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Productivity and Nutrient Quality of *Lemna minor* as Affected by Microbiome, CO_2 Level, and Nutrient Supply

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Abstract: Rising atmospheric carbon dioxide (CO_2) levels can impact plant photosynthesis and productivity and threaten food security, especially when combined with additional environmental stressors. This study addresses the effects of elevated CO_2 in combination with low nutrient supply on *Lemna minor* (common duckweed). We quantified plant growth rate and nutritional quality (protein content) and evaluated whether any adverse effects of elevated CO_2, low nutrients, or the combination of the two could be mitigated by plant-microbe interaction. Plants were grown under controlled conditions and were either uninoculated or inoculated with microorganisms from a local pond that supported *L. minor* populations. Under low nutrients in combination with high CO_2, growth (plant area expansion rate) decreased and biomass accumulation increased, albeit with lower nutritional quality (lower percentage of protein per plant biomass). Inoculation with plant-associated microorganisms restored area expansion rate and further stimulated biomass accumulation while supporting a high protein-to-biomass ratio and, thus, a high nutritional quality. These findings indicate that plant-microbe interaction can support a higher nutritional quality of plant biomass under elevated atmospheric CO_2 levels, an important finding for both human and non-human consumers during a time of rapid environmental change.

Keywords: biomass; carotenoids; duckweed; growth; inoculation; Lemnaceae; nutrition; pigments; protein

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1. Introduction

The current rapidly changing environmental conditions pose threats to food security [1]. The changing climate has intensified both physical (abiotic) stresses (e.g., droughts, floods, extreme temperatures) and biological (biotic) stresses (pests and pathogens). These changing conditions impact photosynthesis and productivity in both agricultural and natural systems [2,3]. While CO_2 is required for photosynthesis and growth of plants and algae, too much CO_2 can have negative effects, especially in combination with drought, extreme temperatures [4], or low nutrient supply [5]. Such adverse effects include diminished plant nutritional quality for human and non-human consumers [6]. Future agriculture will require climate-resilient crops [7] capable of maintaining both productivity and nutritional quality under changing environmental conditions, as well as identification of mitigating factors that can support crop cultivation. In the following introductory paragraphs, we briefly review the effects of atmospheric CO_2 level under different nutrient supply on plant metabolism and possible mitigating effects of plant-microbe interaction. We then introduce the plant system used here to facilitate multi-factorial analysis of the impact of ambient versus elevated CO_2 level, low versus ample nutrient supply, and absence or presence of the plant microbiome.

The level of available nitrogen influences the effects of elevated CO_2 on plant growth and nutritional quality [8,9]. Growth of new tissues requires uptake of nitrogen from the

environment for protein synthesis [10] as well as sugars produced in photosynthesis. The demand from growing tissues for sugar production exerts substantial feedback control over plant photosynthetic capacity [11]. Because elevated CO_2 increases sugar production and low nutrient supply decreases sugar consumption, an imbalance results between sugar source (photosynthetic tissue) and sugar sinks (sugar-consuming tissues) under a combination of these abiotic conditions [12,13]. The resulting excessively high source-to-sink ratio can trigger feedback downregulation of photosynthesis, slow carbohydrate production [14], and accelerate onset of plant senescence [15–17]. Specifically, foliar carbohydrate build-up leads to a back-up of electrons in the photosynthetic electron transport chain and subsequent electron transfer to oxygen that results in production of reactive oxygen species (ROS) [18].

These ROS serve as gene regulators and can suppress the synthesis of proteins responsible for CO_2 fixation and light harvesting in photosynthesis [19,20]. Because a lower number of photosynthetic proteins is sufficient to support the same rate of photosynthesis and growth under elevated CO_2 in C_3 plants, this feedback downregulation of photosynthesis allows precious resources to be redirected under elevated CO_2 [21]. However, because photosynthetic protein constitutes a considerable fraction of leaf protein, this more economic use of photosynthetic protein results in a lower protein-to-biomass ratio, i.e., a reduced nutritional quality for the consumer. In terrestrial crops, this protein loss affects not only leaf protein [22,23] but also the protein content of grains [24]. Thus, source-sink imbalance is often associated with an increased carbon to nitrogen (C:N) ratio [25,26]. An additional contributing factor to the decline in plant mineral nutrition under elevated CO_2 is an apparent inhibition of nitrate uptake and metabolism [27].

In addition to its potential to alter protein-to-biomass ratio, elevated CO_2 can also alter plant micronutrient-to-biomass ratios because feedback downregulation of photosynthetic protein affects carotenoids. For example, chlorophyll-binding proteins also bind several carotenoids that serve as essential human micronutrients. These carotenoids include provitamin A (β-carotene) as well as lutein and the xanthophyll cycle pool, a set of rapidly inter-convertible xanthophylls that produce zeaxanthin under exposure to excess light [18]. Carotenoids are an important part of plant nutritional quality because they serve as essential human micronutrients needed to support human vision, immune health, and cognitive performance [28–31]. We recently reported that a combination of elevated CO_2 and continuous exposure to high intensity light strongly depressed carotenoid-to-biomass ratios for β-carotene, lutein, and zeaxanthin [18]. Previous reports on the effect of elevated CO_2 on foliar carotenoid levels have varied by plant species and growth conditions [32].

It has been proposed that plant-microbe interaction may be able to counteract source-sink and C:N imbalances as well as the resulting photosynthetic downregulation [33–35]. The effect of plant-microbe interaction depends on environmental factors [36,37], including CO_2 level [33] and nitrogen availability [38]. Specifically, the plant microbiome has the potential to (i) increase plant nitrogen content in support of new growth, which increases sink strength [39], (ii) lessen build-up of carbohydrates by serving as an additional carbohydrate sink, thus counteracting electron back-up and excess ROS formation [40,41], (iii) produce growth-stimulating plant hormones, which also increases sink strength [42–44], and/or (iv) induce routing of electrons into alternative pathways in various compartments, thus also lowering ROS production [45] (for a recent general overview, see [34]).

For the present study, a small floating aquatic plant, *Lemna minor*, in the Lemnaceae (duckweed) family was used. Duckweeds are C_3 plants consisting of small green structures called fronds [46] that carry out both photosynthesis and nutrient uptake under most conditions [47]. These plants grow exceptionally fast and can both double frond number and area in 1–3 days [48], which allows changes in growth rates to manifest rapidly [49]. Duckweeds accumulate unusually large amounts of vegetative protein throughout the plant [50,51]. Per cultivation area, duckweed can produce up to 20× the high-quality edible protein compared to soybean [52]. Moreover, duckweed accumulates high concentrations of essential human micronutrients [50], including carotenoids [53]. Because aquatic plants

store considerable levels of vegetative storage protein in their fronds that is not actively involved in the process of photosynthesis [18], we postulate an only modest effect of feedback downregulation under elevated CO_2 on frond protein content but a more marked effect on photosynthesis-associated carotenoids.

In addition to its high nutritional quality, duckweed has properties that support environmental sustainability. Duckweed supports high rates of CO_2 sequestration via its high growth rate and can remove excess nitrogen and phosphorus [52,54–56] as well as heavy metals and other toxins [57,58] from freshwater bodies [59,60]. Duckweed can also reduce agricultural fertilizer runoff and increase crop yield. For example, duckweed grown in rice paddies increased grain yield and decreased nitrogen loss, thus lessening the need for fertilization [61].

The duckweed microbiome includes bacteria (such as *Ensifer adhaerens*) that fix atmospheric nitrogen (N_2) into forms of nitrogen usable by plants [36]. Like soybean and other legumes, *Lemna* also associates with N_2-fixing *Rhizobium* species (*R. lemnae;* [62]). Plant-associated microorganisms can also enhance the uptake of nitrogen compounds dissolved in the growth medium. Moreover, *Lemna* associates with plant-growth promoting microorganisms [63] such as *Pseudomonas* [41,42] and *Acinetobacter* [64]. Manipulation of the duckweed microbiome is relatively facile because the plant floats freely on water with no soil-embedded roots. Duckweed's fast growth rate, high nutritional quality, and microbial associations made it a suitable candidate for this multi-factorial study of the effect of plant-microbe interaction on plant growth and nutritional quality under various combinations of CO_2 level and nutrient supply.

Plant growth rate was assessed as both plant-area expansion rate and biomass accumulation rate rather than either alone [65–67]. This approach allows detection of possible differential effects of elevated CO_2 on the rate of new tissue growth versus dry biomass accumulation. The purpose of this study is to assess effects of atmospheric CO_2 concentration, nutrient supply, and inoculation with microorganisms on area growth, biomass production, protein content, and carotenoid-micronutrient level. We used the floating plant *Lemna minor* because it allows facile manipulation of nutrient supply and microbiome. Figure 1 illustrates the multi-factorial experimental design of the study with two levels of nutrient supply, two levels of CO_2, and absence or presence of microbial inoculation.

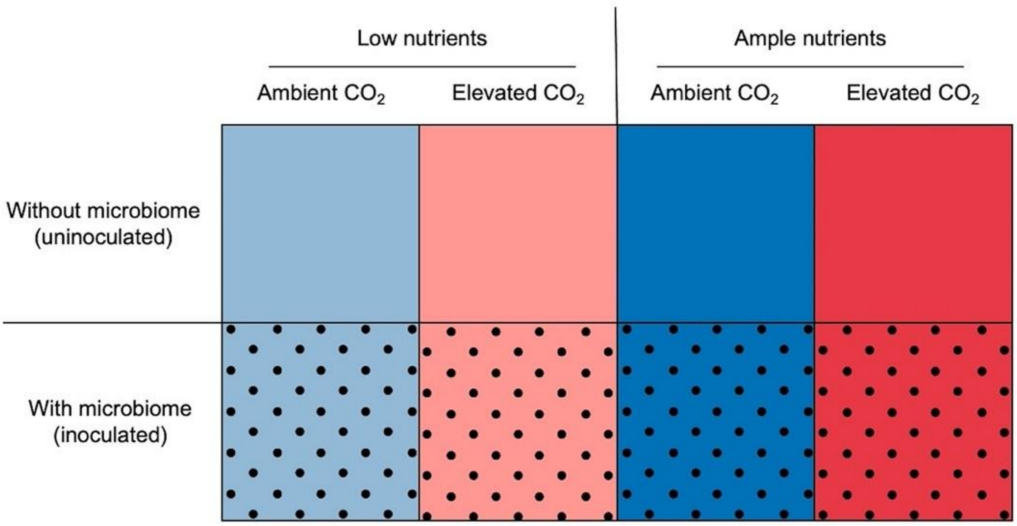

Figure 1. Schematic of the multi-factorial experimental design. Lighter and darker colors represent low and ample nutrient treatments, respectively. Ambient CO_2 conditions are blue and elevated CO_2 conditions are red. The dotted fill pattern represents inoculated treatments.

2. Results and Discussion

As predicted, there was considerable interaction between nutrient supply and CO_2 level, which was further substantially impacted by the plant microbiome. In each of the following sections, we first highlight the main effects for each series of combinations (see Figure 1) of low (light-color columns) versus ample (dark-color columns) nutrient supply, ambient (blue columns) versus elevated (red columns) CO_2 as well as uninoculated (solid columns) fronds versus inoculated (dotted columns). We subsequently summarize any additional, more minor, effects of specific environmental factors.

2.1. Effects of Growth Environment and Inoculation on Plant Growth Rate

The most striking results for area expansion (via new growth; Figure 2A,B) over the course of the experimental phase were a strong inhibition of area-expansion rate by the combination of elevated CO_2 and low nutrient supply in uninoculated fronds (Figure 2A, light-red solid column) and the complete prevention of this inhibition by inoculation (Figure 2A, light-red dotted column).

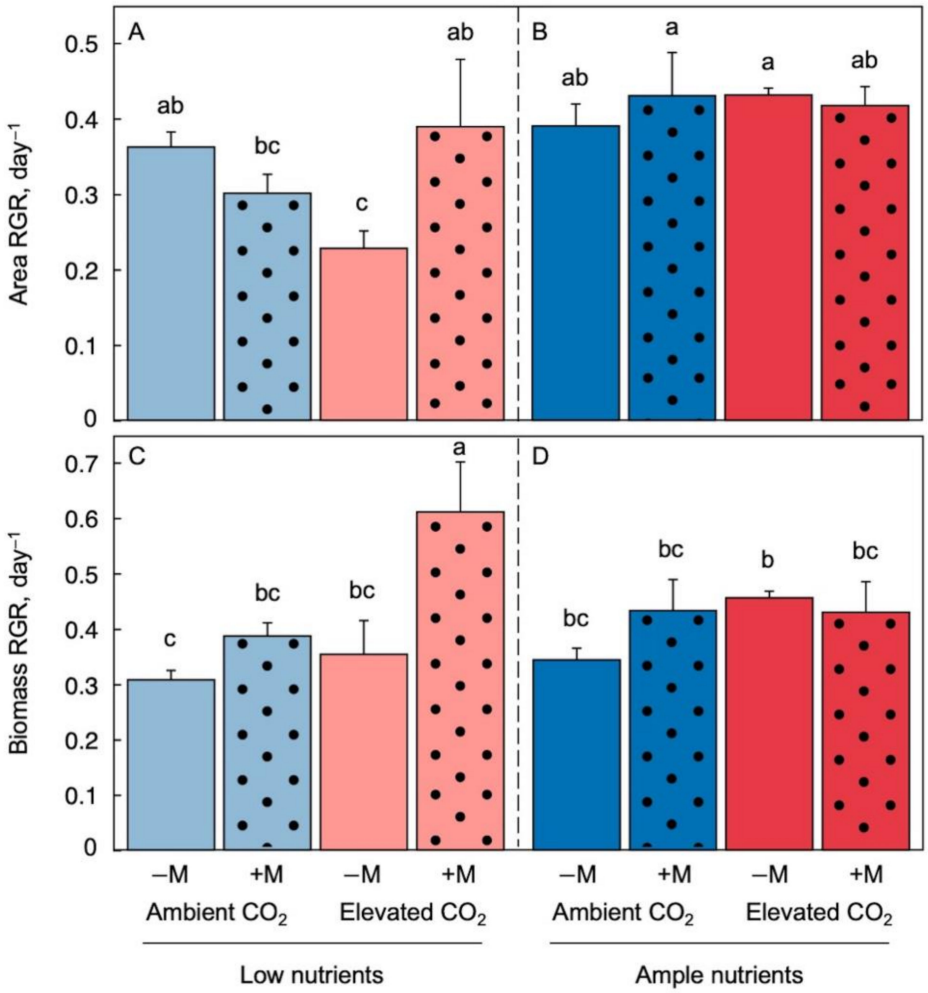

Figure 2. Relative growth rate (RGR) of frond area expansion under low (**A**) and ample (**B**) nutrients as well as rate (RGR) of biomass production under low (**C**) and ample (**D**) nutrients for *Lemna minor* grown in 1/20 (light blue or light red) or 1/2 (dark blue or red) strength Schenk & Hildebrandt medium under either ambient (blue) or elevated (red) CO_2 levels. Blue vs. red colored bars correspond to ambient versus elevated CO_2 conditions, respectively. Bars with solid fill represent groups that were not inoculated (−M) compared to bars with dotted fill that were inoculated (+M). Mean values ± standard deviations; $n = 3$. Different lower-case letters represent significant differences at $p < 0.05$.

The rate of biomass accumulation (Figure 2C,D) exhibited both similar and different effects compared to area-expansion rate. On the one hand, under low nutrient supply (Figure 2C) biomass accumulation was not significantly different under elevated CO_2 (light-red solid column) compared to ambient CO_2 (light-blue solid column), which was a different response from area-expansion rate (Figure 2A). On the other hand, inoculation caused a significant stimulation of biomass accumulation rate (Figure 2C, light-red dotted column compared to light-red solid column), as also seen for area-expansion rate (Figure 2A).

All these findings are consistent with altered source-sink and carbon to nitrogen relationships in duckweed grown under elevated CO_2, as has been reported for other plants [68,69]. Conversely, the plant microbiome may have allowed new area growth by restoring balance in these systems via (i) consumption of carbohydrates supplied by the fronds and (ii) possible improvement in nitrogen uptake from the medium and/or increased nitrogen availability to the fronds through microbial N_2 fixation [34]. Moreover, Figure 2B,D show that neither elevated (versus ambient) CO_2 nor inoculation (versus uninoculated fronds) significantly affected area-expansion rate or biomass accumulation, respectively, under ample nutrient supply. This result further emphasizes that it is specifically the *combination* of elevated CO_2 with low nutrient supply that caused apparent imbalances [5].

The effect of elevated CO_2 on plants varies considerably among species and environmental conditions and can also have a different impact on growth versus nutritional quality. Plants with high growth rates and/or a large storage capacity for carbohydrates will not show any growth penalties under elevated CO_2 [22]. Conversely, other species exhibit reduced growth rates and other growth penalties under elevated CO_2 [70]. However, all C_3 plants tend to exhibit a loss of nutritional quality because elevated CO_2 levels allow plants to perform photosynthesis at the same rate as they would under ambient CO_2, but with fewer photosynthetic proteins and thus a lower protein content [21]. These effects are assessed in the following section on biomass and protein content.

Additional points to note include no impact on area-expansion rate by experimental transfer to low nutrient supply (Figure 2A, light-blue solid column) compared to continued ample nutrient supply (Figure 2B, dark-blue solid column) over the duration of the experimental phase. Because mineral nutrients (especially nitrogen) are needed for protein synthesis and growth of new tissue [10], this result suggests that internal protein stores [50,52] may have contributed to the unabated area expansion via new growth under ambient CO_2 over this time period despite the lower external nutrient supply [54]. However, inoculated fronds exhibited a lower area-expansion rate under ambient CO_2 with low nutrient supply (Figure 2A, light-blue dotted column) compared to ample nutrient supply (Figure 2B, dark-blue dotted column). This effect represents an apparent cost of inoculation under low nutrient supply in ambient CO_2. Whereas the plant microbiome can enhance plant nitrogen status, microorganisms may also compete with plants for various other mineral nutrients. For example, a bacterial strain that strongly promoted duckweed growth under ample nutrient supply instead reduced plant growth under limiting levels of mineral nutrients other than nitrogen [42,67,71]. It should be noted that low nutrient supply in our experiments represented dilution of all mineral nutrients, and not just nitrogen compounds. In this context, it is also noteworthy that phosphorus is a "regulator of nitrogen biogeochemistry" [72] and stimulates biological nitrogen fixation in biomes dominated by legume trees [73]. Furthermore, additional phosphorus is required to support greater rates of N_2 fixation under elevated atmospheric CO_2 levels [74]. It is thus possible that the apparent cost of inoculation under low overall nutrient supply seen in our study may be alleviated by increased phosphorus supply.

2.2. Effect of Growth Environment and Inoculation on Biomass per Area and Protein per Biomass

The most notable, and consistent, trends on this topic include an increased level of dry biomass per unit frond area under elevated CO_2 especially in uninoculated fronds and irrespective of nutrient supply in the medium (Figure 3A,B, light-red and dark-red solid columns compared to light-blue and dark-blue solid columns, respectively). This

additional dry biomass exhibited a non-significant trend to have a somewhat lower ratio of protein to biomass (Figure 3C,D, light-red and dark-red solid columns compared to light-blue and dark-blue solid columns, respectively). The protein to biomass ratio can be used as a proxy for the relative proportion of nitrogen to carbon in the biomass, and as a sign of high-quality biomass.

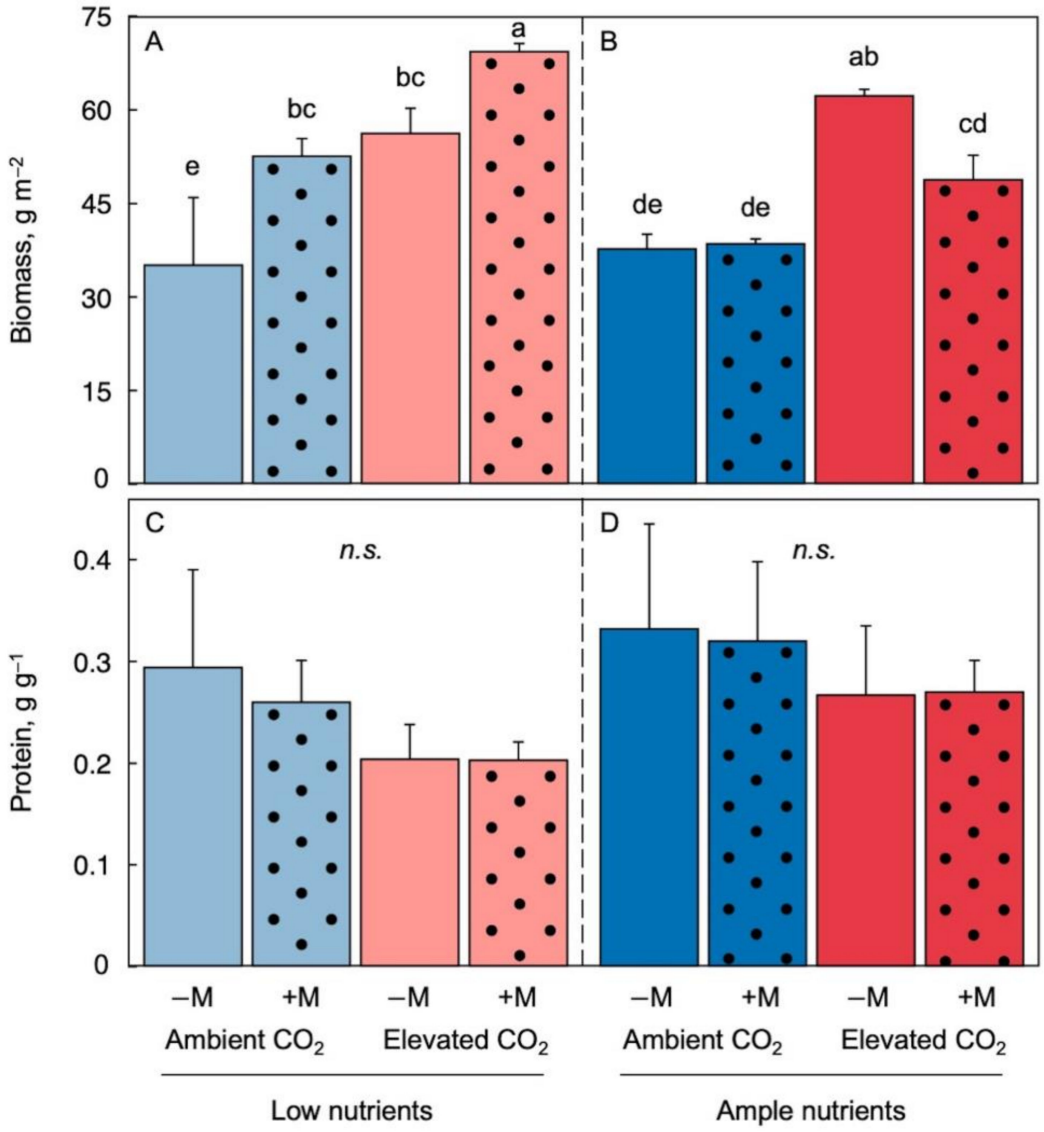

Figure 3. Biomass production per frond area under low (**A**) and ample (**B**) nutrients as well as protein to biomass ratio under low (**C**) and ample (**D**) nutrients for *Lemna minor* grown in 1/20 (light blue or light red) or 1/2 strength (dark blue or red) Schenk & Hildebrandt medium and either ambient (blue) or elevated (red) CO_2 levels. Bars with solid fill represent fronds that were not inoculated (−M), compared to bars with dotted fill that were inoculated (+M). Mean values ± standard deviations; $n = 3$. Different lower-case letters represent significant differences at $p < 0.05$.

Figure 3 panels C and D illustrate that there were only relatively minor, insignificant differences in protein to biomass ratio in response to nutrient supply, elevated CO_2, or inoculation. This finding is consistent with duckweed's ability to store large quantities of vegetative protein that is relatively insensitive to feedback downregulation and may also be able to provide nitrogen for new growth for some time upon transfer to low nutrient supply [54]. In contrast, terrestrial plants often exhibit pronounced effects of elevated CO_2 on foliar protein, especially under limiting nutrient supply, with resulting feedback downregulation of photosynthetic proteins [75,76]. Elevated CO_2 under low nitrogen

supply can also lead to a lowering of protein content in barley, wheat, and rice grains [8,77]. Whereas biomass production may be enhanced, the nutritional quality of this biomass is thus typically diminished under elevated CO_2 and low nutrient supply [78,79].

Under low nutrient supply, production of additional biomass (Figure 3A) with the same quality was seen in inoculated fronds (light-red dotted column) compared to uninoculated fronds (light-red solid column) under both elevated CO_2 and ambient CO_2 levels (Figure 3A,C, light-blue dotted versus light-blue solid columns). These findings indicate a benefit of plant-microbe interaction on the nutritional quality of plant biomass (protein-to-biomass ratio) under low nutrient supply irrespective of CO_2 level.

Trends were somewhat different under ample nutrient supply with respect to the effect of inoculation under elevated CO_2 levels (Figure 3B). The only treatment showing significantly greater biomass per area was elevated CO_2 in uninoculated fronds (Figure 3B, dark-red solid column). Protein-to-biomass ratio showed a nonsignificant trend to be lower under elevated (Figure 3D, dark-red solid and dotted columns) compared to ambient (Figure 3D, dark-blue solid and dotted columns) CO_2 but to be higher than in elevated CO_2 under low nutrient supply (Figure 3C, dark-red solid and dotted columns).

Taken together, these findings suggest that association of a microbiome with the duckweed *L. minor* is beneficial for production of high-quality biomass with respect to protein-to-biomass ratio under the combination of elevated CO_2 and low nutrient supply.

2.3. Effect of Growth Environment and Inoculation on Chlorophyll and Carotenoid Micronutrients

Figures 4 and 5 show chlorophyll and β-carotene (Figure 4A–D) and lutein and xanthophyll cycle pool (Figure 5A–D) on a dry biomass basis. There was a consistent trend for greater chlorophyll-to-biomass and carotenoid-to-biomass ratios under ample nutrient (Figure 4B,D and Figure 5B,D) compared to low nutrient (Figure 4A,C and Figure 5A,C) supply for both ambient and elevated CO_2 as well as inoculated and uninoculated fronds.

The two most notable additional results were the following effects of inoculation that varied with respect to nutrient supply and CO_2 level. Inoculation resulted in greater ratios of chlorophyll-to-biomass and carotenoid-to-biomass only under elevated CO_2 and ample nutrient supply (Figure 4B,D and Figure 5B,D, dark-red dotted columns compared to dark-red solid columns) but not under the combination of elevated CO_2 and low nutrient supply (Figure 4A,C and Figure 5A,C, light-red dotted columns compared to light-red solid columns). There were only very minor differences in the degree of these responses among the different pigments. In addition, there was a consistent trend for chlorophyll-to-biomass and carotenoid-to-biomass ratios to be higher under ambient CO_2 in inoculated fronds growing with ample nutrients (Figure 4B,D and Figure 5B,D, dark-blue dotted columns) compared to low nutrient supply (Figure 4A,C and Figure 5A,C, light-blue dotted columns).

Elevated CO_2 conditions have shown to affect carotenoid levels in ways that vary by plant species and growth conditions [32]. Specifically, it has been suggested that the combination of elevated CO_2 and long photoperiod (high light supply) may lead to particularly pronounced downregulation [34,80] associated with source-sink imbalance. This outcome is also consistent with the effect reported for *L. minor* grown under a combination of elevated CO_2 with a 24 h photoperiod of 1000 µmol photons m^{-2} s^{-1} [18]. Even in a plant like duckweed with its feedback-downregulation-insensitive storage protein, one can expect a decline in the content of the proteins that bind chlorophylls and carotenoids under conditions that trigger feedback downregulation. Overall, the content of chlorophyll and carotenoids associated with chlorophyll-binding proteins declined relative to dry biomass (i) upon transfer to low-nutrient medium compared to continuous growth with ample nutrient supply (Figure 4A,C and Figure 5A,C, compared to Figure 4B,D and Figure 5B,D) and (ii) during growth under elevated versus ambient CO_2 (red columns compared to blue columns in Figures 4 and 5). Whereas inoculation tended to limit or prevent elevated CO_2-induced declines in chlorophyll-to-biomass or carotenoid-to-biomass ratios under ample nutrient supply (Figure 4B,D and Figure 5B,D, dotted columns compared to solid columns), inoculation tended to instead exacerbate declines in carotenoid-to-biomass ratio

caused by low nutrient supply (Figure 4A,C and Figure 5A,C, dotted compared to solid columns). This complex response may, once again, be associated with the potential for both competition for, and provision of, mineral nutrients by microorganisms [42,81,82].

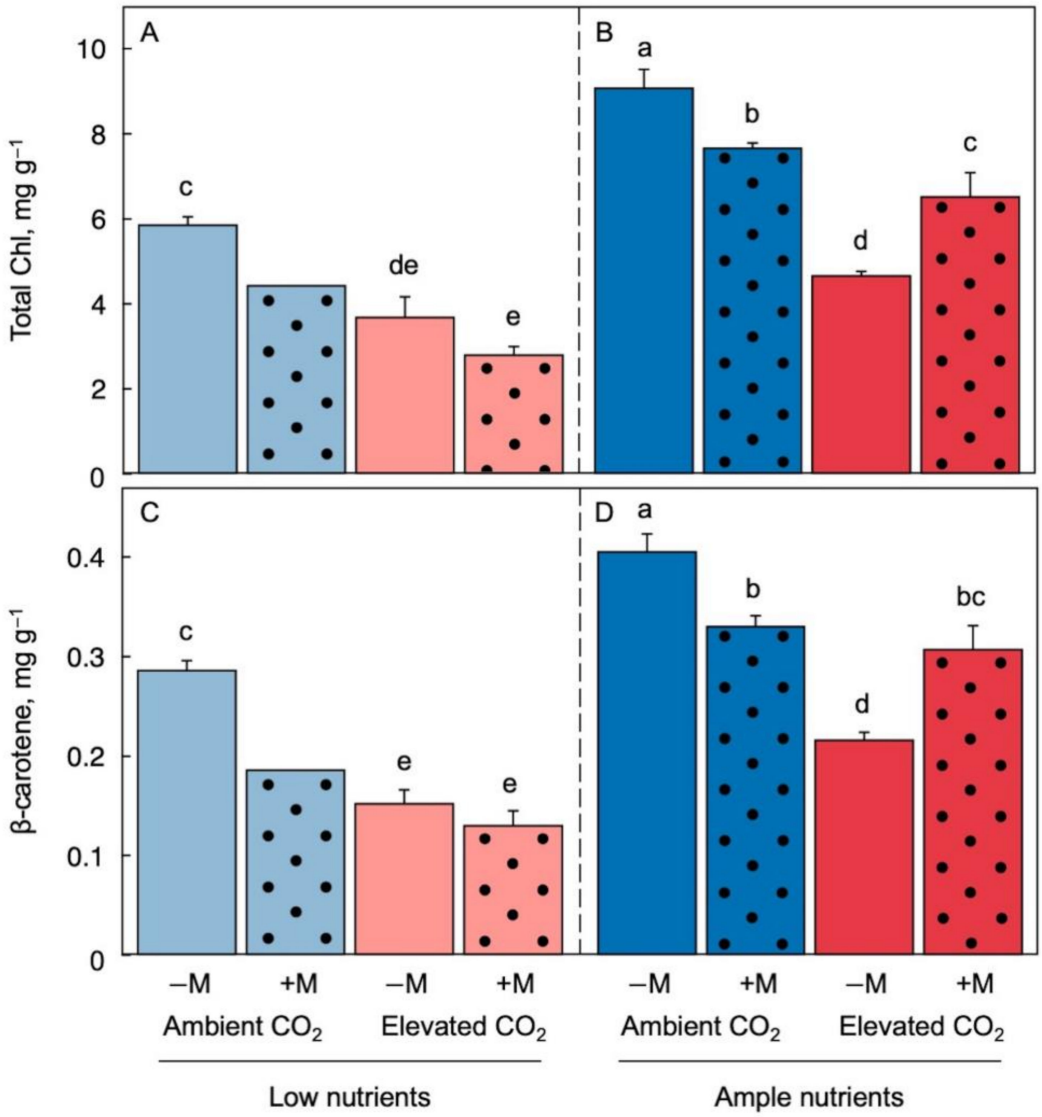

Figure 4. Total chlorophyll (*a* + *b*) content under low (**A**) and ample (**B**) nutrients as well as β-carotene to biomass ratio under low (**C**) and ample (**D**) nutrients for *Lemna minor* grown in 1/20 (light blue or light red) or 1/2 strength (dark blue or red) Schenk & Hildebrandt medium and either ambient (blue) or elevated (red) CO_2 levels. Bars with solid fill represent groups that were not inoculated (−M), compared to bars with dotted fill that were inoculated (+M). Mean values ± standard deviations; $n = 3$ under all conditions except for $n = 2$ in the treatment of inoculated fronds in ambient CO_2 and low nutrients. Different lower-case letters represent significant differences at $p < 0.05$.

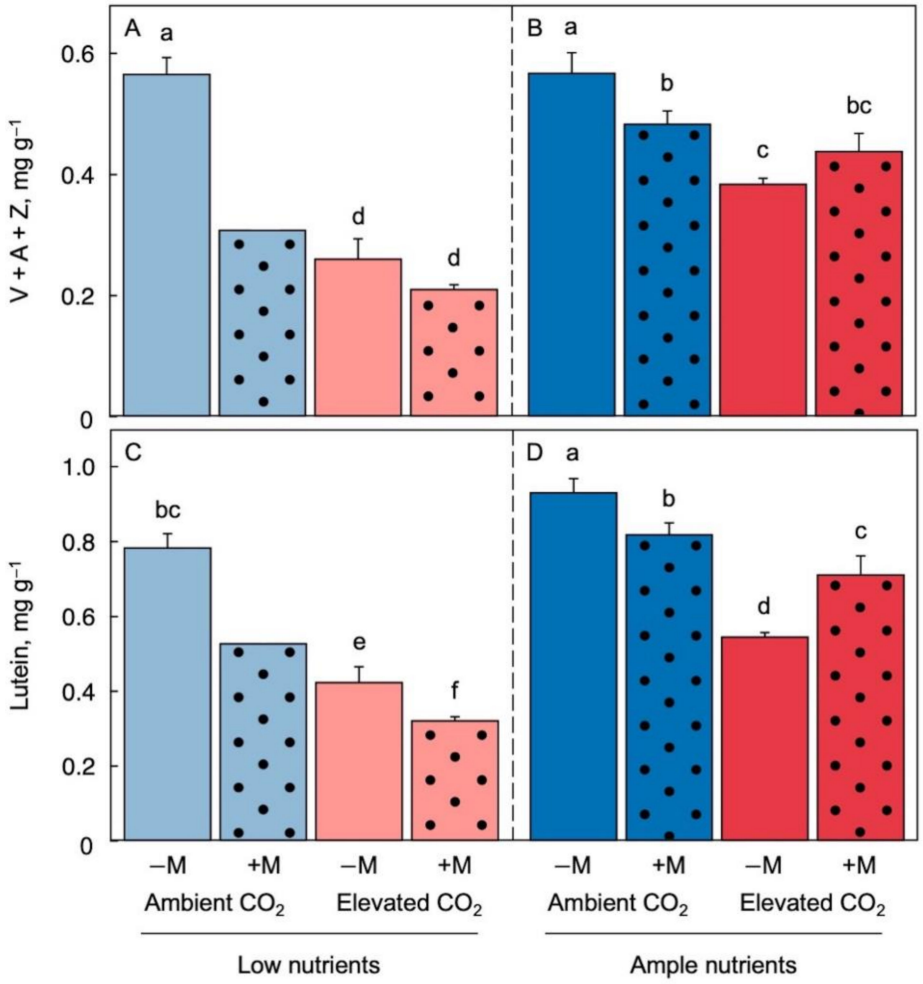

Figure 5. Ratios of the three carotenoids of the xanthophyll cycle to biomass under low (**A**) and ample (**B**) nutrients as well as lutein to biomass under low (**C**) and ample (**D**) nutrients for *Lemna minor* grown in 1/20 (light blue or light red) or 1/2 strength (dark blue or red) Schenk & Hildebrandt medium and either ambient (blue) or elevated (red) CO_2 levels. Bars with solid fill represent groups that were not inoculated (−M) compared to bars with dotted fill that were inoculated (+M). A, antheraxanthin; V, violaxanthin; Z, zeaxanthin. Mean values ± standard deviations; $n = 3$ under all conditions except for $n = 2$ in the treatment of inoculated fronds in ambient CO_2 and low nutrients. Different lower-case letters represent significant differences at $p < 0.05$.

3. Materials and Methods

3.1. Plant Species and Growth Conditions

The duckweed species *Lemna minor* L. 7136 was obtained from Rutgers Duckweed Stock Cooperative (http://www.ruduckweed.org; accessed on 20 December 2022). A single duckweed colony consists of a larger mother frond in the center, two (initially smaller) daughter fronds emerging from the mother frond, and a small root-like structure that connects to the center of the mother frond and extends downward perpendicular to the water surface [83].

A stock culture of duckweed was maintained in Conviron PGR15 growth chambers under a 14 h photoperiod of 200 µmol photons m^{-2} s^{-1} with an air temperature of 25 °C in 1000 mL of 1⁄2 strength Schenk and Hildebrandt (SH) nutrient medium (bioWORLD, Dublin, OH, USA) in PYREX crystallizing dishes (Corning Inc., Corning, NY, USA).

Experimental *L. minor* plants were grown as described by Stewart and coworkers [84] under 200 µmol photons m^{-2} s^{-1} at 25 °C in 270 mL dishes in growth chambers under either ambient (430 ppm) or elevated (860 ppm) atmospheric CO_2 and in either replete

(1/2 strength) or low (1/20 strength) nutrient concentration under a 24 h photoperiod. Furthermore, plant lines used were those obtained from Rutgers (that had previously been sterilized by Rutgers) and were either untreated (uninoculated) or subjected to inoculation with microorganisms from a local pond that supported duckweed populations. Each experimental dish was initially seeded with four duckweed colonies.

Experiments were conducted in two phases. The acclimation phase was an initial pre-adaptation phase of 48 h and was followed by an experimental phase of 72 h. Upon the completion of the acclimation phase, fresh medium was supplied, and the number of colonies was thinned back to four before the start of the experimental phase.

3.2. Light and CO_2 Supply for Plant Growth

The lighting system was comprised of 23 rows of light strips (each 22.3 inches long) with 240 white light emitting diodes (LEDs) per meter (PN 4000K-CC2835LM-240-14-reel; Environmental Lights, San Diego, CA, USA). The LEDs were mounted to a 24 × 24-inch white honeycomb panel that acted as a reflector and heat dissipation plate. The LEDs had a color of 4000 K, a color-rendering index of 92, with a sharp peak between 420 and 480 nm, and a broad peak from 500 to 700 nm (Figure 6). Photon flux density (PFD) was controlled by a Space Lab® (Space Lab Technologies, LLC, Boulder, CO, USA) graphical user interface via a variable DC power supply with photoperiod and pulsing capability. The panel was placed at a height of approximately 36 inches from the plant samples to ensure uniform light distribution.

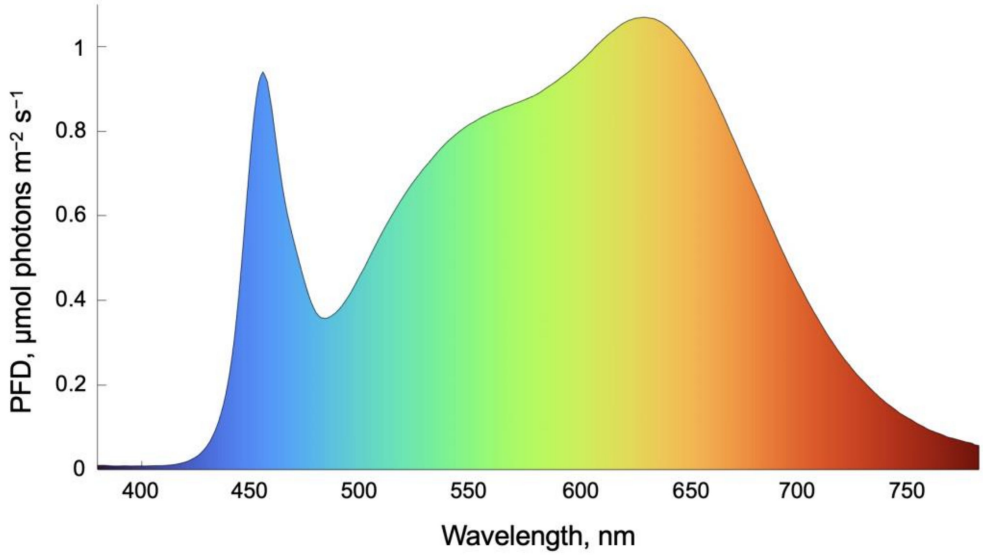

Figure 6. Spectral quality for Environmental Lights PN 4000k-CC2835LM-240-14-reel as PFD in µmol photons m^{-2} s^{-1} as a function of wavelength (nm). Integrated PFDs (total µmol photons m^{-2} s^{-1}) for the following wavelength windows were 0.02 (UV: 380–400 nm), 34.4 (blue: 400–500 nm), 78.7 (green: 500–600 nm), 87.6 (red: 600–700 nm), and 14.60 (far-red: 700–780 nm).

Space Lab® upgraded the PGR15 growth chamber with the capability to control CO_2 concentrations from ambient to 20,000 ppm to within 2% of set-point value. The primary control system included a regulated solenoid valve to regulate CO_2 flow into the chamber from a compressed gas source. The control system utilized a proportional-differential control scheme with minimal overshoot (<1%). For CO_2 concentration measurements and control system feedback, a GMP343 non-dispersive infrared probe (Vaisala, Vantaa, Finland) was utilized, which compensates for temperature, pressure, relative humidity, and oxygen concentration effects in real-time.

3.3. Inoculation Treatment

Sterilized *Lemna minor* plants were inoculated (following the protocol of [41]) with the microbiome associated with *L. minor* populations growing on a pond near the University of Colorado. Within 24 h of pond-water collection fronds were transferred from sterile/sanitized stock cultures to 1 L of pond water containing microorganisms and floated on the water surface for four hours permitting colonization of the plant with microorganisms.

3.4. Protein Extraction and Analysis

Protein content was analyzed using the Total Protein Kit, Micro Lowry, Peterson's Modification (Sigma-Aldrich, Saint Louis, MO, USA) based on the Lowry method recognized for its simplicity and accuracy [85–87]. This protein assay kit allows for rapid recovery of proteins and minimizes potential interferences of phenolics with protein detection [88]. Protein concentration was assessed spectrophotometrically (Beckman DU 640 Spectrophotometer) using a calibration curve with bovine serum albumin. Samples for protein analysis were collected on the last day of the experimental phase and immediately frozen in liquid nitrogen until analysis.

Duckweed samples were removed from liquid nitrogen and six fronds were ground using a glass mortar and pestle. Crushed duckweed samples were combined with 1 mL of deionized water in a microcentrifuge tube and processed for protein analysis as described previously [84]. Absorbance at 660 nm was measured spectrophotometrically (Beckman DU 640 Spectrophotometer; Beckman Instruments, Inc., Fullerton, CA, USA) and values converted to protein levels using a standard calibration curve based on a gradient of bovine serum albumin.

3.5. Dry Frond Mass and Frond Area

From among a total of nine dishes, three were dedicated to daily assessment of relative growth rate (RGR of frond area expansion) during the experimental phase and six dishes were used for daily frond collection for determination of frond dry biomass accumulation (RGR of dry biomass as well as biomass per frond area), samples for protein content and photosynthetic pigment content were collected once at the end of the experimental phase.

To determine dry biomass accumulation, samples of known frond area were placed for a minimum of 48 h into an oven kept at 70 °C after frond area was assessed for these samples from photographs taken from directly above. Frond area was also assessed on a daily basis from the photographs using ImageJ software as described by Stewart and coworkers [84]. Total frond area per dish was determined as the measured percentage of total water surface containing fronds multiplied by the surface area of a crystallizing dish (90 mm inner diameter).

3.6. Relative Growth Rate

Daily relative growth rate was obtained as the difference in ln-adjusted frond area or frond mass divided by the time elapsed (approximately 24 h) between two measurements (see [89]) using the equation below where X2 is frond area or frond mass on the selected day, X1 is frond area or frond mass on the previous day, and Δt is time elapsed between the two measurements. Averages of daily relative growth rates were also calculated over the entire duration of the experiment.

$$\text{Relative Growth Rate} = \frac{ln(\text{X2}) - ln(\text{X1})}{\Delta \text{t}} \quad (1)$$

3.7. Pigment Extraction and Analysis

Levels of chlorophylls *a* & *b*, β-carotene, and the xanthophylls lutein, zeaxanthin (Z), antheraxanthin (A), and violaxanthin (V) were quantified via high-performance liquid chromatography (HPLC) as previously described [90]. Multiple intact fronds were collected under the respective growth conditions, imaged (for quantification of frond area), and

then frozen and stored in liquid nitrogen [53]. Pigments were extracted with acetone as described previously [91] and then separated and quantified with a bonded silica C30 Carotenoid 3-µm column (YMC America Inc., Devens, MA, USA) and a Shimadzu (Shimadzu Corporation, Kyoto, Japan) HPLC system. At a flow rate of 1 mL min^{-1}, a linear gradient from 100% solvent A [92] to 100% solvent B (4:1 mixture of methanol and hexanes, respectively) was used to elute the xanthophylls and chlorophylls, and then solvent B was run isocratically to elute β-carotene. Solvents were prepared fresh with HPLC-grade constituents (Thermo Fisher Scientific Inc., Waltham, MA, USA).

3.8. Statistical Analysis

Statistically significant differences were determined via one-way analysis of variance (ANOVA) and post hoc Tukey–Kramer test for honestly significant differences. Sample size was three replicates per parameter and sampling day. One-way ANOVA analyses were conducted using JPM Pro 16 software (SAS Institute Inc., Cary, NC, USA).

4. Conclusions and Recommendations

4.1. Costs and Benefits of Plant-Microbiome Interaction at a Glance

Specific outcomes varied for the interaction between inoculation, abiotic conditions, and the specific functional feature considered (area-expansion, biomass accumulation, and protein versus micronutrients).

Minor apparent costs of inoculation (somewhat lower area-expansion rate and nutritional content) in low versus high nutrient supply under ambient CO_2 may be associated with competition between plants and microorganisms over limiting mineral nutrients, perhaps primarily those other than nitrogen. It is likely that similar effects may occur in land plants as seen here in duckweed.

Clear benefits of plant-microbiome interaction were seen predominantly under the combination of elevated CO_2 with low nutrient supply where inoculation (i) prevented area-growth penalties and (ii) allowed greater accumulation of biomass with an unaltered protein-to-biomass ratio. It is likely that the rather low sensitivity of biomass quality to elevated CO_2 with respect to protein content is unique to duckweed as an aquatic floating plant with a high capacity for vegetative protein storage. Inoculation also lessened or prevented decreases in carotenoid/biomass ratios for several carotenoids that are essential human micronutrients. It is likely that land plants will experience similar effects as demonstrated here for duckweed.

Overall, the findings from this study are consistent with other reports that beneficial microorganisms maintain growth of new tissue and counteract imbalances in source-sink ratio and C:N ratio by improving plant nitrogen acquisition and consuming carbohydrates supplied by the plant partner. Duckweed may be of particular interest due to the insensitivity of its high protein content to modulation by environmental conditions and the ability of inoculation to maintain micronutrient-to-biomass ratios under the combination of elevated CO_2 and ample nutrient supply (either in agricultural settings or during fertilization of natural communities; [93,94]).

4.2. Future Research

This study further demonstrates the profound and complex interactions among multiple abiotic factors and plant-microbe interactions, and the need for multi-factorial analysis. Duckweed is a suitable model organism for such analyses due to its fast growth rate, small size, and ease of inoculation. Duckweed is also an attractive crop candidate with a remarkable degree of resilience under elevated CO_2, especially when supported by its microbiome. It should be noted that our inoculation used a mixed community of microorganisms from a pond that supports duckweed. The effect of different microbial strains on plant growth may vary and complex interactions among microorganisms may also occur [41]. Future research should identify the microbial strains present and their effects as well as interactions. One

can envision a future approach with customized communities for specific agricultural goals and growth conditions [95].

Specific targets for future research thus include characterization of links between specific microbial clades and modulation of specific plant processes. Such efforts should include differentiation among different mineral nutrients (especially nitrogen and phosphorus) with respect to uptake by the plant and resulting internal concentrations as well as C:N ratios and frond anatomical features like thickness and number of chloroplast-rich cell layers per area. Moreover, the effect of light supply (both light intensity and photoperiod) as an additional environmental factor deserves further attention, especially when high light supply is combined with low mineral nutrient supply and elevated CO_2.

We previously reported that a combination of elevated CO_2 and continuous very high light supply (1000 µmol photons m^{-2} s^{-1}) caused pronounced declines in pigment content, including excess-light-induced zeaxanthin, under ample nutrient supply [18]. As expected, the moderate light supply used here resulted in negligible amounts of zeaxanthin (not shown) under any of the conditions tested. Future studies should address the plant microbiome's effect on zeaxanthin content under combinations of elevated CO_2 with a range of light, temperature, and nutrient levels.

Lastly, long-term response to elevated CO_2 and/or low nutrient supply is of interest. It has been reported that long-term exposure to elevated CO_2 shortens plant lifespan because the latter is regulated by internal carbohydrate supply [17]. The fact that duckweed undergoes rapid vegetative propagation allows study of aging/senescence at the population level as affected by elevated CO_2 in combination with other abiotic factors as well as plant-microbe interaction.

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