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Organic Carbon Is Ineffective in Enhancing the Growth of *Dunaliella*

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Abstract: (1) Background: Mixotrophic growth is commonly associated with higher biomass productivity and lower energy consumption. This paper evaluates the impact of using different carbon sources on growth, protein profile, and nutrient uptake for *Dunaliella tertiolecta* CCAP 19/30 to assess the potential for mixotrophic growth. (2) Methods: Two experimental sets were conducted. The first assessed the contribution of atmospheric carbon to *D. tertiolecta* growth and the microalgae capacity to grow heterotrophically with an organic carbon source to provide both carbon and energy. The second set evaluated the impact of using different carbon sources on its growth, protein yield and quality. (3) Results: *D. tertiolecta* could not grow heterotrophically. Cell and optical density, ash-free dry weight, and essential amino acids index were inferior for all treatments using organic carbon compared to $NaHCO_3$. Neither cell nor optical density presented significant differences among the treatments containing organic carbon, demonstrating that organic carbon does not boost *D. tertiolecta* growth. All the treatments presented similar nitrogen, phosphorus, sulfur recovery, and relative carbohydrate content. (4) Conclusions: Based on the results of this paper, *D. tertiolecta* CCAP 19/30 is an obligated autotroph that cannot grow mixotrophically using organic carbon.

Keywords: mixotrophic growth; wastewater reuse; protein; essential amino acids; *Dunaliella tertiolecta*

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1. Introduction

Dunaliella is a genus of halophilic chlorophyte microalgae distinguished by the absence of a cell wall. It is well known for its ability to cope with extreme environments and to yield bioproducts with industrial relevance, such as carotenoids [1], lipids [2], and protein [3]. In the 1970s and early 1980s, a great deal of research was undertaken with two distinct commercial objectives: (i) to produce glycerol in commercial quantity and (ii) to produce nutritional chemicals, notably carotenoids and particularly β-carotene, and key patents were established [4,5]. *Dunaliella* has also been cultivated for the colorless carotenoids, phytoene and phytofluene [6]. However, the protein content of *Dunaliella* can reach over 50% on an ash-free dry weight basis [7]. In recent years, interest in the potential of *Dunaliella* to serve as a protein source with a high-quality amino acid profile has gained momentum in response to the increasing demand for alternative food, feed, and health supplement sources [8]. In 2020, the food additives sector represented 24.26% of the global market for *D. salina* [9]. Large-scale *Dunaliella* cultivation already exists with satisfactory productivity [10]; however, some factors still limit its worldwide cultivation, such as local

policies and consumer acceptability. One approach is to lessen the cultivation costs to make *Dunaliella* bioproducts more accessible by using alternative and cheaper medium or substrate sources, e.g., treated wastewater [11,12]. Although, the use of wastewater for that purpose depends on its source and local legislation.

Most microalgae are photoautotrophic-restricted microorganisms because of the absence of genes encoding organic carbon transporters [13], i.e., they fixate inorganic carbon through photosynthesis. However, photoautotrophic growth may not be efficient enough to support high-density cultivation because of self-shading [14]. Large-scale *Dunaliella* production is currently based on photoautotrophic growth in media containing inorganic nutrients with CO_2 as exclusive carbon sources. However, since cell density and biomass are important factors for large-scale production, biomass accumulation might be improved by mixotrophic cultivation [15]. In mixotrophic growth, metabolic processes [16] do not suffer from light limitation, and both organic and inorganic carbon are assimilated. Several mechanisms participate in organic carbon assimilation, such as phosphorylation for sugars, diffusion for glycerol, and membrane transporter proteins for organic acids [17]. This feature permits microalgae to treat and grow in wastewater where organic carbon source is abundant [18,19], such as in dairy wastewater [20]. The combination of such mixotrophic growth of microalgae and nutrient recovery from food industrial wastewater offers great opportunity in the context of the circular economy, sustainable and efficient microalgal cultivation, and bioproducts generation [21]. Additionally, the residual biomass can go under different biochemical processes, such as fermentation and anaerobic digestion, to recover methane and biohydrogen after the extraction of higher-value microalgal bioproducts [22], increasing even further the potential for a bio-based economy. Mixotrophic growth is commonly associated with higher biomass productivity [23] and lower energy consumption considering the need for lower light intensity [24]. Moreover, protein content can be directly affected by the carbon source. Abreu et al. [25] identified the best protein productivity for *Chlorella vulgaris* under mixotrophic growth in dairy wastewater. Improvement in protein productivity and changes in amino acid profile in mixotrophic and heterotrophic systems have also been described for other microalgae species (e.g., *Chlamydomonas reinhardtii* [26], *Euglena gracilis* [27], and *Microcystis aeruginosa* [28]).

Mixotrophic metabolic activity has recently been documented mainly for *D. salina*, and glucose is the most reported organic carbon source to cultivate *Dunaliella* mixotrophically, followed by glycerol, acetate, food waste, and other sources (Figure 1).

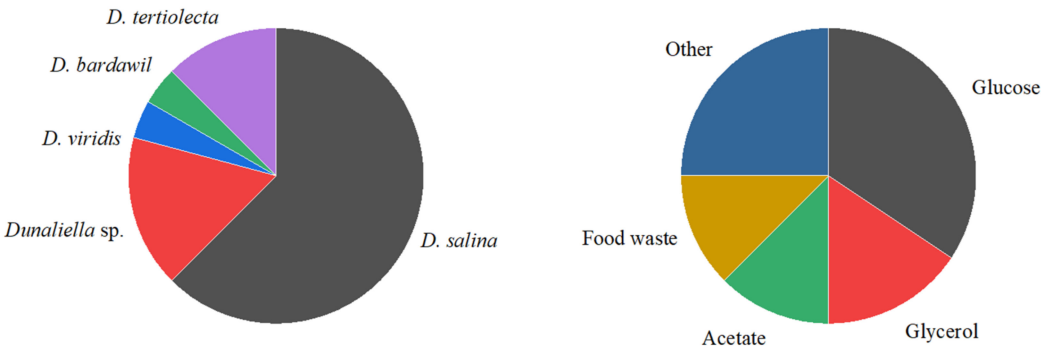

Figure 1. Distribution of papers on the platforms Scopus and Web of Science (1996–2022) regarding the use of different species of *Dunaliella* (**left**) and organic carbon sources (**right**) [14,16,24,29–46]. References were collected on 25 February 2022.

Kadkhodaei et al. [29] cultivated *D. salina* under mixotrophic conditions and obtained the highest protein productivity using 5 g L^{-1} glucose as a carbon source, although the authors did not assess *D. salina* photoautotrophic growth. Acetyl-CoA and NADPH processes, essential pathways for the accumulation of protein and lipids, are reported to be triggered for some species when cultivated in glucose [33], which could explain the results obtained by the previous authors. However, Wan et al. [33] achieved a minimal increase in

protein and lipids for *D. salina* FACHB 435 in mixotrophic growth using glucose, despite the increase in the biomass. This demonstrates that the role of Acetyl-CoA and NADPH in the fate of organic carbon is not clearly understood and is sometimes contradictory. In addition, most of the glucose remained in the medium after 12-day cultivation, demonstrating that the increase in growth is not necessarily associated with organic carbon uptake [33]. These studies contrast with the widely held view that *Dunaliella* is a photoautotrophic-obligated genus [47]. Kim et al. [14] tried to cultivate *D. salina* and *Dunaliella* sp. mixotrophically in different organic carbon sources (glucose, xylose, rhamnose, fructose, sucrose, and galactose). Their results showed that glucose did not affect their growth, and the other organic carbon compounds inhibited it.

Clearly, details pertaining to mixotrophic growth in *Dunaliella* sp. are far from clear, and its strain dependence [17] urges further research. The research on the topic is recent, and many gaps are yet to be filled. For instance, the study on growth and protein quality and quantity must be broadened to include other organic carbon sources to explore the potential of cultivating *Dunaliella* in different wastewater sources, since most papers are limited to glucose [29,30] and glycerol [38,48]. This paper aims to evaluate the ability of mixotrophic growth in *Dunaliella tertiolecta* CCAP 19/30 by analyzing its growth, amino acid profile, and nutrient uptake using different carbon sources (bicarbonate, lactose, glucose, galactose, fructose, maltose, glycerol, and malonic acid).

2. Materials and Methods

2.1. Microalga Strain and Cultivation Method

D. tertiolecta CCAP 19/30 was purchased from the Culture Collection of Algae and Protozoa (CCAP, Scotland, UK). The stock culture was cultivated in sterilized Modified Johnson's Medium [49] (pH 7.5, salinity 1.5 M NaCl), refreshed monthly in a controlled temperature chamber (Varicon Aqua, Worcester, UK) at 25 °C under average 100 µmol photons m^{-2} s^{-1} LED-white light prior to use.

2.2. Preliminary Experiment

The Modified Johnson's Medium [49] used in the preliminary experiment was the same used for the stock solution (pH 7.5, salinity 1.5 M NaCl) with some modifications regarding the carbon source: either 0.84 g L^{-1} of $NaHCO_3$ or 0.285 g L^{-1} of lactose to have a final carbon concentration of 0.12 g L^{-1} was used. The final carbon concentration of 0.12 g L^{-1} was chosen to be equivalent to the Modified Johnson's Medium [49]. To assess the contribution of atmospheric CO_2, one treatment using Modified Johnson's Medium without any carbon source was applied. One further treatment using Modified Jonhson's Medium containing lactose under 24 h dark was also applied to assess the capacity of heterotrophic growth. This resulted in four treatments, namely $NaHCO_3$, Lactose, $AtCO_2$, and Lactose_Dark. All the treatments were conducted in triplicate, inoculated with an initial density of around 1.81×10^5 cell mL^{-1}, and placed on a magnetic-stir plate in a controlled temperature chamber (Varicon Aqua, Worcester, UK) (130 rpm, 25 °C, 100 µmol photons m^{-2} s^{-1}; continuous LED-white light). Lactose_Dark was placed under the same conditions but without light throughout the cultivation period. The cultivation was stopped when the growth reached the stationary phase.

2.3. Mixotrophic Experiment

As described by Sui, et al. [50], Modified Johnson's Medium (pH 7.5, salinity 1.5 M NaCl) was used as a base for each treatment containing only one carbon source (0.84 g L^{-1} of $NaHCO_3$, 0.285 g L^{-1} of lactose, 0.3 g L^{-1} of glucose, 0.3 g L^{-1} of galactose, 0.3 g L^{-1} of fructose, 0.347 g L^{-1} of malonic acid, 0.3 g L^{-1} of maltose, or 0.307 g L^{-1} of glycerol) to have a final carbon concentration of 0.12 g L^{-1}, as discussed in Section 2.2. All treatments were conducted in triplicate in a controlled environment (25 °C; 200 µmol photons m^{-2} s^{-1}; continuous LED-white light; 100 rpm agitation) using Algem® HT24 photobioreactor from

Algenuity (Stewartby, Bedfordshire, UK) (https://www.algenuity.com/; accessed on 20 May 2022). The cultivation was stopped when the growth reached the stationary phase.

2.4. Sample Analysis

2.4.1. Cell Growth

For both experiments, samples were collected daily to count the cells in a hemocytometer using a Motic BA310 microscopy (40× magnification). For the preliminary experiment, optical density at 740 nm (OD740) was recorded daily by placing 0.3 mL sample in a 96-well microplate and reading in a microplate reader (Thermo Scientific Multiskan GO, Oxford, UK). For the mixotrophic experiment, OD740 was automatically recorded every 10 min by the Algem® HT24 photobioreactor (the data was converted into daily results for better visualization).

2.4.2. Ash-Free Dry Weight

At the end of both experiments, an aliquot of microalgal suspension from all treatments was filtered using glass fiber filters (Millipore, Ø 24 mm, pore size 0.7 µm); dried at 105 °C overnight; cooled in a desiccator at room temperature; weighted (A); placed in a 550 °C muffle furnace (Vecstar Ltd., Chesterfield, UK) for two hours; cooled in a desiccator at room temperature; and weighted again (B) to gravimetrically assess the ash-free dry weight (AFDW) (Equation (1)):

$$\text{AFDW}\left(\text{g L}^{-1}\right) = \frac{\text{A} - \text{B}}{\text{V}} \times 1000 \quad (1)$$

A and B are the initial and final weight (g), respectively, and V is the volume of microalgal suspension (L).

2.4.3. Nutrient Analysis

Samples before inoculation and the filtrate from the mixotrophic experiment at the end of the treatment were stored at −20 °C and later used to quantify NO_3-N, PO_4-P, and SO_4-S concentrations using test kits (Merck, Darmstadt, Germany) for nitrate (1.09713.0002), for phosphate (1.14848.0002), and for sulfate (1.01812.0001) following the manufacture instructions. Nutrient removal efficiency ($R_{efficiency}$) was calculated by comparing the nutrient concentration remaining in the medium at end of the treatment to the initial concentration (Equation (2)).

$$R_{efficiency}(\%) = \frac{\text{FC} - \text{IC}}{\text{IC}} \times 100 \quad (2)$$

FC and IC are the final and initial nutrient concentration.

2.4.4. Amino Acids

Microalgal suspension from the mixotrophic experiment was centrifuged at 5000× *g* for 5 min to pelletize the biomass, and hydrolyzed with 6 mol L^{-1} HCl solution in a vacuum-sealed ampule glass tube for 22 h at 110 °C before sample preparation according to the instructions of the Phenomenex EZ:faast amino acid analysis kit [51]. Essential amino acids (EAA) were determined by a gas chromatography system with a flame ionization detector (GC-FID) (Agilent 7890A, Stockport, UK) and the essential amino acid index (EAAI) was calculated by Equation (3):

$$\text{EAAI} = \sqrt[n]{\frac{\text{aa1}}{\text{AA1}} \times \frac{\text{aa2}}{\text{AA2}} \times \ldots\ldots \times \frac{\text{aan}}{\text{AAn}}} \quad (3)$$

aan and AAn are the EAA content (mg EAA g^{-1} total protein) in the sample and are referenced by the Food and Agriculture Organization/World Health Organization (FAO/WHO), respectively, and protein quality was classified according to Table 1. Bovine serum albumin (BSA) was used as a control to calculate the amino acid recovery.

Table 1. Protein quality classification based on the essential amino acid index (EAAI) [52].

Quality	Range
High	EAAI > 0.95
Good	0.86 < EAAI ≤ 0.95
Useful	0.75 < EAAI ≤ 0.86
Inadequate	EAAI ≤ 0.75

2.4.5. Organic Carbon Analysis

Filtered samples from the start and the end of the mixotrophic experiment were diluted with acetonitrile (1:1, *v*:*v*) before analysis. The relative content of organic carbon was quantified according to peak areas normalized to internal control (Equation (4)) based on the methodology proposed by Liu et al. [53]:

$$RC = \frac{Af}{Ai} \quad (4)$$

where RC is the relative content; Af and Ai are the peak areas of samples spiked with the internal standard at the end and the start of the experiment.

A high-performance liquid chromatography equipped with a refractive index detector (HPLC-RID; Agilent Technologies 1100 series, Agilent, Santa Clara, CA, USA), a Zorbax Carbohydrate column (4.6 × 250 mm, 5 µm) (Agilent, Santa Clara, CA, USA) set at 40 °C. The isocratic elution with acetonitrile:water (75:25, *v*/*v*) and a flow rate of 1.8 mL min^{-1} were used. The carbohydrates were identified and peak integrated areas computed by the ChemStation for LC Rev. A.10.02 software.

2.5. Statistical Analysis

All the treatments from both experiments were conducted in triplicate, and the results were expressed as mean ± standard deviation; for better readability, the standard deviation was omitted within the text. OriginPro 2022 (OriginLab Corporation, Northampton, MA, USA) was used to plot the graphs and to compare the groups using Tukey's test with a significance level of $p \leq 0.05$. Past v. 4.07 software [54] was used to perform the correlation analysis among the parameters, and the correlation strength was classified according to Table 2.

Table 2. Summary of Pearson correlation coefficient (r) strengths [55].

Strength of Correlation	Range of Absolute Correlation Coefficient (r)
Very strong	0.8–1.0
Strong	0.6–0.79
Moderate	0.4–0.59
Weak	0.2–0.39
Very weak	0–0.19

3. Results and Discussion

3.1. Preliminary Experiments

D. tertiolecta presented different growth profiles among the treatments. It reached the stationary phase earlier for $NaHCO_3$ treatment (day 6) than for Lactose and $AtCO_2$ (day 7) (Figure 2a). One reason could be the higher bioavailability of carbon within the medium in the treatment with $NaHCO_3$, favoring a faster microalgal growth. The treatments Lactose and $AtCO_2$ presented similar growth profiles, and no significant difference in cell density, optical density, and AFDW was reported, which could indicate that the carbon source was the same, i.e., atmospheric carbon. Since the carbon demand is proportional to cell density, until day 7, the carbon transferred from the atmosphere was enough to support microalgal growth in both treatments. However, on day 8, the carbon transfer could not support *D. tertiolecta* growth any longer, explaining the sudden drop in the optical density.

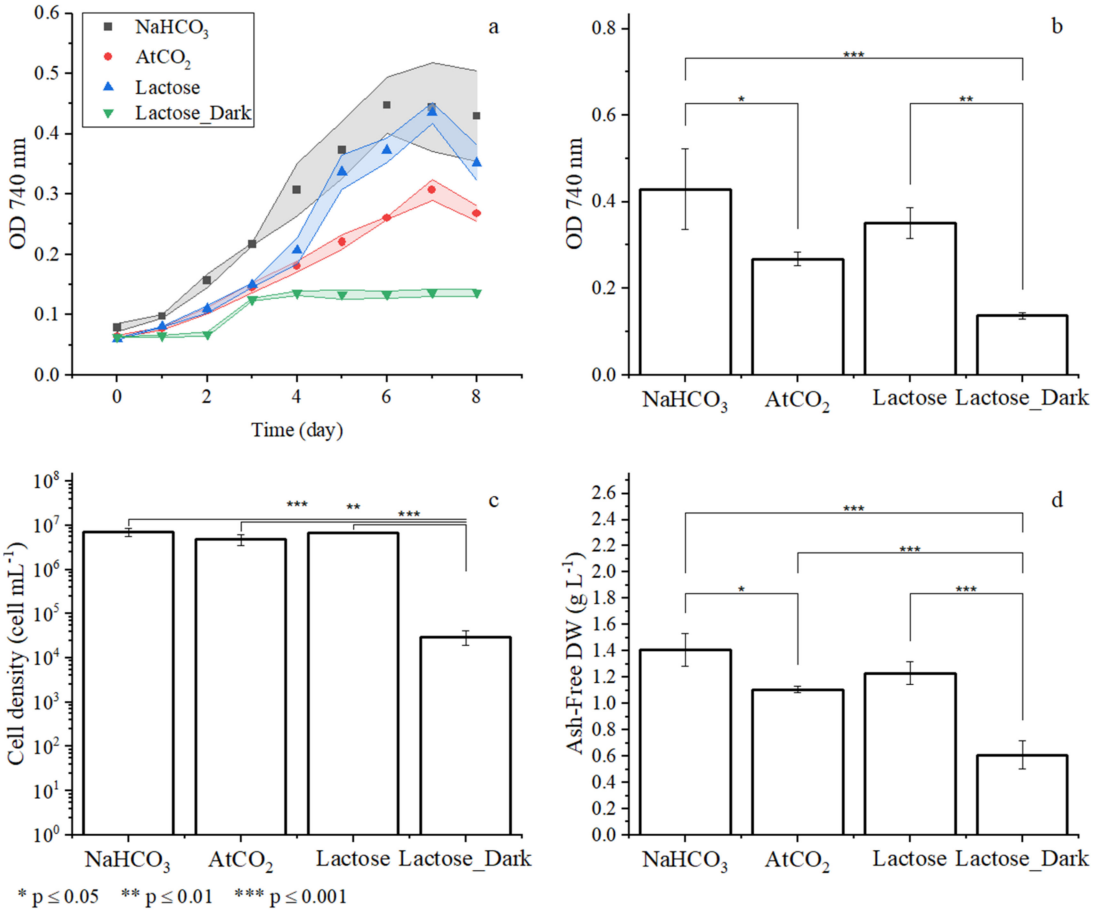

Figure 2. *D. tertiolecta* growth profile in the preliminary experiment. Growth curve (**a**), optical density (**b**), cell density (**c**), and ash-free dry weight (AFDW) (**d**) at the end of the cultivation period.

Lactose_Dark optical density slightly increased on day 3 and maintained roughly the same value for the rest of the cultivation period, significantly lower than the other treatments (Figure 2a,b). The cell density maintained a similar concentration during the first days, increasing on day 3, and then progressively died toward day 7 (data not shown). This growth behavior had been described for *Dunaliella parva* CCAP 19/9 under similar conditions [56]. This shows that some species may store energy and carbon to cope with light-absence periods [57,58] and then start losing biomass due to respiration in the dark [59]. *D. tertiolecta* seems to not possess the required features to grow heterotrophically, such as the capacity of (a) cell division and active metabolisms in dark conditions and (b) growing on inexpensive and easy to sterilize organic substrate [60], features that *D. tertiolecta* does not seem to possess, contrasting previous research [61]. A moderate negative correlation was observed between the cell and optical density throughout the cultivation period for the treatment Lactose_Dark ($r = -0.438$, $p = 0.02$). This demonstrates that the optical density must be associated with bacterial growth. Under dark conditions and organic carbon supply, bacterial growth is expected to outperform microalgal growth [17]. Axenic cultures of *Dunaliella* strains are practically impossible despite laboratory painstaking efforts to purify the cultures. Co-habitant heterotrophic halophilic prokaryote and archaea are consistently transferred through *Dunaliella* cultures [62].

A strong positive correlation ($r > 0.89$, $p = 0.0001$) was recorded between cell and optical density for the remaining treatments. This indicates that the bacterial growth was minimal in the presence of light, and the optical density was associated with *D. tertiolecta* growth. The optical density recorded for the treatment Lactose most likely was a combination of microalgal (supported by atmospheric carbon) and microbial (supported by the organic carbon) growth.

Xie, Lin and Luo [40] showed that co-cultivation of *Dunaliella* sp. and the bacteria *Cellvibrio pealriver* using organic carbon promoted better microalgal growth than mono cultivation of the microalga. One plausible explanation is that *C. pealriver* consumed organic carbon for growth and catalyzed it into some active substrate that *Dunaliella* sp. could metabolize. Cho et al. [63] assessed a bacteria-microalga consortium and demonstrated that heterotrophic bacteria released inorganic carbon that was later utilized by *Chlorella vulgaris*, enhancing its growth. Although *Dunaliella*'s interaction with inherent heterotrophic microorganisms regarding mixotrophic growth remains a grey area, the growth of *Dunaliella* in organic carbon might be a result of symbiotic microalga-bacteria interaction. Consequently, neglecting the possible contribution of other microorganisms in the medium may mislead the conclusion that *Dunaliella* sp. can grow mixotrophically.

At the end of the experiment (day 8), the treatment $NaHCO_3$ promoted the best *D. tertiolecta* growth among all the treatments regarding optical density (0.430; Figure 2a), cell density (7.22×10^6 cell mL^{-1}; Figure 2c), and AFDW (1.44 g L^{-1}; Figure 2d).

3.2. Mixotrophic Experiment

3.2.1. Biomass Growth

Although the results from the preliminary experiment suggested the inability of *D. tertiolecta* to use lactose, a mixotrophic experiment was conducted to evaluate the effect of other organic carbon sources. The growth profiles for all the treatments were similar, reaching the stationary phase on day 7 (Figure 3). No significant difference in the optical density was identified except for the Malonic Acid treatment, which presented no growth; thus, it was recorded only until day 4 and not assessed for nutrient, carbon removal, and essential amino acid.

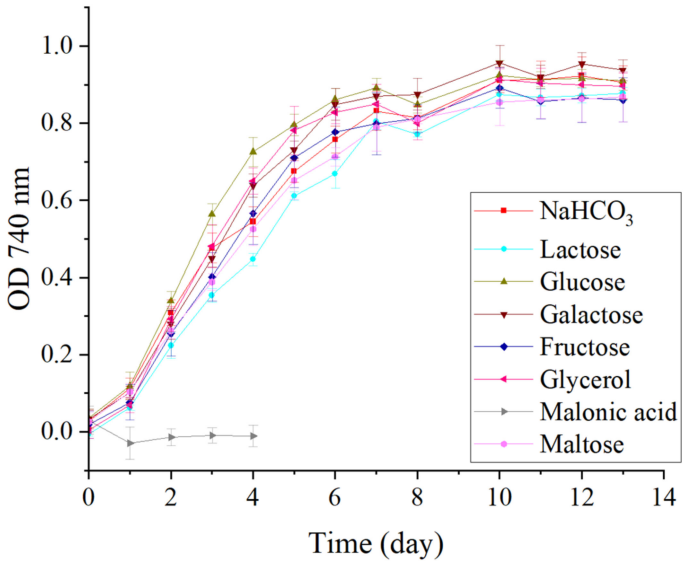

Figure 3. Growth curve of *D. tertiolecta* using different carbon sources in the mixotrophic experiment.

$NaHCO_3$ presented the highest cell density (4.64×10^6 cells mL^{-1}) and the highest AFDW (1.43 g L^{-1}) among all the treatments at the end of the cultivation (Table 3). The basic disposition of microalgae is photoautotrophic, i.e., the supply of CO_2 is required for growth; however, under low-CO_2 availability, carbon accumulation mechanisms (CCM) are triggered, and inorganic carbon from the medium is assimilated [64]. The effective incorporation of bicarbonate into *D. salina* biomass had been described (>90% carbon utilization efficiency) [65]. The only source of CO_2 for *D. tertiolecta* in our experiments was the atmospheric carbon diffused into the medium. Thus, in the presence of $NaHCO_3$, *D. tertiolecta* may have had its CCM activated, resulting in higher cell growth.

Table 3. Cell density and AFDW using different carbon sources in the mixotrophic experiment.

Treatment	Cell Density Cell mL^{-1}	AFDW $g\ L^{-1}$
$NaHCO_3$	$4.64 \times 10^6 \pm 1.81 \times 10^5$ [a]	1.43 ± 0.02 [a]
Lactose	$3.85 \times 10^6 \pm 4.89 \times 10^5$ [ab]	1.29 ± 0.05 [b]
Glucose	$3.12 \times 10^6 \pm 4.61 \times 10^5$ [b]	1.10 ± 0.02 [c]
Galactose	$3.65 \times 10^6 \pm 4.98 \times 10^5$ [ab]	1.17 ± 0.03 [bc]
Fructose	$3.40 \times 10^6 \pm 1.94 \times 10^5$ [b]	1.12 ± 0.05 [c]
Glycerol	$3.25 \times 10^6 \pm 2.10 \times 10^5$ [b]	1.12 ± 0.03 [c]
Maltose	$3.64 \times 10^6 \pm 4.76 \times 10^5$ [ab]	1.11 ± 0.05 [c]

Values within the same column with different superscript letters represent significant differences ($p \leq 0.05$).

The mechanisms involved in bicarbonate assimilation by marine microalgae reflects a long process of adaptation, and two of them are widely found: (a) HCO_3^- is catalyzed to CO_2 by extracellular carbonic anhydrase at the cell surface, then, passive diffusion into the cell takes place; (b) anion exchange transports HCO_3^- across the cell membrane, followed by its dehydration by intracellular carbonic anhydrase [66]. *D. tertiolecta* presents higher extracellular carbonic anhydrase activity than intracellular at low-CO_2, especially toward higher salinities [67–69], which increases the affinity for inorganic carbon [70]. Thus, low-energy consuming passive diffusion must be the mechanism responsible for assimilating $NaHCO_3$ in this study.

Neither cell nor optical density presented significant differences among the treatments containing organic carbon. These demonstrate that organic carbon does not boost *D. tertiolecta* growth, which could also be linked to atmospheric carbon transfer into the medium. Although we did not assess different organic carbon concentrations, it is expected that higher concentrations would result in similar behavior or present an inhibitory effect on *D. tertiolecta* growth, as demonstrated by Rizwan, Mujtaba and Lee [61]. Kim et al. [14] cultivated *D. salina* DCCBC2 and *Dunaliella* sp. mixotrophically in a medium containing 10 mM of different organic compounds (glucose, xylose, rhamnose, fructose, sucrose, or galactose), and they found that neither of the microalgae had their growth enhanced by glucose and that the other sugars inhibited them. Andreeva et al. [71] found similar results; the addition of 0.05–5 g L^{-1} of glucose, sucrose, and fructose led to a negative impact on *D. salina* biomass that could be associated with substrate inhibition. These results contrast a previous study where species of *Dunaliella* presented selectivity regarding different carbon sources [31]. Liang et al. [45] found that glycerol shorted the time *D. tertiolecta* reached the stationary phase compared to autotrophic growth. Nevertheless, they reported that the different glycerol concentrations added to the medium (1–5 g L^{-1}), and the control using $NaHCO_3$ reached similar cell densities (about 7×10^7 cell mL^{-1}) after 16 days of treatment. They also found that the pigment contents (chlorophyll a and b, and carotenoid) increased. This could be a response toward higher osmotic pressure [72] induced by extracellular glycerol, reflecting a faster photoautotrophic inorganic carbon uptake. Bacterial growth could also mislead the results, as they estimated the cell density based on OD rather than cell number.

3.2.2. Nutrient Recovery

Nitrogen is one of the most abundant intracellular elements in microalgae and a pivotal component to building protein [73,74]. Nitrogen removal was similar for all the treatments, except glycerol (99.81%) and maltose (61.11%). Phosphorus was totally removed in all treatments, most likely due to luxury uptake, which has been demonstrated by Andreotti et al. [75] for *D. tertiolecta*. Thus, the nutrient recovery was satisfactory, indicating that *D. tertiolecta* could potentially be used to remove nitrogen and phosphorus from wastewater. Wu et al. [12] had obtained about 80% nitrate and phosphate recovery by *D. tertiolecta* in diluted food waste leachate (FWL) (5% *v*/*v*). Later, Wu et al. [76] obtained 98.99% and 65% recovery for nitrate and phosphate, respectively, by *D. tertiolecta*

in anaerobic-digested FWL (50% *v*/*v*) and an increase in growth. Sulfur participates in the formation of sulfolipids and amino acids such as cysteine and methionine and influences nitrogen metabolism [12]. *D. tertiolecta* poorly removed sulfur from the medium with maximum sulfur removal for glycerol (11.91%). However, no significant difference among the treatments was recorded. Low sulfur removal had been documented for *Chlorococcum* sp. by Lv et al. [77], which was inversely related to initial sulfur concentration, maximum removal of 68.11% and a minimum of 4.07% for an initial sulfate concentration of 18 and 271 mg L^{-1}, respectively. Zhou et al. [78] also reported low sulfur uptake (20–25%) by a community of green microalgae, particularly *C. vulgaris*, cultivated in bubble reactors containing acid-mine-drainage synthetic wastewater. Sulfur is the least abundant macronutrient of plants [79], explaining the low removal.

Regarding organic carbon consumption, there was no significant difference in the relative content (Table 4), following previous results [29,33]. Carbon participates in the formation of proteins, lipids, carbohydrates, pigments, and other small molecules [80]; thus, using different carbon sources is expected to affect other nutrients uptake that participate in the formation of these bioproducts [81,82]. That was not the case with nutrients uptake reported in this research. At the end of the cultivation period, no significant difference was recorded among most treatments regarding the nitrogen, phosphorus, and sulfur recovery, and relative carbohydrate content. Only the treatments glycerol and maltose presented a significant difference in nitrogen recovery; however, since there was no significant difference in optical density, AFDW, cell density, or amino acids profile between these two treatments, it is most likely that the reported nitrogen removal is associated with some other factors instead of microalgal assimilation or analytical errors.

Table 4. Nutrient recovery and organic carbon relative content at the end of the cultivation period of the mixotrophic experiment.

Treatment	Recovery (%)			Organic Carbon Relative Content
	PO_4-P	NO_3-N	SO_4-S	
$NaHCO_3$	100	91.76 ± 2.71 ^{ab}	4.46 ± 6.30	-
Lactose	100	90.03 ± 8.47 ^{ab}	ND	0.97 ± 0.07
Glucose	100	90.03 ± 8.47 ^{ab}	6.67 ± 5.46	0.97 ± 0.04
Galactose	100	74.52 ± 3.58 ^{ab}	9.69 ± 13.71	0.99 ± 0.23
Fructose	100	96.93 ± 4.34 ^{ab}	5.92 ± 4.57	0.98 ± 0.23
Glycerol	100	99.81 ± 0.27 ^{a}	11.91 ± 12.44	0.96 ± 0.07
Maltose	100	61.11 ± 25.84 ^{b}	1.47 ± 2.07	0.95 ± 0.09

Values within the same column with different superscript letters represent significant differences ($p \leq 0.05$). ND—not detected.

3.2.3. Essential Amino Acids

Overall, all the treatments presented high-quality protein (EAAI > 1.00), with EAA over 46% of the total amino acid (AA) content (Figure 4 and Table 5), being suitable to the application of *D. tertiolecta* in a food context. Sui, Mazzucchi, Acharya, Xu, Morgan and Harvey [50] reached EAA/Total AA content around 50% and EAAI varying from 0.59 to 0.8 for *D. tertiolecta* CCAP 19/30 and from 0.79 to 0.86 for *D. salina* 19/41. Sui and Harvey [7] obtained similar EAA/Total AA content and a maximum EAAI of 0.99 for *D. salina* 19/41. The $NaHCO_3$ treatment presented superior protein quality (EAAI = 1.83 ± 0.02) and EAA/Total AA (51%). There were no significant differences in the EAAI for the treatments using different organic carbons ($p > 0.05$). This was expected because the nutrient uptake was similar for the treatments. The amino acid distribution was broadly similar among all the treatments (Table 5 and Figure 5), and the individual EAAI was above the FAO/WHO threshold for all the treatments (Figure 4). Nevertheless, the treatment with $NaHCO_3$ presented the highest protein quality, which could indicate a different pathway/storage of the assimilated nitrogen that is triggered/enhanced by the presence of inorganic carbon [83,84]. Favorable results in biomass productivity and beta-carotene accumulation had been associated with $NaHCO_3$ supplemented as a carbon source [85].

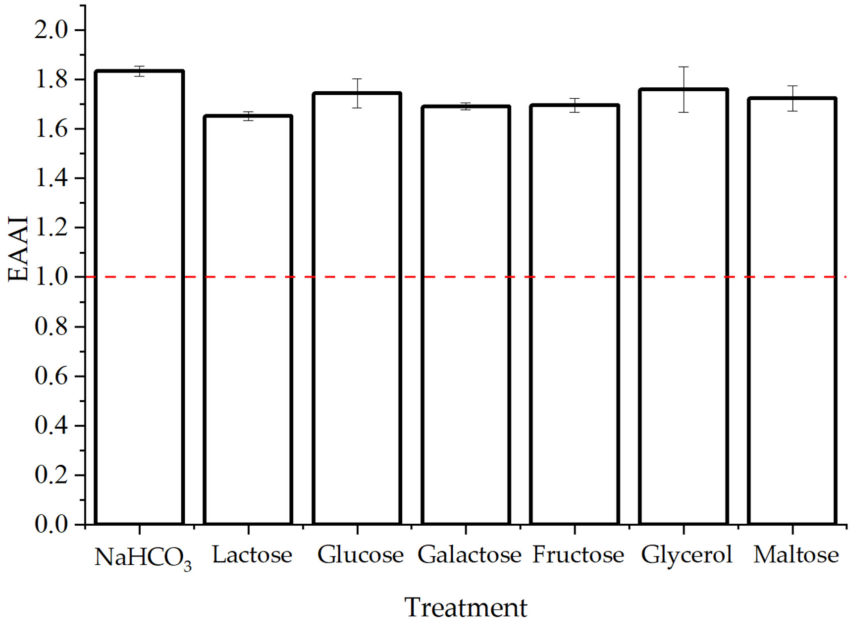

Figure 4. Essential amino acid index for each treatment in the mixotrophic experiment. The horizontal dashed red line represents the FAO/WHO threshold.

Table 5. Essential amino acid from the mixotrophic experiment. Concentrations are given in mg total protein g^{-1}.

Treatment	EAA Concentration (mg g^{-1})								EAA (%AA)
	Valine	Leucine	Isoleucine	Threonine	Methionine	Phenylalanine + Tyrosine	Lysine	Histidine	
$NaHCO_3$	56	101	54	59	18	103	74	47	51
Lactose	57	98	57	59	18	96	55	28	47
Glucose	58	97	60	61	20	101	56	33	49
Galactose	55	97	58	61	17	96	65	30	48
Fructose	62	98	61	55	20	95	54	30	47
Glycerol	54	87	58	55	20	115	62	41	49
Maltose	59	97	53	59	19	93	66	34	48
FAO/WHO reference	39	59	30	23	16	38	45	15	-

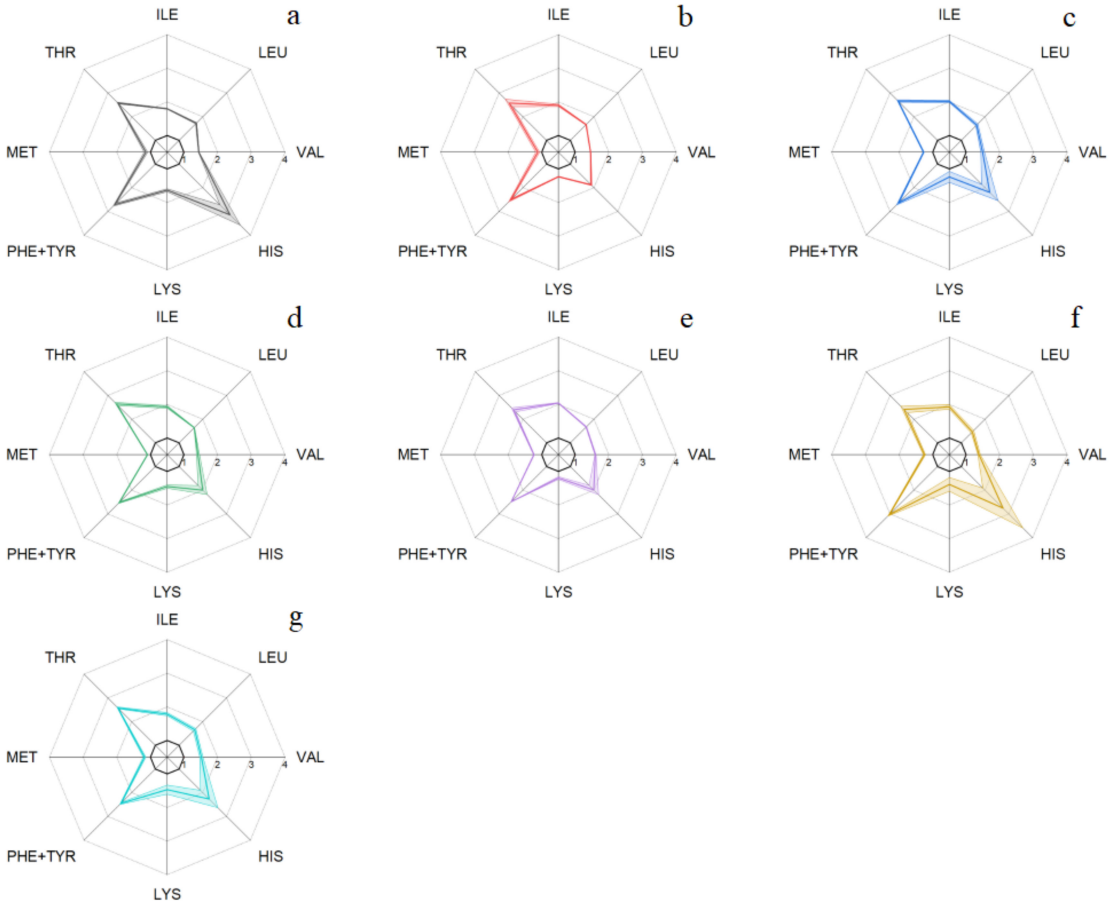

Figure 5. Amino acid profile for each treatment in the mixotrophic experiment. (**a**) $NaHCO_3$; (**b**) Lactose; (**c**) Glucose; (**d**) Galactose; (**e**) Fructose; (**f**) Glycerol; (**g**) Maltose. ILE—Isoleucine; LEU—Leucine; VAL—Valine; HIS—Histidine; LYS—Lysine; PHE + TYR—Phenylalanine + Tyrosine; MET—Methionine; THR—Threonine. FAO/WHO individual EAAI threshold is represented as the bold line in the middle of each radar graph.

4. Conclusions

Our experiment demonstrated that *D. tertiolecta* does not possess features that enable it to grow heterotrophically. Moreover, no results supported the existence of mixotrophic growth of *D. tertiolecta* since using different carbon sources did not reflect in different growth profiles, nutrient assimilation, or protein quality in our experiments. Its growth in a medium containing organic carbon is most likely associated with atmospheric carbon uptake that was passively transferred into the medium. However, we suggest that other different organic carbon concentrations be used to assess their impact on growth, protein yield and profile to identify the inhibitory effect on *D. tertiolecta*, as they were not part of the scope of our research. These results do not exclude the feasibility of using wastewater, especially treated wastewater, to cultivate *D. tertiolecta*, as it can offer a good source of other nutrients needed for its growth. Furthermore, the amino acid content and quality make possible the use of *D. tertiolecta* in a food context.

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