal and cyanobacterial biomass. *Bioreas. Technol.* 2010, 101, 7587–7591. [CrossRef] [PubMed]
21. Folich, J.; Lees, M.; Stanley, G.H. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. *Cancer* 1957, 226, 497–509. [CrossRef]

22. Kumar, A.; Ergas, S.; Yuan, X.; Sahu, A.; Zhang, Q.; Dewulf, J.; Malcata, F.X.; van Langenhove, H. Enhanced CO₂ fixation and biofuel production via microalgae: Recent developments and future directions. *Trends Biotechnol.* 2010, 28, 371–380. [CrossRef] [PubMed]
23. Spalding, M.H. Microalgal-carbon-dioxide-concentrating mechanisms: Chlamydomonas inorganic carbon transporters. *J. Exp. Bot.* 2008, 59, 1463–1473. [CrossRef] [PubMed]

24. Anjos, M.; Fernandes, B.D.; Vicente, A.A.; Teixeira, J.A.; Dragone, G. Optimization of CO₂ bio-mitigation by Chlorella vulgaris. *Bioreas. Technol.* 2013, 133, 149–154. [CrossRef] [PubMed]
25. Yang, J.; Zhang, C.; Hu, H. Screening High CO₂-Tolerant Oleaginous Microalgae from Genera Desmodesmus and Scenedesmus. *Appl. Biochem. Biotechnol.* 2020, 192, 211–229. [CrossRef]

26. Lohman, E.J.; Gardner, R.D.; Pedersen, T.; Peyton, B.M.; Cooksey, K.E.; Gerlach, R. Optimized inorganic carbon regime for enhanced growth and lipid accumulation in Chlorella vulgaris Luisa Gouveia. *Biotechnol. Biofuels* 2015, 8, 1–13. [CrossRef] [PubMed]
27. Zhao, B.; Su, Y. Process effect of microalgal-carbon dioxide fixation and biomass production: A review. *Renew. Sustain. Energy Rev.* 2014, 31, 121–132. [CrossRef]
28. Mangan, N.; Brenner, M. Systems analysis of the CO₂ concentrating mechanism in cyanobacteria. *Elife* 2014, 3, e02043. [CrossRef] [PubMed]

29. Zhu, X.-G.; Long, S.P.; Ort, D.R. Improving Photosynthetic Efficiency for Greater Yield. *Annu. Rev. Plant Biol.* 2010, 61, 235–261. [CrossRef] [PubMed]
30. Bowes, G. Growth at elevated CO₂: Photosynthetic responses mediated through Rubisco. *Plant Cell Environ.* 1991, 14, 795–806. [CrossRef]

31. Yadav, R.R.; Krishnamurthi, K.; Mudliar, S.N.; Devi, S.S.; Naoghare, P.K.; Bafana, A.; Chakrabarti, T. Carbonic anhydrase mediated carbon dioxide sequestration: Promises, challenges and future prospects. *J. Basic Microbiol.* 1998, 38, 497–509. [CrossRef]
32. Sültemeyer, D. Carbonic anhydrase in eukaryotic algae: Characterization, regulation, and possible function during photosynthesis. *Can. J. Bot.* 1998, 76, 962–972. [CrossRef]
33. Lee, J.S.; Lee, J.P. Review of advances in biological CO₂ mitigation technology. *Biotechnol. Bioprocess Eng.* 2003, 8, 354–359. [CrossRef]
34. Contreras-Angulo, J.R.; Mata, T.M.; Cuellar-Bermudez, S.P.; Caetano, N.S.; Chandra, R.; Garcia-Perez, J.S.; Muylaert, K.; Parrasaldivar, R. Symbiotic co-culture of Scenedesmus sp. and Azospirillum brasilense on N-deficient media with biomass production for biofuels. *Sustainability* 2019, 11, 707. [CrossRef]
35. El-Katony, T.M.; El-Adl, M.F. Salt response of the freshwater microalga Scenedesmus obliquus (Turp.) Kutz is modulated by the algal growth phase. *J. Oceanol. Limnol.* 2020, 38, 802–815. [CrossRef]
36. Ali, I.; Naqvi, S.R.; Bahadar, A. Kinetic analysis of Botryococcus braunii pyrolysis using model-free and model fitting methods. *Fuel* 2018, 214, 369–380. [CrossRef]
37. Basu, S.; Roy, A.S.; Mohanty, K.; Ghoshal, A.K. CO₂ biofixation and carbonic anhydrase activity in Scenedesmus obliquus SA1 cultivated in large scale open system. *Bioreas. Technol.* 2014, 164, 323–330. [CrossRef] [PubMed]
38. Varshney, P.; Beardall, J.; Bhattacharya, S.; Wangikar, P.P. Effect of elevated carbon dioxide and nitric oxide on the physiological responses of two green algae, Asterarcys quadricellulare and Chlorella sorokiniana. *J. Appl. Phycol.* 2020, 32, 189–204. [CrossRef]
39. Arora, N.; Pienkos, P.T.; Pruthi, V.; Poluri, K.M.; Guarnieri, M.T. Leveraging algal omics to reveal potential targets for augmenting TAG accumulation. *Biotechnol. Adv.* 2018, 36, 1274–1292. [CrossRef] [PubMed]
40. Tan, X.; Zhu, J.; Wakisaka, M. Effect of protocatechuic acid on Euglena gracilis growth and accumulation of metabolites. *Sustainability* 2020, 12, 9158. [CrossRef]

41. Sake, C.L.; Metcalf, A.J.; Boyle, N.R. The challenge and potential of photosynthesis: Unique considerations for metabolic flux measurements in photosynthetic microorganisms. *Biotechnol. Lett.* 2019, 41, 35–45. [CrossRef] [PubMed]
42. Livne, A.; Sukonik, A. Lipid Synthesis and Abundance of Acetol CoA Carboxylase in *Isochrysis galbana* (Prymnesiophyceae) Following Nitrogen Starvation. *Plant Cell Physiol.* 1992, 33, 1175–1181. [CrossRef]