water

MDPI

Article

Optimization of Cultivation Conditions for *Tetraselmis striata* and Biomass Quality Evaluation for Fish Feed Production

Vasiliki Patrinou [1], Alexandra Daskalaki [2], Dimitris Kampantais [3], Dimitris C. Kanakis [3], Christina N. Economou [4], Dimitris Bokas [5], Yannis Kotzamanis [3], George Aggelis [2], Dimitris V. Vayenas [4] and Athanasia G. Tekerlekopoulou [1,*]

[1] Department of Environmental Engineering, School of Engineering, University of Patras, 30100 Agrinio, Greece
[2] Department of Biology, School of Natural Sciences, University of Patras, 26500 Patras, Greece
[3] Hellenic Centre for Marine Research/Institute of Marine Biology, Biotechnology and Aquaculture, 19013 Anavyssos, Greece
[4] Department of Chemical Engineering, School of Engineering, University of Patras, 26500 Patras, Greece
[5] PLAGTON S.A., 30019 Xiromero, Greece
* Correspondence: atekerle@upatras.gr; Tel.: +30-26410-74204

Abstract: The marine microalgae *Tetraselmis striata* was cultivated in drilling waters with different salinities. Growth substrate optimization was performed while the effects of different pH, temperature, photoperiod and CO_2 flow rate on biomass productivity and its composition were studied. Results showed that the strain grew better in 2.8% drilling waters employing the fertilizer Nutri-Leaf together with $NaHCO_3$. A pH value of 8 resulted in high biomass productivity (79.8 mg L^{-1} d^{-1}) and biomass composition (proteins 51.2% d.w., carbohydrates 14.6% d.w., lipids 27.8% d.w. and total chlorophylls 5.1% d.w.). The optimum cultivation temperature was found to be 25 ± 1 °C which further enhanced biomass productivity (93.7 mg L^{-1} d^{-1}) and composition (proteins 38.7% d.w., carbohydrates 20.4% d.w., lipids 30.2% d.w., total chlorophylls 5.1% d.w.). Photoperiod experiments showed that continuous illumination was essential for biomass production. A 10 mL min^{-1} flow rate of CO_2 lead to biomass productivity of 87.5 mg L^{-1} d^{-1} and high intracellular content (proteins 44.6% d.w., carbohydrates 10.3% d.w., lipids 27.3% d.w., total chlorophylls 5.2% d.w.). Applying the optimum growth conditions, the produced biomass presented high protein content with adequate amino acids and high percentages of eicosapentaenoic acid (EPA), indicating its suitability for incorporation into conventional fish feeds. In addition, this study analyzed how functional parameters may influence the uptake of nutrients by *Tetraselmis*.

Keywords: *Tetraselmis striata*; salinity; growth substrate; pH; temperature; photoperiod; CO_2 flow rate

Citation: Patrinou, V.; Daskalaki, A.; Kampantais, D.; Kanakis, D.C.; Economou, C.N.; Bokas, D.; Kotzamanis, Y.; Aggelis, G.; Vayenas, D.V.; Tekerlekopoulou, A.G. Optimization of Cultivation Conditions for *Tetraselmis striata* and Biomass Quality Evaluation for Fish Feed Production. *Water* **2022**, *14*, 3162. https://doi.org/10.3390/w14193162

Academic Editor: Guangyi Wang

Received: 18 August 2022
Accepted: 3 October 2022
Published: 7 October 2022

1. Introduction

Meat, including fish, is the main source of protein in human diets. The increasing world population is predicted to reach 9.7 billion by 2050, which means that the demand for food resources is expected to increase by over 60%. Additionally, human activities associated with industrialization are causing marine pollution and the accumulation of chemicals and heavy metals in waters and combined with overfishing, all contribute to the depletion of natural fish resources [1].

Aquaculture is a fast-growing industry with a significant role in protein production for consumers worldwide. The farming of aquatic organisms is predicted to surpass the production of wild fisheries by 2025 while, according to the Food and Agriculture Organization (FAO), by the year 2030, 109 million tons of fish will be produced by aquaculture [2]. This industry is developing three times faster than the animal production industries to meet the current demand for healthy and nutritious foods in a growing population. Consequently, significant challenges related to the high costs and reduced availability of fishmeal and fish oils for inclusion in fish diets have emerged.

The concept of using microalgae in fish diets as a source of high-quality bioactive compounds is a promising solution for sustainable aquaculture. Recently, microalgae have been recognized as a natural alternative source of nutrition. Specifically, marine microalgae are reported to accumulate a wide range of high added value products and typically contain higher percentages of long chain polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and higher ratios of ω-3/ω-6 PUFAs, than freshwater microalgae [1]. Their main application in aquaculture is related to nutrition as live feeds for early growth stages and they can be used as a sole component or can be incorporated into conventional on-growing fish feeds. Depending on their pigment and nutritional content they are also used to enhance the color of fish flesh and skin or to induce other biological activities (i.e., antioxidant properties). Utilization of microalgal biomass in fish diets is beneficial for fish growth as it leads to increased deposition of proteins in the muscle tissue, improved resistance to diseases [3] and also positively impacts the consumer's health [1].

Several studies refer that the most common microalgae species applied in aquaculture include the strains *Nannochloropsis*, *Tetraselmis*, *Isochrysis*, *Chlorella*, *Pavlova*, *Phaeodactylum*, *Chaetoceros*, *Skeletonema* and *Thalassiosira* [3,4]. In order to be used in aquaculture, each strain should lack toxicity, have high nutritional value, and have suitable cell size and shape. They should also possess a digestible cell wall that allows nutrient bioavailability [5]. Of the above-mentioned microalgae, *Tetraselmis* sp. has a wide spectrum of antimicrobial activities and probiotic properties, constitutes a good source of vitamin E for human and animal consumption, and stands out as food for the larvae of commercially important organisms such as mussels and oysters [4].

The nutritional value of biomass is determined by its protein, amino acid, vitamin and PUFA content, while EPA, DHA and arachidonic acid (ARA) are the most important fatty acids for the aquaculture nutrition. Different growth strategies can be applied to improve the biomass production of microalgae and increase the contents of those specific nutrients. Modification of the growth conditions is a simple way to direct microalgal metabolism towards the targeted nutrients [6]. Specifically, pH, temperature, salinity, light and dark exposure period, and the presence of CO_2 are the major parameters that affect both microalgal biomass growth and its composition.

According to the literature, *Tetraselmis chui*, *Tetraselmis suecica* and *Tetraselmis* sp. are the most commonly studied strains and the majority of research is mainly focused on lipid production, although only some of these studies have had an emphasis on fatty acid analysis [7–9]. The effect of medium composition and its strength (employing mainly F/2, also F and Walne medium, as well as aquaculture wastewaters combined with chemical media) on biomass growth, lipid, protein, carbohydrate and pigment production has been studied on the above-mentioned strains [8–12]. Additionally, the effect of salinity on the biomass growth of *Tetraselmis* sp. was examined by Kim et al. [7] and Khatoon et al. [13], while the effects of temperature on biomass growth and fatty acid composition of the same strain was studied by Rukminasari et al. [14]. Chaisutyakorn et al. [15] studied the effect of temperature on the growth of *T. chui* biomass, while only Sas et al. [16] studied the effect of temperature and medium strength (using F/2) on both the growth and biomass composition of *T. suecica*. Photoperiod optimization [17–19], pigment content [17], protein content [18] and nutrient accumulation [19] have been studied for the *T. chui* strain. Regarding the effect of CO_2 on biomass growth and chemical composition, all the above-mentioned strains have been investigated by applying a variety of volumetric CO_2 flow rates [20–23].

Tetraselmis striata is one of the lesser studied species of the *Tetraselmis* genus as research is limited to the optimization of its growth parameters [4,24]. Specifically, Boopathy et al. [25] studied the effect of different strength F/2 media and the modified CFRR1 ABRR1 substrate on its growth and lipid production. O3 medium was used by Patidar et al. [26] to examine the effects of pH, salinity, temperature and light intensity, however their research focused only on biomass and lipid production. Imamoglu et al. [4] tested the effects of agitation rate, temperature and light intensity on *T. striata* growth and its protein

and pigment contents, while Zevallos-Feria [27] studied the parameters of temperature and photoperiod but they exclusively focused on biomass growth. To the best of the authors' knowledge, until now, no previous research has been performed on the effects of different CO_2 concentrations on the growth and composition of *T. striata* biomass. Moreover, existing studies dealing with the optimization of many growth parameters do not include detailed and comprehensive evaluations of all intracellular products or fatty acid or amino acid profiles.

The aim of this work was to optimize the important functional parameters affecting the growth of the marine microalgae *Tetraselmis striata* which was cultivated in drilling waters. The waters presented different salinities of 3.9 ± 0.1% and 2.8 ± 0.1%. Initially, efforts were made to optimize the growth substrate. The drilling waters were supplemented only with N, P or enriched substrates such as modified F/2 and the commercial fertilizer Nutri-Leaf (30%-TN, 10%-P, 10%-K), while the addition of C in the form of $NaHCO_3$ was also studied. The effects of pH, temperature, photoperiod and CO_2 flow rate were then examined using the commercial fertilizer. Biomass quality analyses evaluated the suitability of the produced biomass for incorporation into conventional fish feeds. To the best of our knowledge, drilling waters have not been used as a substrate for microalgae cultivation while this study is one of the few that attempts to optimize multiple growth conditions for this specific microalga and include a full biomass composition analysis. In addition, this work analyzed how functional parameters may influence the uptake of nutrients by *Tetraselmis*, considering possible reuse of the growth medium.

2. Materials and Methods

2.1. Microorganism and Culture Conditions

A pure culture of *T. striata* was purchased from the Culture Collection of Algae at Goettingen University in Germany (SAG). The strain gradually established and was maintained phototrophically in 4 L glass aquariums (29 cm length × 10 cm width × 15 cm height) under stable, growth conditions. Drilling waters of 2.8 ± 0.1% salinity were used as growth media for the stock culture and supplemented with a modified F/2 substrate. The modified F/2 substrate (in which sodium nitrate was replaced by ammonium sulfate) contained (in g L^{-1}): 0.212 $(NH_4)_2SO_4$, 0.024 $NaH_2PO_4.H_2O$ and 1 mL L^{-1} of mixed solution (trace element stocks with solution B, see below), while vitamins (solution C) were not added to reduce the cost of the culture medium. Each trace element stock was prepared separately. $CuSO_4.5H_2O$ stock contained 10 g L^{-1}, $ZnSO_4.7H_2O$ stock contained 22 g L^{-1}, $CoCl_2.6H_2O$ stock contained 10 g L^{-1}, $MnCl_2.4H_2O$ stock contained 180 g L^{-1} and $Na_2MoO_4.2H_2O$ stock contained 6 g L^{-1}. Solution B consisted of 4.36 g L^{-1} Na_2EDTA and 3.15 g L^{-1} $FeCl_3.6H_2O$, while for the preparation of the mixed solution, 1 mL of each trace element stock was added. Stock cultures were maintained under continuous illumination using two fluorescent lamps (25–29 Wm^{-2}) which provided a light intensity of 56 μmol photons m^{-2} s^{-1}. Temperature was in the range of 24–27 °C and pH values were in the range of 7.5–8.5 (uncontrolled conditions). Additionally, the medium was recirculated using a submerged centrifugal mini air pump (flow rate 380 L h^{-1}).

The growth substrates were autoclaved before the experiments and the strain was cultivated under non aseptic conditions. The inoculum had a constant biomass concentration of 80 ± 30 mg L^{-1} in all experimental sets. Growth medium optimization experiments were conducted in 0.5 L Erlenmeyer flasks under the same cultivation conditions as the stock culture, however recirculation of the medium was provided by a magnetic stirrer. Experiments on the optimization of growth parameters (pH, temperature, photoperiod, CO_2 addition) were conducted in different bioreactors (4 L glass aquariums). Regarding the pH optimization experiments, pH was regulated manually using either NaOH or HCl. Desired temperature was achieved using thermostats (Diversa Heater Thermo Plus, 25 W) placed inside the bioreactors. To test the different photoperiods, the fluorescent lamps were turned off using a timer, while flow meters were used to ensure continuous, steady, flow of pure CO_2.

2.2. Tested Growth Substrates

Drilling waters with different salinity levels were obtained from the commercial fish farm PLAGTON S.A. (Western Greece). High salinity (3.9 ± 0.1%, drill 1) and lower salinity drilling waters (2.8 ± 0.1%, drill 2) were used to study the effect of salinity on biomass productivity and the specific growth rate of *T. striata*. As the drills presented no nutrient load (Table S1), nutrient supplementation was necessary to sustain growth.

In this work, six different experimental sets (exp. sets A, B, C, D, E and F) were performed examining various substrates to determine the most efficient and low-cost growth medium for *T. striata* cultivation. The composition of each substrate tested is given in detail in Table S2. Initially, high salinity water (drill 1) was supplemented only with nutrients critical for growth (N and P at a ratio of about 5:1, using NH_4^+-N as a nitrogen source; Exp. set A). The N:P ratio of 5:1 was selected based on research in the literature that indicates that for optimal growth conditions the N:P ratio should range from 5:1 to 19:1, with many of them being below the Redfield ratio of 16:1 [9,28]. Experimental set B also had high salinity water but with a N:P nutrient ratio of about 12:1. Lower salinity (2.8 ± 0.1%) drilling waters were also studied (Exp. sets C to F). Experimental set C had an N:P ratio of about 12:1. Substrates further enriched with modified F/2 (Exp. set D, N:P≈10) and the commercial fertilizer Nutri-Leaf (30% TN-10% P- 10% K, N:P≈7) without and with the addition of a low-cost inorganic carbon source ($NaHCO_3$) were also studied (Exp. sets E and F, respectively).

2.3. Optimization of Growth Conditions

Experiments were performed for the optimization of pH, temperature, photoperiod and CO_2 supplementation aiming to further enhance *T. striata's* biomass productivity and specific growth rate, as well as the biochemical composition of the produced biomass. pH values of 7 and 8 were tested under continuous illumination of 56 μmol photons m^{-2} s^{-1} and uncontrolled temperature (24–27 °C). The pH value of 9 was not tested as all the growth substrates contained NH_4^+-N aiming to avoid toxic accumulations of NH_3 and pH 6 was not tested based on the low biomass yields previously achieved at pH 7. Temperature optimization experiments were performed while applying the determined optimum pH value which not only enhanced biomass production but also biomass quality. Temperature experiments took place under conditions of continuous illumination and three temperatures were employed (19 ± 1 °C, 25 ± 1 °C, 28 ± 1 °C). Three photoperiods of 20:4 h, 18:6 h, 12:12 h light (L):dark (D) were then studied. Regarding carbon addition, pure CO_2 (100%) of flow rates 10, 20 and 90 mL min^{-1} was also applied to enrich the culture medium.

2.4. Analytical Methods and Calculations

2.4.1. Medium Analyses

Samples of 50 mL were taken every 48 h from the photobioreactors. The samples were centrifuged (4200× *g* rpm, 20 min) to separate the biomass from the chemical medium. The centrifuged medium was filtered through 0.45 μm Whattman filter papers and then was used for the measurement of all nutrient parameters. NH_4^+-N was estimated using the modified indophenol blue method [29]. The samples containing NH_4^+-N were estimated spectrophotometrically at 640 nm. PO_4^{3-} were measured at 880 nm using the ascorbic acid method (4500-P E.) [30]. Total Kjeldahl nitrogen (TKN), was also measured using the Standard Methods for the Examination of Water and Wastewater [30]. To estimate TKN it is necessary to digest the samples for 1 h at 420 °C using a digestion block. Total Nitrogen (TN) was estimated as the sum of TKN (organic form) and NO_3^--N, NO_2-N (inorganic forms). Both inorganic N forms were determined following the spectrophotometric methods of 4500-NO_3-B and 4500-NO_2-B as described in the Standard Methods for the Examination of Water and Wastewater [30].

Dissolved chemical oxygen demand (d-COD) was determined according to Standard Methods for the Examination of Water and Wastewater using the colorimetric closed reflux method (5220 D) [30] after modification. Specifically, to determine d-COD in the marine samples a pre-treatment step was necessary for the complete removal of halides, employing

3 gr of Ag_2SO_4 for 30 mL of sample [31]. The sample is subjected to stirring with Ag_2SO_4 for 60 to 90 min until the sediment appears lilac in color. This color indicates that the halides have been completely removed. Exossacharides (measured as starch equivalents) were determined following the Dubois method [32]. The samples were hydrolyzed using H_2SO_4 in the presence of phenol and were then spectrophotometered at 490 nm.

2.4.2. Biomass Analyses

Estimation of Dry Biomass and Biomass Yields

Samples were taken every 48 h to determine microalgal growth and characterize the nutritional metabolites. The biomass was harvested by centrifuging at 4200× *g* rpm for 20 min, then re-suspending in distilled water and rinsing thoroughly to remove all excess salts. Wet biomass was left to dry at 80 °C in a pre-weighed vial. The dry cell biomass was determined gravimetrically (mg L^{-1}) as total suspended solids (TSS) according to Standard Methods for the Examination of Water and Wastewater [33]. Biomass productivity (P) expressed in mg L^{-1} d^{-1} was measured from the variation in biomass concentration (X) within the time (t) as $P = (X_f - X_i)/(t_f - t_i)$. Specific growth rate ($d^{-1}$) was calculated as μ (day^{-1}) = $\ln (X_f - X_i)/(t_f - t_i)$. X_f and X_i correspond to two consecutive different biomass concentrations at their respective t_f and t_i times.

Lipid and Carbohydrate Estimation

Lipid content of the dry biomass was estimated gravimetrically and following Folch's method [34]. Lipids were extracted from the dried biomass employing chloroform and methanol 2:1 *v*/*v* as solvents. Using a separating funnel, the extracted lipids were washed with 0.88% *w*/*v* KCl solution. The washed extract was then dried over Na_2SO_4, recollected using filter paper in a pre-weighted round-bottomed flask and finally the solvent was removed by evaporation. Lipids were then expressed as a percentage of the dry cell weight (% d.w.).

Intracellular carbohydrates were determined following the Dubois method [32]. To measure intracellular carbohydrate content, 1 mg of dry biomass was mixed with 5 mL distilled water. From this solution, 1 mL was used as the sample together with 1 mL of 5% *w*/*v* phenol solution and 5 mL sulfuric acid (95–97% purity).

Lipid Fractionation and Fatty Acid Analysis

For fractionation, microalgal lipids (approximately 100 mg) were dissolved in 1 mL chloroform and fractionated using a column (25 × 100 mm) containing 1 g silicic acid (Fluka). The column was washed successively with 100 mL dichloromethane (Sigma-Aldrich, Steinheim, Germany) to obtain neutral lipids (NL), 100 mL acetone (Fluka) to obtain glycolipids plus sphingolipids (G + S), and 50 mL methanol (Sigma-Aldrich, Steinheim, Germany) to obtain phospholipids (P) [35]. The resulting fractions (NL, G + S, and P) were quantified gravimetrically.

The fatty acid composition of total lipids and lipid fractions was determined by Gas Chromatography (GC) after transmethylation of the fatty acid moieties in a two-stage reaction to avoid trans-isomerization using CH_3O–Na+ and CH_3OH/HCl according to AFNOR [36]. An Agilent Technologies 7890 A GC apparatus (Shanghai, China), equipped with a flame ionization detector and a HP-88 (60 m × 0.32 mm) column (J&W Scientific, Folsom, CA, USA) was used. Helium was used as the carrier gas, at 1 mL min^{-1} flow rate. The analyses were run at injection temperature 250 °C, oven temperature 200 °C, and flame ionization detector temperature 280 °C. Peaks of methyl esters were identified by reference to authentic standards.

Protein and Amino Acid Profile Estimation

Crude protein content was determined with the Kjeldahl method (N × 6.25) following AOAC [37]. Amino acid composition of the lyophilized biomass after acid hydrolysis (6N, 110 °C, 24 h) was calculated by AccQ-Tag™ Ultra according to the amino acid analy-

sis application solution (Waters Corporation, Milford, MA, USA). DL-Norvaline (Sigma) 2.5 mM was used as an internal standard. Ultra Performance Liquid Chromatography (UPLC) was performed on an ACQUITY system (Waters Corporation, Milford, MA, USA) equipped with PDA detector and the detection wavelength was set at 260 nm. The column used was a BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm) from Waters. The flow rate was 0.7 mL min^{-1} and column temperature was maintained at 55 °C. Peak identification and integration were performed by the software Empower v.2.0 (Waters Corporation, Millford, MA, USA) using an Amino Acid Standard H (Pierce) as an external standard [38].

Pigment Estimation

For pigment determination, total chlorophylls as the sum of chlorophyll-a and chlorophyll-b were estimated using 5 mL of culture which was washed twice with cold distilled water. The biomass was then re-suspended in 1 mL distilled water and transferred into glass tubes with 4 mL pure acetone. The tubes were covered with aluminum foil and left at 4 °C until all pigments were extracted and the biomass was left colorless. Finally, pigment concentration in wet biomass was measured spectrophotometrically (at 663 and 647 nm) and calculated using the equations of Lichtenthaler and Buschmann [39].

The wet microalgal biomass was centrifuged while a clean-up protocol was applied to remove seawater salts [40]. Determination and quantification of the targeted carotenoids (astaxanthin, lutein, zeaxanthin, canthaxanthin, b-cryptoxanthin, echinenone, lycopene and b-carotene) were performed using a UPLC H-Class -QTOF-MS system (Waters Corp., Millford, MA, USA). Additional information on carotenoid analysis is provided in the Supplementary Materials.

2.5. Statistical Analysis

All experimental sets were performed in duplicate and the results are presented as mean values ± standard deviation (SD). Standard deviations are presented in all figures as error bars. Mean values are derived from two samples taken from two different bioreactors with the same operating conditions. Statistically significant differences in nutrient reduction rates, biomass production and concentration of intracellular compounds, were analyzed using one-way ANOVA analysis of variance. The value of $p \leq 0.05$ was considered statistically significant.

3. Results and Discussion

3.1. Substrate Optimization

Salinity is essential for microalgal growth and under conditions of saline stress modifications of metabolism allow cell adaptation. Additionally, salinity stress is a well-known strategy to enhance lipid content [6]. Initially, the marine microalga *Tetraselmis striata* was cultivated in drilling waters with two different salinities: high salinity of 3.9 ± 0.1% and lower salinity of 2.8 ± 0.1%. At the same time, the effect of substrate composition was also studied employing the growth media as described in Section 2.2. It should be noted that during these experiments, extended biomass quality characterization was not performed, although biomass growth, nutrient reduction rates and maximum lipid contents were monitored in order to determine the optimum medium leading to enhanced biomass productivity and specific growth rate. Figure 1 presents the evolution of biomass production for all the tested substrates, while all initial nutrient concentrations are given in Table 1. The lowest biomass productivities of *Tetraselmis striata* were achieved in the high saline growth media with 37.5 and 40.0 mg L^{-1} d^{-1} for experimental sets A and B, respectively (Table 2). Experimental set A presented higher lipid content (16.0% d.w.) than set B (10.3% d.w.), however biomass productivity and specific growth rate were higher in set B with the N:P ratio of about 12. Although *T. striata* has been characterized by Imamoglu et al. [4] as a euryhaline strain, the other limited research on this microalga has shown that salinity values close to 3% produce significant biomass growth [41,42]. Therefore, drilling water of 2.8 ± 0.1% salinity was used in all subsequent experimental sets. The

composition of growth media was also evaluated with these waters, examining both simple substrates (containing only N:P at the ratio of about 12:1 (set C)), as well as those further enriched with modified F/2 (set D), and the commercial fertilizer Nutri-Leaf 30-10-10 (set E). The results revealed that the highest biomass productivities (58.2, 62.0 mg L^{-1} d^{-1}) and specific growth rates (0.227, 0.200 d^{-1}) were recorded in experimental sets D and E, respectively. Statistically significant differences were not observed among biomass productivities (p = 0.1513) and specific growth rates (p = 0.78232) for sets D and E. Sets D and E also presented enhanced lipid contents compared to set C and the higher salinity substrates of sets A and B. Statistically significant differences were observed between lipid contents of sets D and E (p = 0.02517) and between sets C, D and E (p = 0.00273).

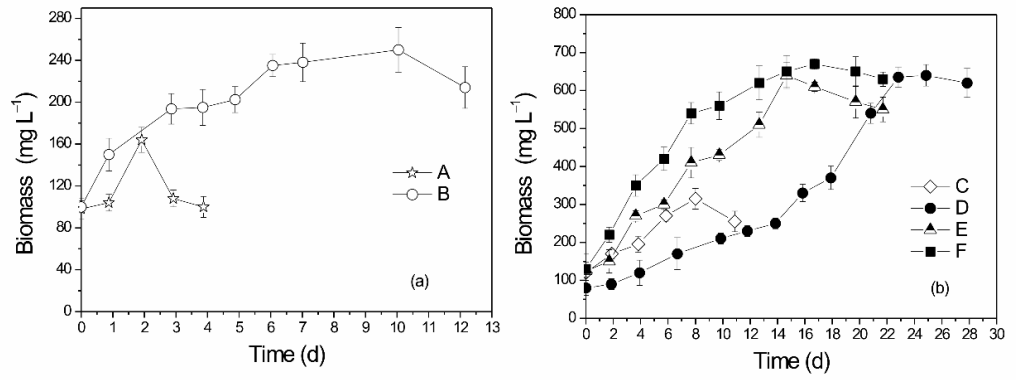

Figure 1. Effect of salinity and substrate composition on the biomass production of *T. striata*. Experimental sets: (**a**) A (salinity 3.9 ± 0.1%, N:P≈5), B (salinity 3.9 ± 0.1%, N:P≈12), (**b**) C (salinity 2.8 ± 0.1%, N:P≈12), D: (salinity 2.8 ± 0.1%, modified F/2), E (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 without $NaHCO_3$), and F (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 with $NaHCO_3$).

Table 1. Initial biomass and nutrient concentrations of all growth substrates tested.

	Initial Concentrations (mg L^{-1})					
Growth Substrate	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**Biomass**	**N:P**
A [1]	18.6 ± 0.2	34.3 ± 2.1	6.5 ± 1.0	19.6 ± 3.3	98 ± 10.0	5:1
B [2]	63.5 ± 1.4	72.4 ± 2.8	6.0 ± 0.2	29.6 ± 0.3	100 ± 5.6	12:1
C [3]	62.7 ± 2.6	75.0 ± 3.3	6.2 ± 0.3	17.2 ± 0.3	120 ± 28.3	12:1
D [4]	30.5 ± 2.3	44.4 ± 3.8	4.4 ± 0.1	8.0 ± 1.1	80 ± 19.7	10:0
E [5]	5.1 ± 2.2	16.1 ± 0.9	2.2 ± 0.1	14.8 ± 0.1	125 ± 16.1	7:3
F [6]	5.4 ± 0.5	16.2 ± 0.8	2.1 ± 0.01	10.1 ± 2.3	130 ± 39.0	7:7

Notes: [1–6] Abbreviations of experimental sets follow those of Figure 1.

Even though set D produced the highest lipid content (24.2% d.w.), the commercial fertilizer Nutri-Leaf 30-10-10 was used as substrate in all the following experiments of parameter optimization. The use of F/2 for large-scale microalgae cultivation will likely increase overall operational costs as it is a complex medium consisting of many components and requires specialized personnel for its preparation. In contrast, employing a ready-to-use soluble fertilizer such as Nutri-Leaf can reduce cultivation costs. Nutri-Leaf together with $NaHCO_3$ (exp. set F) was examined to study whether the addition of a low-cost inorganic carbon source could further enhance biomass and lipid production. Nutri-Leaf together with $NaHCO_3$ produced the highest biomass productivity (70.1 mg L^{-1} d^{-1}) observed in all the tested growth media and also a significant lipid content of 16.5% d.w. Between sets E and F, the p-value for biomass productivities was 0.052, while for lipid content p = 0.34427.

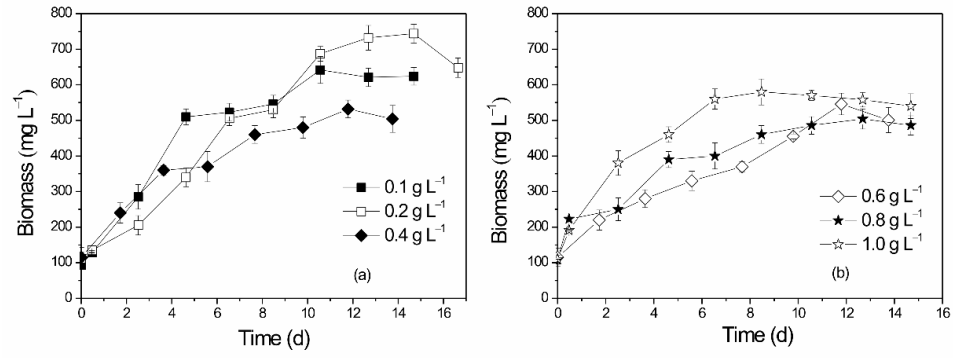

Figure 2. Effect of fertilizer quantities (**a**) 0.1, 0.2, 0.4 g L^{-1}, (**b**) 0.6, 0.8, 1 g L^{-1} on biomass production of *T. striata*.

Table 2. Nutrient reduction rates, lipid content, biomass productivity and specific growth rates achieved in all substrates tested.

Growth Substrate	% Removal Rate				Maximum Biomass Productivity (mg L^{-1} d^{-1})	Specific Growth Rate (d^{-1})	% Maximum Lipid Content
	NH_4^+-N	TN	PO_4^{3-}	Total Sugars			
A [1]	89.0 ± 0.3	34.8 ± 1.3	58.3 ± 1.2	0.0	37.5 ± 4.4	0.101 ± 0.01	16.0 ± 2.02
B [2]	85.4 ± 2.2	52.5 ± 4.3	87.2 ± 0.1	0.0	40.0 ± 2.3	0.129 ± 0.01	10.3 ± 0.8
C [3]	76.2 ± 1.0	51.1 ± 3.2	94.9 ± 0.8	0.0	47.6 ± 9.8	0.184 ± 0.01	13.7 ± 2.4
D [4]	98.4 ± 2.3	51.0 ± 0.4	97.8 ± 0.2	0.0	58.2 ± 10.7	0.227 ± 0.03	24.2 ± 2.8
E [5]	95.1 ± 0.1	54.6 ± 2.4	95.5 ± 0.1	0.0	62.0 ± 13.8	0.200 ± 0.05	16.4 ± 4.6
F [6]	97.2 ± 0.6	56.0 ± 0.5	97.5 ± 0.1	0.0	70.1 ± 11.2	0.240 ± 0.04	16.5 ± 2.3

Notes: [1–6] Abbreviations of experimental sets follow those of Figure 2.

Regarding nutrient reduction rates (Table 2, Figures S1–S3), higher NH_4^+-N, TN and PO_4^{3-} reductions were recorded in sets D, E and F that had enhanced biomass production, than in sets A and B. Total sugar concentrations were not reduced in any of the substrates tested and this is because autotrophic microalgae excrete exopolysaccharides during photosynthesis [43]. Considering biomass productivity, lipid content and nutrient reduction, Nutri-Leaf together with $NaHCO_3$ (exp. set F) was selected as the most efficient growth medium.

Dammak et al. [12] reported that not only the crucial nutrients N and P are significant in a growth medium, but metals, ions and vitamins can also define biomass production and its biochemical composition. Similarly, in our study higher biomass efficiencies were recorded in the most enriched substrates. In experimental sets E and F, Nutri-Leaf was applied at the recommended dosage (0.05 g L^{-1}) for liquid fertilizer, together with 0.18 g L^{-1} $NaHCO_3$ (Table S2). As nutrient availability [6] in addition to medium composition can affect microalgal growth, further optimization experiments of the supplemented fertilizer were conducted. Quantities of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 g Nutri-Leaf per litre were tested under uncontrolled pH and temperature conditions, while the same stable quantity of 0.18 g L^{-1} $NaHCO_3$ was added to all experimental sets.

Figure 2 presents the effect of fertilizer quantity on the growth of *T. striata*. Initial biomass concentrations ranged between 92.5 and 115 mg L^{-1}, while final biomass concentrations reached values of between 504 and 732 mg L^{-1}. Of all the tested fertilizer quantities, the highest biomass concentration of 732 mg L^{-1} was recorded at 0.2 g L^{-1}. High biomass productivities (81.8 and 84.2 mg L^{-1} d^{-1}) and specific growth rates (0.236 and 0.240 d^{-1}) were recorded when fertilizer quantities of 0.1 and 0.2 g L^{-1} were applied, although the highest biomass yield and lipid content recorded was at 0.2 g L^{-1} (Table 3). Kamil [44] observed a similar specific growth rate of 0.23 d^{-1} with the strain *Tetraselmis chuii*, when a commercial fertilizer with urea (as the main nitrogen source) was employed. Statistically significant differences were not observed for biomass productivities (p = 0.4768), specific growth rates (p = 0.39951) or lipid contents (p = 0.33461) between the fertilizer quantities of 0.1 and 0.2 g L^{-1}.

Table 3. Ammonium nitrogen removal rates, biomass productivities, specific growth rates and lipid contents achieved under the different fertilizer quantities.

Fertilizer Quantity (g L^{-1})	Initial Concentration of NH_4^+-N (mg L^{-1})	% Removal Rate of NH_4^+-N	Initial Biomass Concentration (mg L^{-1})	Maximum Biomass Productivity (mg L^{-1} d^{-1})	Specific Growth Rate (d^{-1})	% Maximum Lipid Content
0.1	6.3 ± 0.2	97.1 ± 0.2	92.5 ± 10.6	81.8 ± 6.8	0.236 ± 0.09	15.9 ± 2.5
0.2	10.0 ± 0.6	97.7 ± 0.1	112.1 ± 17.1	84.2 ± 3.2	0.240 ± 0.05	16.3 ± 2.1
0.4	16.0 ± 2.8	88.7± 0.3	115.0 ± 57.8	62.8 ± 4.3	0.212 ± 0.07	16.1 ± 1.2
0.6	25.8 ± 2.0	90.0 ± 0.3	115.0 ± 45.4	60.7 ± 3.3	0.127 ± 0.02	14.7 ± 0.7
0.8	33.0 ± 3.5	41.6 ± 0.6	108.5 ± 12.0	66.7 ± 2.8	0.213 ± 0.04	12.7 ± 5.1
1.0	43.1 ± 2.7	41.0 ± 0.5	109.8 ± 0.3	59.7 ± 3.1	0.183 ± 0.02	9.8 ± 0.7

In contrast, when fertilizer quantities exceeded 0.4 g L^{-1}, biomass yields gradually reduced and showed productivities ranging from 59.7 to 62.8 mg L^{-1} d^{-1} and specific growth rates of between 0.127 d^{-1} and 0.212 d^{-1}. A similar pattern was also observed for NH_4^+-N removal rates that ranged from 88.7 to 41% (Table 3, Figure S4). Regarding fertilizer composition, TN is present at 30% and increasing the quantity of fertilizer resulted in high initial TN concentrations. This may have inhibited NH_4^+-N assimilation and also affected biomass production. Farahin et al. [45] examined the effect of increasing N concentrations on the growth of *Tetraselmis tetrathele*, employing 0.31 to 0.87 g NH_4^+-N L^{-1}. They found that the strain acclimatized to each concentration, although the highest biomass production of 1.25 g L^{-1} was recorded at the lowest NH_4^+-N concentration tested (0.31 g NH_4^+-N L^{-1}). In a similar study, *Spirulina platensis* FACHB-431 was studied under N concentrations ranging from 50 to 200 mg NH_4^+-N L^{-1} using a modified Zarrouk medium [46]. It was observed that *Spirulina* growth was significantly affected by increasing N concentrations and maximum biomass production was recorded at the lowest NH_4^+-N concentration of 50 mg L^{-1}.

Lipid contents ranged from 9.8 to 16.3% d.w. in all the substrates tested. A similar pattern to that of biomass growth and NH_4^+-N removal was also observed for lipid yields, indicating that lipid content decreased as fertilizer increased (Table 3). Thus, the optimum fertilizer quantity in these experiments was found to be 0.2 g L^{-1} which led to the highest biomass productivity, significant NH_4^+-N removal rates (97.7%) and enhanced lipid content (16.3%). The commercial fertilizer Nutri-Leaf 30-10-10 was also studied by Kokkinos et al. [47] for the cultivation of fresh water microalgae (Chlorophyte and indigenous freshwater strains) and marine strains (a strain of Chlorella). In their research, 0.07 g m^{-3} of Nutri-Leaf produced lipids ranging from 2.2 to 10.6%, contents lower than those of the present study. Further optimization of all the other functional parameters was conducted employing 0.2 g L^{-1} of fertilizer together with 0.18 g L^{-1} $NaHCO_3$ as growth medium.

3.2. Optimization of Substrate pH

Two pH values (7 and 8) were tested using the determined optimum fertilizer quantity. Experiments were performed under continuous illumination and uncontrolled temperature (within the range of 24–27 °C). pH was adjusted to the desired level and maintained at 7 ± 0.2 or 8 ± 0.2. Figure 3 illustrates the effect of pH on the growth of *T. striata* and Table 4 presents all initial nutrient concentrations. The results show that the strain grew well at both pH values. Final biomass concentration reached 759 mg L^{-1} at pH 8, but did not exceed 544 mg L^{-1} at pH 7. Consequently, higher biomass productivity was recorded at pH 8 (79.8 mg L^{-1} d^{-1} corresponding to a growth rate of 0.266 d^{-1}), than pH 7 (biomass productivity 60.1 mg L^{-1} d^{-1} corresponding to a growth rate of 0.248 d^{-1}) (Table 5). Statistically significant differences of biomass productivities (p = 0.04439) were recorded between the two pH values, but significant differences were not observed for specific growth rates (p = 0.42629).

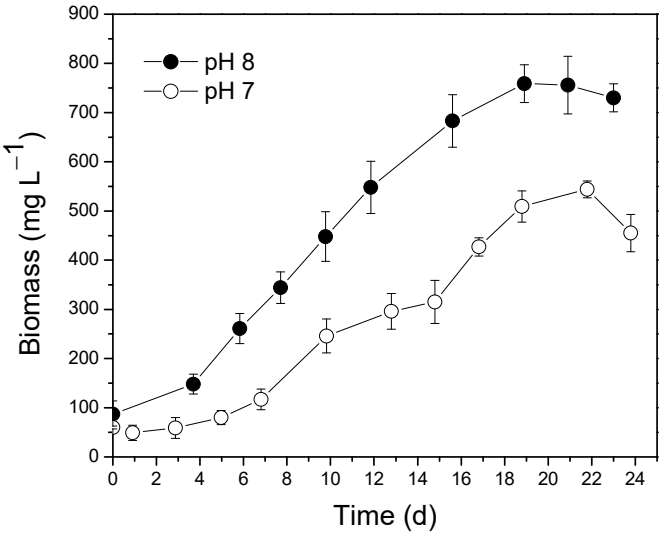

Figure 3. Effect of pH on the biomass production of *T. striata*.

Table 4. Initial biomass and nutrient concentrations at experimental sets for optimum pH investigation.

Initial Concentrations (mg L^{-1})								
pH Value	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**d-COD**	**Biomass**	**C:N**	**N:P**
8	24.2 ± 0.6	93.9 ± 4.0	12.3 ± 0.2	5.8 ± 1.5	115.3 ± 5.6	87.0 ± 26.8	1.23	7.64
7	23.1 ± 0.1	82.1 ± 0.3	12.1 ± 0.1	6.0 ± 0.1	94.6 ± 16.9	60.0 ± 2.8	1.15	6.78

Table 5. Nutrient reduction rates, biomass productivity and specific growth rates achieved at pH 7 and 8.

	% Removal Rate					Maximum Biomass Productivity (mg L^{-1} d^{-1})	Specific Growth Rate (d^{-1})
pH Value	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total sugars**	**d-COD**		
8	98.7 ± 0.1	79.1± 2.5	99 ± 0.2	0.0	94.8 ± 2.8	79.8 ± 1.4	0.266 ± 0.06
7	94.6 ± 0.4	76.4 ± 0.1	97.8 ± 0.4	0.0	56.3 ± 9.1	60.1 ± 5.5	0.248 ± 0.05

It should be noted that most studies using *Tetraselmis strains* without pH optimization used pH values of 7.5–8.5 [25,44]. *Tetraselmis* sp. was cultivated by Khatoon et al. [13] in both indoor and outdoor (natural) conditions testing pH values between 5.5 and 9.5. They found the strain could grow well at pH values of 7.5 and 8.5 in both indoor and outdoor conditions and recorded significant specific growth rates that ranged from 0.346 to 0.435 d^{-1} and from 0.352 to 0.359 d^{-1}, respectively, while reduced growth rates were recorded with pH values of 5.5 and 9.5. Patidar et al. [26] cultivated *Tetraselmis striata* at pH values of 4, 6, 8, and 10, under axenic and symbiotic conditions with the bacteria *Pelagibaca bermudensis*. Similar to the present study, they found that the highest biomass productivities (43.5 mg L^{-1} d^{-1}) were recorded at pH 8.

It should be mentioned that differences in initial nutrient concentrations were attributed to nutrients originating from the initial inoculum. Profiles of nutrient removal rates are presented in Figures S5–S7. As shown in Table 5, higher nutrient removal rates were recorded at pH 8 than at pH 7. Total sugars increased with time since organic substances were produced during photosynthesis [43]. The concentration of organic matter was also monitored through d-COD measurements. At the end of the growth cycle, higher d-COD removal was recorded at pH 8 than pH 7.

Effect of pH on Biomass Composition

Although pH optimization experiments showed that pH 8 resulted in higher biomass yields and nutrient removal rates than pH 7, biomass analysis showed that lipids (p = 0.643) and carbohydrates (p = 0.502) were not affected significantly by pH (Table 6). Specifically, pH 8 resulted in lipid and carbohydrate contents of 27.8% d.w. and 14.6% d.w., respectively, while pH 7 produced contents of 26.4% d.w. and 14.7% d.w., respectively. The lipid content achieved here with *T. striata* is among the highest recorded in the relevant bibliography for Tetraselmis species, although carbohydrate contents of up to 30% d.w. have been recorded. Specifically, Boopathy at al. [25] examined *T. striata* under laboratory conditions and recorded lipid contents and carbohydrates of about 15% and 17%, respectively, at pH 8.5. Khatoon et al. [13] examined *Tetraselmis* sp. and found that pH 7.5 enhanced lipid content (over 20% d.w.) but not carbohydrates (about 15% d.w.), while pH 8.5 favored carbohydrates (about 30% d.w.) more than lipids (about 15% d.w.).

Table 6. Effect of pH on biomass biochemical composition.

	% d.w. Content				
pH Value	**Proteins**	**Lipids**	**Carbohydrates**	**Total Chlorophylls**	**Total Carotenoids**
8	51.2 ± 2.0	27.8 ± 1.0	14.6 ± 1.5	5.1 ± 0.1	0.20 ± 0.01
7	47.7 ± 0.5	26.4 ± 0.4	14.7 ± 0.8	4.6 ± 0.1	0.88 ± 0.10

Dammak et al. [41] studied the effect of pH on a *Tetraselmis* species closely related to *T. striata* and recorded enhanced lipid content at both pH 7 (49% d.w.) and pH 8 (39.3% d.w.). As shown in Table 6, *T. striata* tended to accumulate more lipids than carbohydrates.

Significant protein content was recorded at both pH values but the highest protein content of 51.2% d.w. was recorded at pH 8. pH can influence microalgal metabolism both directly and indirectly as it affects nutrient solubility and cellular morphology. Protein accumulation can be determined by the availability of nitrogen in the growth media as well as the growth stage [48]. Increased pH values also can interfere with the NH_4^+/NH_3 buffer system by inducing free ammonia in the cultivation medium to negatively affect photosynthesis and the accumulation of proteins and pigments [6]. The results of the present study suggest that the optimum pH for maximum biomass production can also improve biomass composition. It should be noted that although proteins comprise fundamental organic components of microalgal cells, they have been much less studied than lipids [49].

Values of total chlorophyll showed similar accumulation rates for both pH values tested, with maximum contents of 5.1% d.w., and 4.6% d.w., for pH 8 and 7, respectively. Lower accumulation rates not exceeding 0.88% d.w. were recorded for total carotenoids, indicating that this particular strain produces mainly chlorophyll. Total recorded carotenoids were higher at pH 7 and this could be attributed to the fact that secondary metabolites tend to accumulate under conditions of stress [6,48]. Dammak et al. [50], reported that under basic environmental growth conditions (pH 7, salinity 4%) Tetraselmis sp. biomass comprises 250 mg proteins g^{-1} d.w., 195 mg lipids g^{-1} d.w., 340 mg starch g^{-1} d.w., 18.08 mg total chlorophylls g^{-1} d.w., and 0.025 mg carotenoids g^{-1} d.w. This research team also studied the effect of pH and salinity on pigment production. At 3% salinity, pH 8 produced higher total chlorophyll (13.26 mg g^{-1} d.w.) and carotenoid contents (0.030 mg g^{-1} d.w.) than pH 7. Higher total chlorophyll (46 to 51 mg g^{-1} d.w.) and total carotenoid (2.2 to 8.8 mg g^{-1} d.w.) contents were achieved at both pH values in the present study.

pH 8 was selected as the optimum pH value in this work as it resulted in enhanced biomass production and high value intracellular components.

3.3. Temperature Optimization

The effect of temperature (19 ± 1 °C, 25 ± 1 °C and 28 ± 1 °C) was examined under conditions of optimized fertilizer quantity and pH (Figure 4). The microalga grew

successfully at 19 ± 1 °C and 25 ± 1 °C recording biomass concentrations of 598 and 620 mg L^{-1}, respectively. The temperature of 28 ± 1 °C significantly affected biomass production resulting in a low concentration of 358 mg L^{-1}. The highest biomass productivity of 93.7 mg L^{-1} d^{-1} (corresponding to a growth rate of 0.283 d^{-1}) was observed at 25 ± 1 °C while the lowest was recorded at 28 ± 1 °C (61.5 mg L^{-1} d^{-1}). Statistically significant differences of biomass productivity (p = 0.04851) and specific growth rate (p = 0.03807) were observed between 25 and 28 ± 1 °C. It should be noted that a growth cycle of 16 days was observed in the experiment of controlled temperature, while in uncontrolled conditions the growth cycle prolonged until the 24th day.

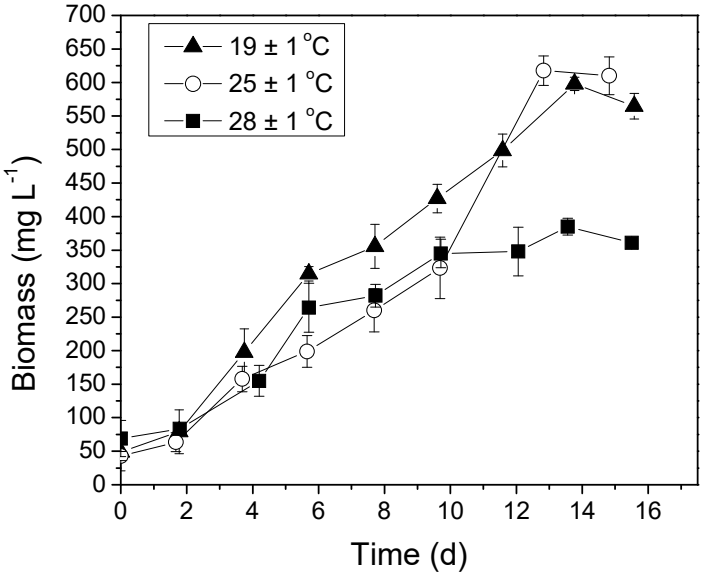

Figure 4. Effect of temperature on the biomass production of *T. striata*.

Temperature is a crucial factor that directly affects microalgal cell growth and death. Moreover, when the optimum temperature value is surpassed, photosynthesis and growth rates can reduce [15]. As mentioned above, only a few studies focus on *Tetraselmis striata*. Therefore, direct comparisons between these experiments and those in the literature are not feasible because of the different strains and experimental conditions employed. Molina et al. [9] reported that *Tetraselmis* sp. presented a linear relationship between specific growth rate and temperatures up to 25 °C and a decrease in growth rate appeared when this value was exceeded.

Tetraselmis sp. was cultivated at 28, 30, 32 and 34 °C by Rukminasari et al. [14] and the highest growth rate of 0.2 cell d^{-1} was recorded at 30 °C. A Tetraselmis strain was also cultivated at 19–21 °C, 24–26 °C, 29–31 °C by Abu-Rezq et al. [42]. They observed the highest growth rate of 0.15 d^{-1} at 19–21 °C, while in our study a specific growth rate of 0.237 d^{-1} was achieved at 19 ± 1 °C. Chaisutyakorn et al. [15] tested the effect of 25, 30, 35 and 40 °C on the growth of *Tetraselmis suecica* FIKU032. In this work, the highest growth rates were recorded at 25 °C (0.353 d^{-1}) and 30 °C (0.378 d^{-1}) while the authors also observed reduced biomass production and growth rates when the temperature was increased. Chen et al. [51] found that *Tetraselmis chui* can grow optimally at 25 °C. Imamoglu et al. [4] cultivated *Tetraselmis striata* in a broad range of temperatures (20–30 °C) and observed that the specific strain recorded the highest growth rate (0.226 d^{-1}) at 25 °C.

Regarding nutrient contents, all initial concentrations are given in Table 7 while nutrient removal rates are presented in Table 8 (and Figures S8–S10). NH_4^+-N showed removal rates of over 99% at 19 and 25 ± 1 °C, while at 28 ± 1 °C NH_4^+-N was removed at a lower rate (94.5%). The same trend was noted for TN, PO_4^{3-} and d-COD removal rates that exhibited increased removal at 19 and 25 ± 1 °C the temperatures at which biomass growth enhanced. At 28 ± 1 °C, d-COD was not reduced, however its initial concentration was

lower here (22 mg L^{-1}) than in the other substrates (Table 7). Total sugar concentrations increased at all tested temperatures.

Table 7. Initial biomass and nutrient concentrations in the experimental sets for optimum temperature investigation.

Initial Concentrations (mg L^{-1})								
Temperature	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**d-COD**	**Biomass**	**C:N**	**N:P**
19 ± 1 °C	30.4 ± 0.4	84.9 ± 3.0	9.9 ± 0.1	2.6 ± 1.1	76.7 ± 4.7	48.8 ± 18.2	0.90	8.51
25 ± 1 °C	34.3 ± 2.8	92.9 ± 2.9	11.5 ± 0.1	2.3 ± 5.2	92.0 ± 0.2	42.9 ± 6.9	1.00	8.10
28 ± 1 °C	31.7 ± 3.3	89.8 ± 3.6	11.7 ± 0.2	2.5 ± 0.01	22.0 ± 0.3	69 ± 26.8	0.24	7.71

Table 8. Nutrient reduction rates, biomass productivity and specific growth rates achieved at experimental sets for optimum temperature investigation.

	% Removal Rate					Maximum Biomass Productivity (mg L^{-1} d^{-1})	Specific Growth Rate (d^{-1})
Temperature	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**d-COD**		
19 ± 1 °C	99.8 ± 0.1	99.1 ± 0.1	99.7 ± 0.2	0.0	53.0 ± 2.3	69.3 ± 3.9	0.237 ±0.07
25 ± 1 °C	99.2 ± 0.1	95.5 ± 1.6	100.0 ± 0.1	0.0	72.5 ± 0.9	93.7 ± 11.5	0.283 ±0.09
28 ± 1 °C	94.5 ±0.1	95.4 ± 2.1	99.6 ± 0.1	0.0	0.0	61.5 ± 1.3	0.223 ±0.06

The temperature of 25 °C was determined as optimum as it produced enhanced biomass yields and also resulted in significant nutrient reduction rates. However, the temperature of 19 °C also appears to be suitable.

Effect of Temperature on Biomass Composition

Biomass quality was also evaluated for the three temperatures examined. Differences in biomass biochemical composition are presented in Table 9. At 19 ± 1 °C the lipid content of the dry biomass reached 26.7% d.w., while carbohydrate content was 16.3% d.w. However, at 25 ± 1 °C, the ability of the cells to accumulate carbohydrates (20.4% d.w.) and lipids (30.2% d.w.) improved significantly. Statistically significant differences were not observed for lipid contents between 19 and 25 ± 1 °C (p = 0.17882) or all tested temperatures (p = 0.16731). However, statistically significant differences were recorded for carbohydrate contents at 19 and 25 ± 1 °C (p = 0.02828) and between all tested temperatures (p = 0.03854). It was also observed that carbohydrate and lipid contents decreased (13.5% d.w. and 26.2% d.w.) when the temperature reached the value of 28 °C.

Table 9. Effect of temperature on biomass biochemical composition.

% d.w. Content					
Temperature	**Proteins**	**Lipids**	**Carbohydrates**	**Total Chlorophylls**	**Total Carotenoids**
19 ± 1 °C	34.8 ± 1.0	26.7 ± 1.7	16.3 ± 1.3	3.4 ± 0.10	0.42 ± 0.03
25 ± 1 °C	38.7 ± 1.2	30.2 ± 1.5	20.4 ± 2.2	5.1 ± 1.20	0.51 ± 0.10
28 ± 1 °C	41.6 ± 2.1	26.2 ± 1.3	13.5 ± 0.4	2.7 ± 0.05	0.27 ± 0.05

Sheng et al. [52] reported that microalgal lipids tend to decrease beyond the optimal temperature due to stress upon the photosynthetic system II. Thus, the present results highlight that optimum temperature for the accumulation of these intracellular components was 25 ± 1 °C. Studies on the effects of temperatures have been performed on many microalgae. Both Converti et al. [53] and Wei et al. [54] concluded that growth and lipid accumulation reduced in *Nannochloropsis oculata* and *Tetraselmis subcordiformis* cultures when the optimum temperature was exceeded. Compared to the existing bibliography for *Tetraselmis strains*, the lipid contents recorded in the present study are among the highest reported. Patidar et al. [26] found that *Tetraselmis striata* exhibited a maximum lipid content

of 20.3% at 20 °C, while at 15 °C the lipid content increased to 25.7%. Arkronrat et al. [55] found that at 25 °C *Tetraselmis* sp. produced a carbohydrate content of 16.6% and a lipid content of 9.4%. In another study, *Tetraselmis suecica* FIKU032 grown at 25 °C produced a lipid content of 10% while at 30 °C lipid contents decreased [15]. Finally, Wei et al. [54] also reported that the optimum temperature for the growth of *Tetraselmis subcordiformis* enhanced lipid production up to the value of 22.2%.

Protein accumulation reached a maximum of 41.6% d.w. at 28 ± 1 °C, while significant protein content (38.7% d.w.) was also produced at 25 ± 1 °C. It should be noted the intracellular components were estimated at the late exponential to early stationary phase in each growth condition examined. During the early exponential phase values of protein content recorded at 19 and 28 ± 1 °C were similar to those observed at the end of the growth cycle, while at 25 ± 1 °C protein content was significantly higher (49.9% d.w.; data not shown). Generally, protein contents in marine microalgae tend to decrease with high temperatures due to protein breakdown and interference with enzyme regulators. Additionally, protein production is strongly affected by the availability of nitrogen within the growth medium and this is described as a growth stage dependent accumulation [56]. Thus, the differences observed in protein contents between the growth phases for 25 and 28 ± 1 °C can be attributed to these effects. In one study, *Tetraselmis striata* CTP4 was cultivated under mesophilic (20 °C, 100 μmol photons m^{-2} s^{-1}) and stress conditions (30 °C, 380 μmol photons m^{-2} s^{-1}) [57]. The results showed a significant reduction in protein from the mesophilic (25.7% d.w.) to the stress (17.5% d.w.) conditions [57]. In another study *Tetraselmis suecica* was cultivated at 20, 25 and 30 °C and the highest protein content was recorded at 20 °C (21.9% d.w.) while protein production reduced as the temperature increased [16].

Total chlorophylls and total carotenoids presented their highest values at 25 ± 1 °C (maximum values of 5.1% d.w. and 0.51% d.w., respectively). At 19 ± 1 °C these values were 3.4% d.w. (chlorophylls) and 0.42% d.w. (carotenoids). As seen in Table 9, the lowest pigment contents were recorded at 28 ± 1 °C. These findings are in accordance with other research on the effect of temperature on *Tetraselmis striata* CTP4 where higher temperatures led to reduced pigment production [57].

The results lead to the conclusion that the optimum temperature for *T. striata* growth is 25 ± 1 °C, and an increase in this value significantly affects not just biomass, but also lipid, carbohydrate and pigment production.

3.4. Optimization of Photoperiod

Experiments were then conducted at pH 8 and 25 ± 1 °C to investigate the effect of the photoperiods 20:4, 18:6, 12:12 h L:D (Table 10). To correlate the effect of photoperiod on biomass production, the previous experiment conducted under conditions of continuous illumination (Section 3.3, pH 8, 25 ± 1 °C, 24:0 h L:D) was selected as the controlled conditions experiment. Figure 5 presents the effect of light and dark cycles on *T. striata* growth. Biomass was significantly affected by the absence of light and reduced as the dark periods prolonged. Specifically, maximum biomass concentrations of 387, 343 and 327 mg L^{-1} d^{-1} were observed for the 20:4, 18:6, 12:12h L:D photoperiods, respectively. Biomass productivities and specific growth rates were notably low, the highest of which was recorded as 46.3 mg L^{-1} d^{-1} (corresponding growth rate of 0.136 d^{-1}) for the photoperiod of 20:4 h L:D (Table 11). Statistically significant differences of biomass concentrations (p = 0.33265) and productivities (p = 0.26995) were not recorded between the three photoperiods; however, significant differences were observed for their specific growth rates (p = 0.0299). Moreover, it was observed that *T. striata*'s growth cycle prolonged until the 20th day. It should be noted that at the beginning of cultivation young microalgae cells presented an adaptation phase in all photoperiods tested, but then microalgae under continuous illumination (20:4 h L:D) grew faster, following the standard sigmoid growth curve, indicating the ability of the biomass to acclimatize and develop better than in the other photoperiods tested. This is probably because insufficient light causes photolimitation of microalgal growth.

Table 10. Initial biomass and nutrient concentrations in the experimental sets for optimum photoperiod investigation.

	Initial Concentrations (mg L^{-1})							
Photoperiod	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**d-COD**	**Biomass**	**C:N**	**N:P**
20:4 h L:D	21.6 ± 1.2	80.4 ± 1.0	9.9 ± 0.1	1.9 ± 0.5	26.0 ± 4.7	70.3 ± 10.6	0.32	8.07
18:6 h L:D	31.7 ± 2.8	84.7 ± 4.2	10.7± 0.1	1.2 ± 0.7	84.7 ± 0.9	48.3 ± 7.1	1.00	7.85
12:12 h L:D	15.6 ± 1.5	77.2 ± 6.1	9.7 ± 0.2	2.1 ± 0.1	77.7 ± 6.1	46.4 ± 4.4	1.00	7.94

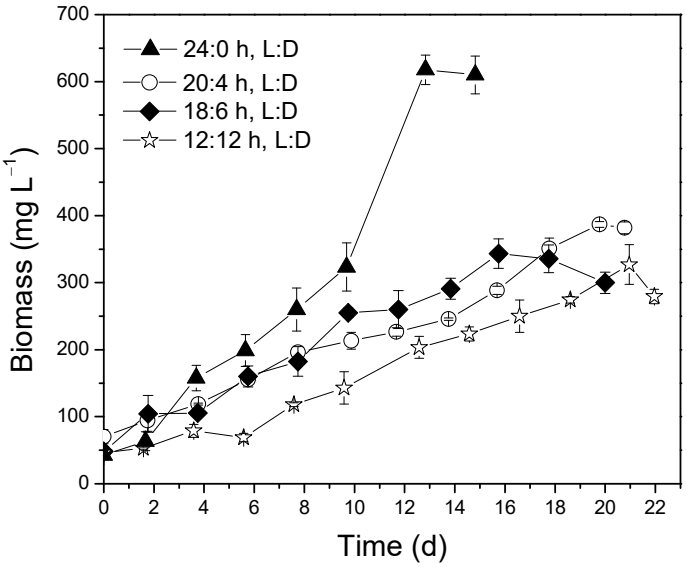

Figure 5. Effect of photoperiod on the biomass production of *T. striata*.

Table 11. Nutrient reduction rates, biomass productivity and specific growth rates in experimental sets for optimum photoperiod investigation.

	% Removal Rate					Maximum Biomass Productivity (mg L^{-1} d^{-1})	Specific Growth Rate (d^{-1})
Photoperiod	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**d-COD**		
20:4 h L:D	62.6 ± 1.8	74.00 ± 2.7	98.7 ± 0.10	0.0	0.0	46.3 ± 3.1	0.136 ± 0.04
18:6 h L:D	52.0 ± 3.8	76.80 ± 6.1	97.7 ± 0.03	0.0	47.2 ± 8.0	36.3 ± 4.2	0.208 ± 0.08
12:12 h L:D	47.8 ± 0.5	61.01 ± 2.5	96.8 ± 0.01	0.0	68.8 ± 2.3	30.0 ± 2.6	0.118 ± 0.03
Control set							
24:0 h L:D	99.2 ± 0.1	95.5 ± 1.6	100.0 ± 0.1	0.0	72.5 ± 0.9	93.7 ± 11.5	0.283 ± 0.09

These results are in accordance with the relevant bibliography. Patidar et al. [26] studied the effect of two photoperiods (24:0 and 12:12 h L:D) on the growth of *T. striata* and concluded that continuous illumination is crucial for enhanced cell growth, recording biomass productivities of up to 79.85 mg L^{-1} d^{-1} under 24:0 h L:D. Zevallos-Feria [27] reported the same trend, as the specific growth rate of *Tetraselmis striata* gradually reduced from 0.44 d^{-1} at 24:0 h L:D, to 0.17 d^{-1} as dark periods were increased. Many other researchers studying the effect of photoperiod on different *Tetraselmis strains* have reached the same conclusion [18,19]. Of these, Pedro [18] also observed a shorter biological cycle under the 24:0 h L:D photoperiod.

Nutrient initial concentrations are given in Table 10 while nutrient removal rates are presented in Table 11 (nutrient removals over time are presented in Figures S11–S13). Low NH_4^+-N reduction rates of below 62.6% were recorded and the lowest reduction rate (47.8%) occurred in the 12:12 h L:D photoperiod. Similarly, TN presented lower removal rates (from 74 to 61%) as the dark periods were prolonged. Compared to the controlled conditions, TN and NH_4^+-N showed higher reduction rates when light was adequate

(Table 8). Generally, nitrogen assimilation is a photo-dependent process [58] and this may explain the lower removal rates recorded in the longer dark periods. Regarding PO_4^{3-}, the photoperiod did not appear to negatively affect its reduction rate. Total sugars also did not show any reduction, although their production rate was slower at 18:6 and 12:12 h L:D. d-COD consumption ranged from 47.2 to 68.8% for the 18:6 and 12:12 photoperiods exhibiting lower removal rates than the controlled conditions (Table 11). At 20:4 h L:D, d-COD was not removed, however its initial concentration was lower here than in the other substrates tested (Table 10).

According to the results of these experiments, high biomass yields and significant nutrient accumulation can be obtained at 24:0 h L:D illumination.

Effect of Photoperiod on Biomass Composition

At 24:0 and 20:4 h L:D the microalgal cells accumulated lipids (30.2% d.w., 28.5% d.w.) and carbohydrates (20.4% d.w., 15.6% d.w.) at higher rates. Statistically significant differences of carbohydrate contents ($p = 0.00179$) were observed between 24:0 and 20:4 h L:D, although significant differences of lipid contents were not observed ($p = 0.46567$). Lipid and carbohydrate contents gradually reduced when the dark periods were prolonged (Table 12). The lowest accumulation rates were recorded at 12:12 h L:D with 24.7% d.w. lipids and 13.8% d.w. carbohydrates. Statistically significant differences of lipid contents between the 24:0 h L:D and the other photoperiods were not recorded ($p = 0.44539$), although significant differences were recorded for carbohydrate contents ($p = 0.000035$). Statistically significant differences were recorded both for lipids ($p = 0.04641$) and carbohydrates ($p = 0.0005749$) between the control set (24:0 h L:D) and the 12:12 h L:D photoperiod. Fakhri et al. [59] suggested that prolonged dark periods are associated with the photolimitation phenomenon. During this phenomenon, cells receive insufficient light. When exposed to dark conditions, microalgae can consume carbon compounds produced during the light regime in order to maintain their cells. Further responses to photolimitation are the reduction in growth rates and biomass yields [60]. Contrary to the present study, Patidar et al. [26] found that T. striata produced lipid contents of 20.3% d.w. at 24:0 h L:D and slightly higher values (22.4% d.w.) at 12:12 h. However, the lipids recorded were lower than those produced in the present study under the same growth conditions (24:0 h L:D, 30.2% d.w.).

Table 12. Effect of photoperiod on biomass biochemical composition.

	% d.w. Content				
Photoperiod	**Proteins**	**Lipids**	**Carbohydrates**	**Total Chlorophylls**	**Total Carotenoids**
20:4 h L:D	53.2 ± 0.5	28.5 ± 3.8	15.6 ± 1.4	4.2 ± 0.1	0.60 ± 0.10
18:6 h L:D	50.4 ± 1.2	25.8 ± 1.1	14.4 ± 1.6	2.8 ± 0.6	0.53 ± 0.01
12:12 h L:D	54.0 ± 1.8	24.7 ± 2.5	13.8 ± 0.5	3.8 ± 0.4	0.59 ± 0.20
Control set 24:0 h L:D	38.7 ± 1.2	30.2 ± 1.5	20.4 ± 2.2	5.1 ± 1.2	0.51 ± 0.10

Protein accumulation was not affected significantly by light absence (Table 12) and the highest value achieved at 12:12 h L:D was 54.0% d.w. As mentioned above, protein production is strongly affected by the availability of nitrogen in the growth medium; therefore, nutrient uptake also reduced as the photoperiod resulted in lower growth rates. A similar effect of photoperiod on nutrient uptake rates was also observed by Meseck et al. [19]. Nitrogen availability in the growth medium at the end of the growth cycle could explain the high protein contents noted in the photoperiod experiments. On the contrary, pigment production showed a reverse trend of gradual reduction. Specifically, total chlorophylls had low accumulation rates both at 18:6 and 12:12 h L:D (2.8% d.w. and 3.8% d.w., respectively), while total carotenoids seem unaffected by the prolonged dark conditions. Fakhri et al. [59] suggested that high pigment content in longer light regimes might be related to cell number and biomass production.

Tetraselmis chuii was studied under the photoperiods of 24:0, 18:6 and 12:12 h L:D by Pedro [18] who observed that similar protein contents were achieved under dark conditions and the highest protein content was recorded at 24:0 h L:D. Yusouf et al. [17] studied the effect of photoperiod (24:0 and 12:12 h L:D) on pigment accumulation in the strain *Tetraselmis chuii* and recorded that both chlorophylls and carotenoids showed higher accumulation rates in continuous illumination, while a significant decrease was recorded at 12:12 h L:D.

High protein contents were recorded in all the photoperiod experiments, however, production of other intracellular products gradually decreased as the dark periods extended. The effect of photoperiod on growth was significant and biomass productivity reduced by almost half compared to controlled conditions. Thus, continuous illumination was selected as the optimum as it resulted in both high biomass and intracellular product production.

3.5. Optimization of CO_2 Flow Rate

Further experiments were conducted using pure CO_2 as the sole carbon source at three different flow rates: 10, 20 and 90 mL min^{-1} (Table 13). It should be noted that the medium was not enriched with $NaHCO_3$ here in order to study just the effect of pure CO_2 on biomass growth. Figure 6 presents the effect of CO_2 flow rate on *T. striata* growth. The previous experiment from Section 3.3 was selected as the control condition of 0 mL CO_2 min^{-1} (pH 8, 25 ± 1 °C, 24:0 h L:D, $NaHCO_3$ only as carbon source). The results revealed that *T. striata* could not tolerate the high flow rate of 90 mL min^{-1}, as it significantly affected the pH of the growth substrate resulting in values of 5.8–6.5. On the other hand, the strong buffering capacity of the CO_2 did not allow the pH to stabilize at the optimum value of 8.0 and the culture almost immediately deteriorated.

Table 13. Initial biomass and nutrient concentrations in the experimental sets using pure CO_2 as carbon source.

	Initial Concentrations (mg L^{-1})							
CO_2 Flow Rate	**NH_4^+-N**	**TN**	**PO_4^{3-}**	**Total Sugars**	**d-COD**	**Biomass**	**C:N**	**N:P**
10 mL min^{-1}	20.2 ± 1.9	87.5 ± 1.3	10.9 ± 0.1	2.4 ± 0.2	28.4 ± 5.2	83.3 ± 7.8	0.32	8.03
20 mL min^{-1}	17.8 ± 2.3	78.4 ± 3.2	9.5 ± 1.4	3.4 ± 0.1	88.9 ± 0.2	45.0 ± 15.0	1.13	8.21
90 mL min^{-1}	14.5 ± 0.4	80.8 ± 2.5	10.7 ± 1.7	2.8 ± 0.7	43.3 ± 4.2	84.0 ± 5.6	0.54	7.60

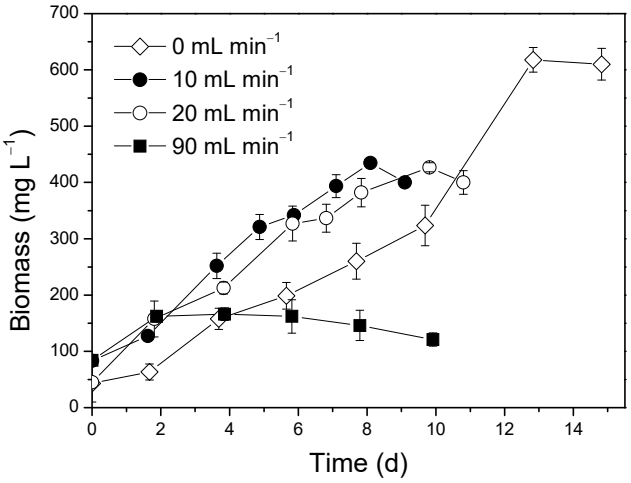

Figure 6. Effect of CO_2 flow rate on the biomass production of *T. striata*.

A high CO_2 level can cause a drop in pH. Depending on the initial CO_2 concentration, if the rate of CO_2 dissolution is higher than the rate of its fixation by the microalgae, constant acidification of the growth substrate may occur [61]. Gradual acidification of the growth substrate was observed at lower flow rates of 10 and 20 mL min^{-1} although pH was adjusted and remained above 7.5. The highest biomass productivity was recorded with

the 10 mL min^{-1} flow rate reaching 87.5 mg L^{-1} d^{-1} with a corresponding growth rate of 0.262 d^{-1}, while biomass yields were also significant at 20 mL min^{-1} (Table 14). CO_2 supplementation not only enhanced biomass growth but also led to a shorter biological cycle of just 11 days. It should be noted that at the optimum temperature of 25 ± 1 °C similar biomass productivity was achieved (93.7 mg L^{-1} d^{-1}) although the growth cycle lasted about 16 days. Thus, CO_2 can also be used as a means of growth acceleration.

Table 14. Nutrient reduction rates, biomass productivity and specific growth rates in the experimental sets using pure CO_2 as carbon source.

	% Removal Rate					Maximum Biomass Productivity (mg L^{-1} d^{-1})	Specific Growth Rate (d^{-1})
CO_2 Flow Rate	NH_4^+-N	TN	PO_4^{3-}	Total Sugars	d-COD		
10 mL min^{-1}	99.6 ± 0.1	91.8 ± 0.4	68.0 ± 0.8	0.0	21.0 ± 3.5	87.5 ± 16.4	0.262 ± 0.01
20 mL min^{-1}	82.8 ± 1.9	78.1 ± 1.2	52.8 ± 0.7	0.0	0.0	73.8 ± 10.1	0.216 ± 0.07
90 mL min^{-1}	0.0	0.0	0.0	0.0	0.0	20.3 ± 2.2	0.050 ± 0.01

Again, direct comparison of the experimental results with other research works is not feasible as there is great diversity in the strains and practices used. However, it is safe to conclude that biomass tolerance and the optimal CO_2 addition is strain specific. Tahir et al. [62] noted that reduced growth rates of Tetraselmis chuii may be attributed to pH decreasing as a result of increasing acidification at elevated CO_2 concentrations. Additionally, the strain recorded the highest specific growth rate (0.096 d^{-1}) at the lowest CO_2 concentration (390 ppm). Olaizola et al. [61] cultivated Tetraselmis chuii with 5% pure CO_2 at different flow rates of 0, 10, 30, 100 and 150 mL min^{-1}. The maximum growth rate of 0.7 doublings/day was recorded at 150 mL min^{-1}. The effect of CO_2 concentration and flow rate on Tetraselmis subcordiformis growth was studied by Zheng et al. [23] who observed a maximum biomass productivity of 34.17 mg L^{-1} h^{-1} at 5% CO_2 and a flow rate of 0.3 VV^{-1} m^{-1}. Tetraselmis suecica was also cultured with 0.04, 5, 15 and 30% CO_2 and results showed that the strain could grow equally well at 5 and 15% with a maximum biomass productivity of 111.2 mg L^{-1} d^{-1} [21].

Initial nutrient concentrations are given in Table 13 and nutrient reduction rates are presented in Table 14 (nutrient removals over time are shown in Figures S14–S16). The highest nutrient removal rates were recorded with the 10 mL min^{-1} flow rate, while no nutrient reductions were recorded with the growth inhibitory flow rate of 90 mL min^{-1}.

Effect of CO_2 on Biomass Composition

Biomass quality degraded significantly at 90 mL min^{-1} (Table 15). All intracellular components showed low rates of accumulation when the high CO_2 flow rate was employed due to biomass collapse. Recorded lipids were lowest at the flow rate of 20 mL min^{-1} but increased to 27.3% at the flow rate of 10 mL min^{-1}. A positive effect between CO_2 addition and lipid enhancement has also been observed by other researchers [63]. Carbohydrate content ranged between 10.3 to 11.5% d.w. and no significant differences between the flow rates were detected. This trend has also been recorded in other studies employing different CO_2 concentrations [21,64]. CO_2 supplementation can influence several key enzymes of carbon metabolism and increased enzymatic activity can enhance photosynthesis and subsequent growth [64]. Additionally, higher CO_2 concentrations may cause stress conditions for cells and carbohydrate production, thus excess lipid production may occur [65]. *Tetraselmis suecica* was cultivated with different CO_2 levels and carbohydrate contents of 20% were recorded, although lipid content further increased at the highest CO_2 [21]. In another study *Tetraselmis subcordiformis* presented a high lipid content of 58.6% when cultured with 2% CO_2 together with 1 g L^{-1} $NaHCO_3$ [66] showing that this combination of the different inorganic C sources can be a promising practice.

Table 15. Effect of CO_2 on biomass biochemical composition.

	% d.w. Content				
CO_2 Flow Rate	**Proteins**	**Lipids**	**Carbohydrates**	**Total Chlorophylls**	**Total Carotenoids**
10 mL min^{-1}	44.6 ± 0.8	27.3 ± 1.5	10.3 ± 2.6	5.2 ± 0.3	1.13 ± 0.3
20 mL min^{-1}	44.5 ± 0.7	22.5 ± 4.5	11.5 ± 1.0	3.5 ± 0.4	0.78 ± 0.1
90 mL min^{-1}	25.4 ± 0.2	19.0 ± 2.6	11.5 ± 0.7	1.4 ± 0.07	0.19 ± 0.1

High protein contents were recorded at the lower CO_2 flow rates (Table 15) and total chlorophylls and carotenoids presented a similar pattern. Pigments showed their maximum values of 5.2 (chlorophylls) and 1.13% d.w. (carotenoids) at the lowest flow rate of 10 mL min^{-1}, while the highest flow rate led to the lowest contents. It should be noted that most research on the effect of CO_2 focuses on lipid and carbohydrate contents rather than pigments and proteins. White et al. [67] found that not only lipids but also proteins can increase in the presence of an inorganic C source. Kassim and Meng [21] studied growth of Tetraselmis suecica at different CO_2 flow rates (0.04,5,15,30%) and they found similar protein production (60%) in all cases. Patil and Kaliwal [68] studied the strain Scenedesmus bajacalifornicus BBKLP-07 at elevated CO_2 levels (5–25% v/v) and recorded the maximum protein content of 30% at 15% v/v CO_2 level, while protein content was significantly reduced at higher CO_2 levels. The same pattern was also observed for total pigments and carotenoids at the higher CO_2 levels and their findings are in accordance with our experimental results.

In conclusion, the flow rate of 10 mL min^{-1} promoted the highest growth yields, nutrient removal and biomass quality. Even though the biological cycle decreased significantly in the presence of CO_2, biomass productivity did not surpass that of the optimized growth conditions with $NaHCO_3$. Additionally, analysis of the biomasses produced using CO_2 and $NaHCO_3$ did not detect significant differences in composition. Therefore, it is possible for CO_2 to be substituted by $NaHCO_3$ in a potential full-scale cultivation scheme. The marine strain *Tetraselmis striata* was found to accumulate significant amounts of metabolic compounds possibly surpassing freshwater stains such as *Chlamydomonas reinhardtii*, *Chlorella* and *Spirulina* sp. that already prevail in global markets as superfoods [69]. Therefore, cultivating this marine strain as a renewable biomass feedstock is feasible, and provides the advantage of using seawater which is almost unlimited (97% of the world's water is seawater) or even saline wastewaters, compared to constantly decreasing freshwater reserves.

3.6. Effect of Different Growth Conditions on Fatty Acid Composition

3.6.1. Effect of pH on Fatty Acid Composition

The fatty acid (FA) profile of the total lipids (TL) and the FA distribution in the different lipid fractions synthesized by *T. striata* seems to be affected by the pH of the growth medium. The results showed that in the case of substrates with pH 8.0, the predominant lipid fraction was glycolipids + sphingolipids (G + S) with a percentage of 47.9% in TL, followed by that of neutral lipids (NL) (i.e., 37.1% in TL) and phospholipids (PL) (i.e., 15.0% in TL) (Table S3). In contrast, in substrates with pH 7.0 the predominant lipid fraction was that of NL (i.e., 48.0% in TL) followed by that of G+S and PL (i.e., 41.8% and 10.2% in TL, respectively). These results are in agreement with those obtained by Almutairi et al. [70] for *T. suecica*, who detected higher NL content at pH 7.0 rather than at pH 8.0. It is commonly accepted that NL predominantly serve as energy storage and their production is triggered under various stress factors such as pH [71]. In cultures grown under pH 8.0, TL mainly comprised unsaturated FAs (i.e., monounsaturated FAs—MUFAs and polyunsaturated FAs—PUFAs) instead of saturated (SFAs) (Table S3). Specifically, the predominant FA was eicosapentaenoic acid (EPA, C20:5 n-3) (i.e., 27.6% of total FAs) followed by palmitoleic (C16:1) (i.e., 24.7% of total FAs) and palmitic acid (C16:0) (i.e., 18.5% of total FAs). Myristic (C14:0), oleic (C18:1 n-9), linoleic (C18:2) and eicosenoic (C20:1 n-9) acids were also detected but in lower levels (4.8%, 8.3%, 5.1% and 3.1% in total FAs, respectively). The accumulated

lipids (NL) were rich in MUFAs (52.0%) and SFAs (27.2%) with C16:1, C18:1 n-9 and C16:0 being the most abundant FAs in this fraction. The amount of PUFAs in this fraction was relatively low (15.6%), with EPA reaching the levels of 10.1%. The PL fraction consisted mainly of MUFAs (39.9%), while it also contained high percentages of PUFAs and SFAs (24.0 and 28.9%, respectively). In this fraction, C16:0 presented the highest percentage compared to other FAs (23.6%), while the monounsaturated FAs C16:1 and C18:1 n-9 also had high values. On the contrary, the percentage of PUFAs was remarkable in GL (36.6%) and of these EPA was the most abundant (35.1%). However, the lower pH condition negatively affected the biosynthesis of PUFAs, especially that of EPA which appeared reduced in both TL and individual lipid fractions (with the exception of PL). In contrast, TL and the aforementioned lipid fractions became more saturated and specifically rich in C16:0. A redistribution of the PL fraction was observed, since the percentage of MUFAs increased by 21.3%, while that of SFAs decreased by 22.2% compared to the pH 8.0 growth condition. The generation of lipids with higher saturated compounds as a response to lower pH conditions has been reported for various microalgal strains. For example, Mandotra et al. [72] showed that low pH values had a negative effect on PUFA concentration accompanied by a significant rise in the SFAs of the microalga Scenedesmus abundans. On the contrary, Sakarika and Kornaros [73] reported that the FA composition of Chlorella vulgaris was unaffected by the pH values of the growth medium. Since PUFAs and especially EPA are essential for fish nutrition, the substrate with pH 8.0 was considered the most suitable for producing microalgal cell mass suitable for fish feed.

3.6.2. Effect of Temperature on Fatty Acid Composition

The impact of temperature fluctuation on FA composition is presented in Table S4. Lipids synthesized at the lowest temperature condition (19 ± 1 °C) mainly consisted of MUFAs (36.5% of the total FAs) and SFAs (32.6% of the total FAs), while PUFAs were present in lower quantities (25.1% of the total FAs). The majority of FAs in the TL were C16:0 (26.0%), C16:1 (24.2%) and EPA (20.4%), while C14:0, C18:1 n-9, C18:2 and C20:1 n-9 were also detected, but at relatively low quantities (<9%). At 25 ± 1 °C, lipids became more saturated as a result of the rise in the levels of C16:0 (34.6%), while the amount of PUFAs decreased (20.7%) due to the reduced biosynthesis of EPA (16.6%). The further increase in temperature to 28 ± 1 °C further inhibited the biosynthesis of PUFAs (16.1%) and especially that of EPA which decreased to 10.2% accompanied with increased quantities of SFAs and MUFAs. Consequently, it appears that the cells increased their relative amount of unsaturated FAs when subjected to lower environmental temperatures. This change in the degree of unsaturation caused by temperature decrease has been noticed in many strains of microalgae and is considered an adaptation to extreme environmental conditions [74]. Generally, when growth occurs at low temperatures, cells tend to synthesize more unsaturated FAs, which have lower melting points than saturated FAs, in order to maintain membrane fluidity and activity. Conversely, at high temperatures increased SFA content is observed as they provide membrane stability [75]. Increasing the ratio of PUFAs as a response to temperature decrease was observed in *T. suecica* FIKU032 and *Nannochloropsis* sp. by Chaisutyakorn et al. [15] in *Chlorella sorokiniana* by Wang et al. [76] and in various other strains by Mai et al. [77]. Our data revealed that the lowest biomass productivity and PUFA contents were recorded at 28 ± 1 °C, therefore this temperature value was considered as an unsuitable condition for the cultivation of *T. striata*.

Fractionation of the lipids synthesized by *T. striata* growing at 19 ± 1 °C and 25 ± 1 °C was performed (Table S4). The results showed that at the lowest temperature (19 ± 1 °C), NL (42.4% in TL) were predominant over the polar lipids G + S and PL (38.8 and 18.8% in TL, respectively), while at 25 ± 1 °C, cells produced lipids in which G+S (45.9% in TL) predominated over NL and PL (44.2 and 8.8% in TL, respectively). Temperature changes slightly affected the NL content which remained almost stable, an observation made also for *Tetraselmis subcordiformis* by Wei et al. [54]. In contrast, *Nannochloropsis oculata* exhibited reduced levels of NL at the lower temperature condition [54]. Chua et al. [78]

reported that the cold-treated cells of *Nannochloropsis oceanica* showed increased chloroplast size compared to untreated cells. This observation might explain the increased amount of PL noted in the low temperature growth condition (almost 2.0-fold higher than the highest temperature), since PL are membrane lipids of various organelles, including chloroplasts. Decreased temperature imposed an exponential increase in the proportion of PUFAs and reduction in C16:0 and C16:1 in all the lipid fractions. Specifically, NL and G contained higher quantities of EPA (2.0-fold higher) at low temperature compared to the higher temperature condition, while PL had increased levels of C18:2. To conclude, the FA composition of membrane and storage lipids responded similarly to the temperature changes and followed the general trend of increased PUFA levels with decreasing growth temperatures.

3.6.3. Effect of Photoperiod on Fatty Acid Composition

The FA composition of the lipids synthesized by T. striata growing on different light and dark cycles (20:4, 18:4 and 12:12 h L:D) is presented in Table S5. The experiment that was previously conducted at 25 ± 1 °C under continuous illumination (Section 3.6.2) was selected as the control condition. In all the evaluated photoperiods, the predominant FAs of TL were: EPA (26.4 to 34.3% in total FAs), followed by C16:0 (18.6 to 20.9% in total FAs) and C16:1 (18.4 to 21.0% in total FAs). Moreover, the FAs C14:0, C18:0, C18:1 n-9, C18:2 and C20:1 n-9 were also detected at lower quantities (<9%). In all cases, TL contained higher amounts of PUFAs and lower amounts of MUFAs and SFAs compared to the cultures under full illumination. Specifically, in continuous illumination conditions PUFAs contributed to the total FAs with 20.7%, while for the photoperiod 20:4 h L:D their percentage increased to 37.9% and reached the maximum value of 41.6% under the photoperiod 12:12 h L:D. This increase was mainly a result of the rise in EPA and not in C18:2 which remained almost constant in all conditions. Polyunsaturation of FAs often occurs in algal cells as a response to decreasing illumination since PUFAs are essential for the maintenance of the photosynthetic membrane and play an important role in adaptation in low light conditions. For example, higher amounts of PUFAs were observed in the microalgae *Chaetoceros calcitrans*, *Chlorella* sp. and *Nannochloropsis* sp. under extended dark periods [79]. Similar results were obtained for *Scenedesmus* sp. [80] and Scenedesmus obliquus [81]. Since the productivity and the lipid content of the biomass decreased as the dark period was prolonged, recording minimum values in the photoperiod 12:12 h L:D, this illumination condition was considered as unsuitable for full-scale application. Hence, the lipids produced by *T. striata* growing under this condition were not further analyzed. As shown in Table S5, the prolonged dark periods in the light/dark cycle favored the biosynthesis of membrane lipids, especially that of PL which exhibited the highest percentage at the photoperiod 20:4 h L:D (20.5% compared to 9.9% in TL under continuous illumination). Our results are in agreement with those of Srirangan et al. [82] who reported that cells grown under continuous illumination contained 25% less membrane lipids (mainly chloroplast membrane lipids) than cells grown under the 12:12 h L:D photoperiod.

Regarding NL, their percentage was lower in both photoperiods (20:4 and 18:6 h L:D) compared to the continuous illumination mode, while G + S remained almost stable (46.9 ± 0.9% in TL). Along with decreased light periods in the light/dark cycle, the proportion of PUFAs (especially of EPA) in NL and G increased, while that of the SFA (especially C16:0) declined. Specifically, the sum of PUFAs in NL increased by 75.5% and 107.8% in photoperiods 20:4 and 18:6 h L:D, respectively, compared to the continuous illumination condition. Similarly, G exhibited a rise of over 100% in PUFA content in both photoperiods as a result of the remarkable increase in EPA levels.

3.6.4. Effect of CO_2 Supply on Fatty Acid Composition

The FA profiles of the lipids produced by the *T. striata* culture supplied with CO_2 at flow rates of 10 mL min^{-1} and 20 mL min^{-1} are presented in Table S6. For the high flow rate (i.e., 20 mL min^{-1}), the FA composition of the TL did not present any significant differences compared to that produced from cells without a CO_2 supply. Specifically, the major fatty acids recorded

were C16:0, C16:1 and EPA which were found in slightly lower quantities. However, supplying the culture with CO_2 at a flow rate of 10 mL min^{-1} induced the biosynthesis of PUFAs, since the amounts of EPA and C18:2 in TL increased at the expense of C16:0 which reduced. In the microalga Parachlorella kessleri higher CO_2 concentrations were seen to favor the production of SFAs and MUFAs, while PUFAs were reduced [83]. Since the highest growth yields and lipid production were achieved by supplementing the culture with CO_2 at a flow rate of 10 mL min^{-1}, only this condition was further analyzed (i.e., fractionation of the lipids and FA profiles of the lipid classes). Enriching the culture with CO_2 at a flow rate of 10 mL min^{-1} enhanced the biosynthesis of polar lipids, while the accumulation of NL was inhibited. The FA profile of NL and PL did not present any significant differences compared to the control condition (i.e., without CO_2 supply). In contrast, the G fraction showed significantly higher PUFA content as a result of the higher content of EPA.

3.7. Effect of Different Growth Conditions on the Amino Acid Profile of T. striata

All samples presented identical essential to non-essential ratio of amino acids (0.96–1.02). Among the essential amino acids (EAA), lysine (2.54–4.06 g/100 g dry microalgae biomass) and leucine (2.83–4.66 g/100 g) showed the highest concentrations, while amidst the non-essential (NEAA), the most noticeable contents were those of glutamic acid and glutamine (3.72–5.76 g/100 g) and acid aspartic acid and asparagine (3.12–4.98 g/100 g). These data are in accordance with the published literature on other Tetraselmis species [84,85] where lysine, leucine, aspartic acid and glutamic acid were the main amino acids found. The *T. striata* cultivated in photoperiodic system of 12:12 h L:D presented the highest concentration of essential amino acids, with lysine and leucine presenting 4.06 and 4.45 g/100 g dry biomass, respectively. Similarly, in another trial, when Scenedesmus obliquus was cultivated under 12:12 h L:D photoperiod at different stages of its cell growth, it exhibited an increased content of lysine, leucine, aspartic acid and glutamic acid compared to the same strain that cultured at 24:0 h L:D [81]. In line with their crude protein, the sum of EAA (14.90–18.44 g/100 g) and NEAA (14.94–18.09 g/100 g) amino acids in *T. striata* cultivated under different temperatures showed a noticeable decrease. The variation in the protein and amino acid content of microalgae cultivated at different temperature varies from species to species. According to the published literature several microalgal strains showed different protein and amino acid content when cultured at elevated or reduced temperatures [86].

3.8. Effect of Different Growth Conditions on Carotenoid Profile of T. striata

Almost all the analyzed carotenoids were detected and quantified in *T. striata* grown on a laboratory scale except lycopene, where β-carotene and lutein and zeaxanthin exhibited the highest concentrations. More specifically, *T. striata* cultivated under photoperiod of 20:4, 12:12 and 18:6 h L:D presented the highest concentrations of β-carotene (12,395.39, 8639.2 and 7644.58 mg/kg dry microalgae biomass, respectively), while at the same time those samples had apparently low content of lutein and zeaxanthin. The highest concentrations of lutein and zeaxanthin were quantified in T. striata cultivated at 25 ± 1 °C (2026 mg/kg), followed by *T. striata* pH = 7 (1454 mg/kg), T. Striata CO_2 20 mL min^{-1} (1153.93 mg/kg) and *T. striata* pH = 8 (916 mg/kg). Likewise, lutein and b-carotene were the major carotenoids that were found in *Tetraselmis* sp. [87]. Even though astaxanthin was observed in all the cultivation conditions, an increase (28 ± 1 °C) and decrease (19 ± 1 °C) in temperature, as well as a decreased pH at value 7, led to increased contents of 428.69, 336.81 and 324.15 mg/kg dry microalgae biomass, respectively. An increase in astaxanthin content was observed when *Haematococcus pluvialis* was cultivated in temperatures that were ranged between 20–27 °C, especially when these were combined with other stress conditions [88]. Similar trials performed by Domınguez-Bocanegra et al. [89] indicated that the optimum temperature for astaxanthin production was at 28 °C, for *H. pluvialis*, whereas others have indicated the 25 °C [90]. Echinenone content amongst the different cultures ranged between 135.42 and 497.32 mg/kg, where the highest concentration was recorded when the strain was cultivated at pH = 7. Beta-cryptoxanthin concentration ranged between 21.46 and 100.58 mg/kg dry biomass. When CO_2 flowed at the rates of 10 and

20 mL min^{-1}, b-cryptoxanthin production was promoted, reaching the highest concentration (99.15–100.58 mg/kg). Lastly, canthaxanthin (3.63–43.54 mg/kg) had the lowest concentration among the analyzed carotenoids, where controlled temperature at 25 ± 1 °C, pH = 8 and continuous illumination presented the highest value.

4. Conclusions

Tetraselmis striata was cultivated in drilling waters obtained from the Greek commercial fish farm of PLAGTON S.A. The effects of salinity, substrate formulation, pH, temperature, photoperiod and CO_2 flow rate on growth and biomass composition were studied. A salinity of 2.8 ± 0.1% with the commercial fertilizer Nutri-Leaf (30%-TN, 10%-P, 10%-K) and $NaHCO_3$ was found to be the most efficient growth substrate. pH experiments showed that pH 8 significantly increased biomass production. The results also showed a high dependence between illumination and growth, while the most suitable cultivation temperature for the strain was 25 °C. When the growth medium was supplemented with CO_2, *T. striata* could not tolerate the highest flow rate of 90 mL.min^{-1}, and the highest biomass productivity was observed with the flow rate of 10 mL.min^{-1}. Biomass quality evaluation showed that the strain is capable of accumulating high protein and amino acid concentrations. High lipid accumulation was also recorded in the biomass and fatty acid analysis showed that this microalga is an important producer of EPA and PUFA. To conclude, under the optimized growth conditions obtained from the laboratory-scale experiments, *T. striata* produced high quality biomass rich in nutrients important for fish growth. Thus, the strain can be considered as a promising ingredient for incorporation into conventional aquafeeds.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14193162/s1, Table S1: physicochemical characterization of the drilling waters; Table S2: nutrient composition of all tested growth substrates; Table S3: fatty acid composition (%) of total lipids (TL), neutral lipids (NL), glycolipids (GL) and phospholipids (PL) synthesized by *T. striata* growing under different pH conditions; Table S4: fatty acid composition (%) of total lipids (TL), neutral lipids (NL), glycolipids (GL) and phospholipids (PL) synthesized by *T. striata* growing under different temperature conditions; Table S5: fatty acid composition (%) of total lipids (TL), neutral lipids (NL), glycolipids (GL) and phospholipids (PL) synthesized by *T. striata* growing under different photoperiods; Table S6: fatty acid composition (%) of total lipids (TL), neutral lipids (NL), glycolipids (GL) and phospholipids (PL) synthesized by *T. striata* supplied with CO_2 at different flow rates; carotenoid analysis; Figure S1: NH_4^+-N removal over time from the different growth substrates. Experimental sets: A (salinity 3.9 ± 0.1%, N:P≈5), B (salinity 3.9 ± 0.1%, N:P≈12), C (salinity 2.8 ± 0.1%, N:P≈12), D: (salinity 2.8 ± 0.1%, modified F/2), E (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 without $NaHCO_3$), and F (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 with $NaHCO_3$); Figure S2: PO_4^{3-} removal over time from the different growth substrates. Experimental sets: A (salinity 3.9 ± 0.1%, N:P≈5), B (salinity 3.9 ± 0.1%, N:P≈12), C (salinity 2.8 ± 0.1%, N:P≈12), D: (salinity 2.8 ± 0.1%, modified F/2), E (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 without $NaHCO_3$), and F (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 with $NaHCO_3$); Figure S3: total sugar production over time from the different growth substrates. Experimental sets: A (salinity 3.9 ± 0.1%, N:P≈5), B (salinity 3.9 ± 0.1%, N:P≈12), C (salinity 2.8 ± 0.1%, N:P≈12), D: (salinity 2.8 ± 0.1%, modified F/2), E (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 without $NaHCO_3$), and F (salinity 2.8 ± 0.1%, Nutri-Leaf 30-10-10 with $NaHCO_3$); Figure S4: NH_4^+-N removal over time applying different initial fertilizer quantities; Figure S5: NH_4^+-N removal over time during the pH experiments; Figure S6: PO_4^{3-} removal over time during the pH experiments; Figure S7: total sugar production over time during the pH experiments; Figure S8: NH_4^+-N removal over time during the temperature experiments; Figure S9: PO_4^{3-} removal over time during the temperature experiments; Figure S10: total sugar production over time during the temperature experiments; Figure S11: NH_4^+-N removal over time during the photoperiod experiments; Figure S12: PO_4^{3-} removal over time during the photoperiod experiments; Figure S13: total sugar production over time during the photoperiod experiments; Figure S14: NH_4^+-N removal over time during the CO_2 experiments; Figure S15: PO_4^{3-} removal over time during the CO_2 experiments; Figure S16: total sugar production over time during the CO_2 experiments. References [91,92] are cited in the "Supplementary Materials".

Author Contributions: Conceptualization, G.A., D.V.V. and A.G.T.; methodology, V.P., A.D., D.K., C.N.E., G.A., D.V.V., Y.K. and A.G.T.; investigation, V.P., A.D. and D.K.; data curation, V.P., A.D., D.K., D.C.K., C.N.E., D.B., G.A., D.V.V., Y.K. and A.G.T.; writing—original draft preparation, V.P. and A.G.T.; writing—review and editing, V.P., A.D., D.K., C.N.E., D.B., G.A., D.V.V., Y.K. and A.G.T.; supervision, A.G.T.; project administration, A.G.T.; funding acquisition, V.P., A.D., D.K., G.A., D.V.V., Y.K. and A.G.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was financially supported by the project "Large-scale cultivation of microalgae and utilization of the biomass produced as alternative raw material in fish feed-AlgaFeed4Fish" (MIS 5045858, FK 80916). The project "AlgaFeed4Fish" was co-funded by European (European Regional Development Fund) and National Resources (General Secretariat for Research and Technology) in the context of the Operational Program "Competitiveness, Entrepreneurship and Innovation" (EPAnEK, NSRF 2014-2020), specifically through the Action "Special Actions"—"Aquacultures"—"Industrial Materials"—"Open Innovation in Culture".

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Chen, F.; Leng, Y.; Lu, Q.; Zhou, W. The application of microalgae biomass and bio-products as aquafeed for aquaculture. *Algal Res.* **2021**, *60*, 102541. [CrossRef]
2. Food and Agriculture Organization. *The State of World Fisheries and Aquaculture. Sustainability in Action*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2020.
3. Shah, M.R.; Lutzu, G.A.; Alam, A.; Sarker, P.; Kabir Chowdhury, M.A.; Parsaeimehr, A.; Liang, Y.; Daroch, M. Microalgae in aquafeeds for a sustainable aquaculture industry. *J. Appl. Phycol.* **2018**, *30*, 197–213. [CrossRef]
4. Imamoglu, E.; Demirel, Z.; Conk Dalay, M. Process optimization and modeling for the cultivation of *Nannochloropsis* sp. and *Tetraselmis striata* via response surface methodology. *J. Phycol.* **2015**, *51*, 442–453. [CrossRef] [PubMed]
5. Kaparapu, J. Application of Microalgae in Aquaculture. *Phykos* **2018**, *48*, 21–26.
6. Ma, R.; Wang, B.; Chua, E.T.; Zhao, X.; Lu, K.; Ho, S.H.; Shi, X.; Liu, L.; Xie, Y.; Lu, Y.; et al. Comprehensive utilization of marine microalgae for enhanced co-production of multiple compounds. *Mar. Drugs* **2020**, *18*, 467. [CrossRef]
7. Kim, G.; Lee, C.H.; Lee, K. Enhancement of lipid production in marine microalga *Tetraselmis* sp. through salinity variation. *Korean J. Chem. Eng.* **2015**, *33*, 230–237. [CrossRef]
8. Kim, G.; Mujtaba, G.; Lee, K. Effects of nitrogen sources on cell growth and biochemical composition of marine chlorophyte *Tetraselmis* sp. for lipid production. *Algae* **2016**, *31*, 257–266. [CrossRef]
9. Molina, E.; Martínez, E.; Sańchez, S.; García, F.; Contreras, A. The influence of temperature and the initial N:P ratio on the growth of microalgae *Tetraselmis* sp. *Process Biochem.* **1991**, *26*, 183–187. [CrossRef]
10. Danquah, M.K.; Harun, R.; Halim, R.; Forde, G.M. Cultivation medium design via elemental balancing for *Tetraselmis suecica*. *Chem. Biochem. Eng. Q.* **2010**, *24*, 361–369.
11. Carneiro, M.; Pôjo, V.; Malcata, F.X.; Otero, A. Lipid accumulation in selected *Tetraselmis* strains. *J. Appl. Phycol.* **2019**, *31*, 2845–2853. [CrossRef]
12. Dammak, M.; Hadrich, B.; Miladi, R.; Barkallah, M.; Hentati, F.; Hachicha, R.; Laroche, C.; Michaud, P.; Fendi, I.; Abdelkafi, S. Effects of nutritional conditions on growth and biochemical composition of *Tetraselmis* sp. *Lipids Health Dis.* **2017**, *16*, 1–13. [CrossRef]
13. Khatoon, H.; Abdu Rahman, N.; Banerjee, S.; Harun, N.; Suleiman, S.S.; Zakaria, N.H.; Lananan, F.; Abdul Hamid, S.H.; Endut, A. Effects of different salinities and pH on the growth and proximate composition of *Nannochloropsis* sp. and *Tetraselmis* sp. isolated from South China Sea cultured under control and natural condition. *Int. Biodeterior. Biodegr.* **2014**, *95*, 11–18. [CrossRef]
14. Rukminasari, N.; Omar, S.B.A.; Lukman, M. Effects of Increasing Temperature and Nitrate Concentration on Cell Abundance, Growth Rate, Biomass and Free Fatty Acid of *Tetraselmis* sp. In Proceedings of the 2nd International Symposium on Marine Science and Fisheries, Makassar, Indonesia, 22 June 2019.
15. Chaisutyakorn, P.; Praiboon, J.; Kaewsuralikhit, C. The effect of temperature on growth and lipid and fatty acid composition on marine microalgae used for biodiesel production. *J. Appl. Phycol.* **2018**, *30*, 37–45. [CrossRef]
16. Sas, A.A.; Turki, A.J.; Affan, A.; Al-Taisan, W.A.; Das, S.K.; Su, S.N.; Cob, Z.C. The Influence of Temperature and Nutrient Concentrations on Growth Rate, Biomass, Chlorophyll-a, and Biochemical Compositions of *Tetraselmis suecica* (Chlorophyta). In Proceedings of the IOP Conference Series: Earth and Environmental Science, Selangor, Malaysia, 10 November 2021.
17. Yusof, N.S.; Yeong, Y.S.; Zakeri, H.A.; Wahid, M.E.A.; Ghafar, S.N.A.; Yusuf, N. Photoperiod influenced the growth and antioxidative responses of *Chlorella vulgaris*, *Isochrysis galbana*, and *Tetraselmis chuii*. *J. Appl. Pharm. Sci.* **2021**, *11*, 125–134.

18. Pedro, S.I.H. Evaluation of Photoperiod Effect on the Growth and Protein Content of Microalgae. Ph.D. Thesis, School of Tourism and Maritime Technology, Polytechnic Institute of Leiria, Peniche, Portugal, 2015.
19. Meseck, S.L.; Alix, J.H.; Wikfors, G.H. Photoperiod and light intensity effects on growth and utilization of nutrients by the aquaculture feed microalga, *Tetraselmis chui* (PLY429). *Aquaculture* **2005**, *246*, 393–404. [CrossRef]
20. Molina, E.; Martinez, M.E.; Sanchez, S.; Garcia, F.; Contreras, A. The influence of flow rate and the composition of the CO_2/air gaseous mixture on discontinuous growth of *Tetraselmis* sp. *Appl. Microbiol. Biotechnol.* **1990**, *34*, 103–107. [CrossRef]
21. Kassim, M.A.; Meng, T.K. Carbon dioxide (CO_2) biofixation by microalgae and its potential for biorefinery and biofuel production. *Sci.Total Environ.* **2016**, *584–585*, 1121–1129. [CrossRef]
22. Ferriols, V.M.E.N.; Saclauso, C.A.; Fortes, N.R.; Toledo, N.A.; Pahila, I.G. Effect of elevated carbon dioxide and phosphorus levels on nitrogen uptake, lipid content and growth of *Tetraselmis* sp. *J. Fish. Aquat. Sci.* **2013**, *8*, 659–672.
23. Zheng, Y.; Chen, Z.; Lu, H.; Zhang, W. Optimization of carbon dioxide fixation and starch accumulation by *Tetraselmis subcordiformis* in a rectangular airlift photobioreactor. *Afr. J. Biotechnol.* **2011**, *10*, 1888–1901.
24. Rico, M.; González, A.G.; Santana-Casiano, M.; González-Dávila, M.; Pérez-Almeida, N.; de Tangil, M.S. Production of Primary and Secondary Metabolites Using Algae. In *Prospects and Challenges in Algal Biotechnology*; Tripathi, B.N., Kumar, D., Eds.; Springer Nature Singapore Pte Ltd.: Singapore, 2017; pp. 311–326.
25. Boopathy, A.B.; Jayakumar, T.; Chinnasamy, S.; Rajaram, M.G.; Mohan, N.; Nagaraj, S.; Rengasamy, R.; Manubolu, M.; Sheu, J.R.; Chang, C.C. Biomass and Lipid Production Potential of an Indian Marine Algal Isolate *Tetraselmis striata* BBRR1. *Energies* **2020**, *13*, 341. [CrossRef]
26. Patidar, S.K.; Kim, S.H.; Kim, J.H.; Park, J.; Park, B.S.; Han, M.S. *Pelagibaca bermudensis* promotes biofuel competence of *Tetraselmis striata* in a broad range of abiotic stressors: Dynamics of quorum-sensing precursors and strategic improvement in lipid productivity. *Biotechnol. Biofuels.* **2018**, *11*, 1–16. [CrossRef]
27. Zevallos-Feria, S. Influence of photoperiod on *Tetraselmis striata* (Chlorodendraceae) in benthic cultivation system. *The Biologist (Lima)* **2017**, *15*, 387–395.
28. Geider, R.; La Roche, J. Redfield revisited: Variability of C:N:P in marine microalgae and its biochemical basis. *Eur. J. Phycol.* **2002**, *57*, 1–17. [CrossRef]
29. Pai, S.C.; Tsau, Y.J.; Yang, T.I. pH and buffering capacity problems involved in the determination of ammonia in saline water using the indophenol blue spectrophotometric method. *Anal. Chim. Acta.* **2001**, *434*, 209–216. [CrossRef]
30. American Public Health Association. *Standard Methods for the Examination of Water and Wastewater*, 21st ed.; American Public Health Association: Washington, DC, USA, 2005.
31. Tsonis, S.P. A modified method for the examination of chemical oxygen demand in sea water. *WIT Trans. Ecol. Environ.* **1993**, *2*, 1743–3541.
32. 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]
33. American Public Health Association. *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; American Public Health Association: Washington, DC, USA, 1998.
34. Folch, J.; Lees, M.; Sloane-Stanley, G.A. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **1951**, *226*, 497–509. [CrossRef]
35. Bellou, S.; Aggelis, G. Biochemical activities in *Chlorella* sp. and *Nannochloropsis salina* during lipid and sugar synthesis in a lab-scale open pond simulating reactor. *J. Biotechnol.* **2012**, *164*, 318–329. [CrossRef]
36. Association Francaise de Normalisation. *Collection of French Standards for Fats, Oleaginous Grains and Derived Products*, 3rd ed.; French Association for Standardization, Paris (AFNOR): Paris, France, 1984; p. 95.
37. Official Methods of Analysis of AOAC International. *Agriculture Chemicals; Contaminants, Drugs*, 16th ed.; AOAC International: Arlington, VA, USA, 1995; Volume 1.
38. Kotzamanis, Y.; Kouroupakis, E.; Ilia, V.; Haralabous, J.; Papaioannou, N.; Papanna, K.; Richards, R.; Gisbert, E. Effects of high-level fishmeal replacement by plant proteins supplemented with different levels of lysine on growth performance and incidence of systemic non-infectious granulomatosis in meagre (*Argyrosomus regius*). *Aquac. Nutr.* **2018**, *24*, 1738–1751. [CrossRef]
39. Lichtenthaler, H.K.; Buschmann, C. Chlorophylls and Carotenoids: Measurement and Characterization by UV-VIS Spectroscopy. In *Handbook of Food Analytical Chemistry*; Wiley Publishes: Hoboken, NJ, USA, 2005; Volume 2, pp. 171–178.
40. Mohamed, M.S.; Tan, J.S.; Kadkhodaei, S.; Mohamad, R.; Mokhtar, M.N.; Ariff, A.B. Kinetics and modeling of microalga *Tetraselmis* sp. FTC 209 growth with respect to its adaptation toward different trophic conditions. *Biochem. Eng. J.* **2014**, *88*, 30–41. [CrossRef]
41. Dammak, M.; Haase, S.M.; Miladi, R.; Ben Amor, F.; Barkallah, M.; Gosset, D.; Pichon, C.; Huchzermyer, B.; Fendri, I.; Denis, M.; et al. Enhanced lipid and biomass production by a newly isolated and identified marine microalga. *Lipids Health Dis.* **2016**, *15*, 1–13. [CrossRef]
42. Abu-Rezq, T.S.; Al-Musallam, L.; Al-Shimmari, J.; Dias, P. Optimum production conditions for different high-quality marine algae. *Hydrobiologia* **1999**, *403*, 97–107. [CrossRef]
43. Laroche, C. Exopolysaccharides from Microalgae and Cyanobacteria: Diversity of Strains, Production Strategies, and Applications. *Mar. Drugs* **2022**, *20*, 336. [CrossRef]
44. Kamil, W.A. Low cost technique for *Tetraselmis chuii* (Butcher) production in different nutritive conditions as a live feed diet for shrimp hatchery. *Egypt. J. Aquat. Res.* **2009**, *35*, 355–362.

45. Farahin, A.W.; Natrah, I.; Nagao, N.; Yusoff, F.M.; Shariff, M.; Banerjee, S.; Katamaya, T.; Nakakuni, M.; Koyama, M.; Nakasaki, K.; et al. Tolerance of *Tetraselmis tetrathele* to High Ammonium Nitrogen and Its Effect on Growth Rate, Carotenoid and Fatty Acids Productivity. *Front. Bioeng. Biotechnol.* **2021**, *9*, 568776. [CrossRef]
46. Li, X.; Li, W.; Zhai, J.; Wei, H.; Wang, Q. Effect of ammonium nitrogen on microalgal growth, biochemical composition and photosynthetic performance in mixotrophic cultivation. *Bioresour. Technol.* **2019**, *273*, 368–376. [CrossRef]
47. Kokkinos, N.; Lazaridou, A.; Stamatis, N.; Orfanidis, S.; Mitropoulos, A.C.; Christoforidis, A.; Nikolaou, N. Biodiesel production from selected microalgae strains and determination of its properties and combustion specific characteristics. *J. Eng. Sci. Technol. Rev.* **2015**, *8*, 1–6. [CrossRef]
48. Kumar, S.; Saramma, A.V. Effect of Salinity and pH Ranges on the Growth and Biochemical Composition of Marine Microalga-*Nannochloropsis salina*. *Int. J. Agric. Environ. Biotechnol.* **2018**, *11*, 651–660. [CrossRef]
49. Zaher, S.S.; Helal, A.M. How culture medium pH range influence phytoplankton growth performance and biochemical content. *Egypt. J. Aquat. Biol. Fish.* **2020**, *24*, 103–116. [CrossRef]
50. Dammak, M.; Hadrich, B.; Barkallah, M.; Hentati, F.; Ben Hlima, H.; Pichon, C.; Denis, M.; Fendi, I.; Michaud, P.; Abdelkafi, S. Modelling *Tetraselmis* sp. growth-kinetics and optimizing bioactive-compound production through environmental conditions. *Bioresour. Technol.* **2018**, *249*, 510–518. [CrossRef]
51. Chen, S.Y.; Pan, L.Y.; Hong, M.J.; Lee, A.C. The effects of temperature on the growth of and ammonia uptake by marine microalgae. *Bot. Stud.* **2012**, *53*, 125–133.
52. Sheng, J.; Kim, H.W.; Badalamenti, J.P.; Zhou, C.; Sridharakrishnan, S.; Krajimalnik-Brown, R.; Rittman, B.E.; Vannela, R. Effects of temperature shifts on growth rate and lipid characteristics of *Synechocystis* sp. PCC6803 in a bench-top photobioreactor. *Bioresour. Technol.* **2011**, *102*, 11218–11225. [CrossRef]
53. Converti, A.; Casazza, A.A.; Ortiz, E.Y.; Perego, P.; Del Borghi, M. Effect of temperature and nitrogen concentration on the growth and lipid content of *Nannochloropsis oculate* and *Chlorella vulgaris* for biodiesel production. *Chem. Eng. Process. Process Intensif.* **2009**, *48*, 1146–1151. [CrossRef]
54. Wei, L.; Huang, X.; Huang, Z. Temperature effects on lipid properties of microalgae *Tetraselmis subcordiformis* and *Nannochloropsis oculata* as biofuel resources. *Chin. J. Oceanol. Limnol.* **2015**, *33*, 99–106. [CrossRef]
55. Arkronrat, W.; Deemark, P.; Oniam, V. Growth performance and proximate composition of mixed cultures of marine microalgae (*Nannochloropsis* sp. & *Tetraselmis* sp.) with monocultures. *Songklanakarin J. Sci. Technol.* **2016**, *38*, 1–5.
56. Schulze, P.S.C.; Carvalho, C.F.; Pereira, H.; Gangadhar, K.N.; Schüler, L.M.; Santos, T.F.; Varela, J.C.; Barreira, L. Urban wastewater treatment by *Tetraselmis* sp. CTP4 (Chlorophyta). *Bioresour. Technol.* **2017**, *223*, 175–183. [CrossRef]
57. Schüler, L.M.; Bombo, G.; Duarte, P.; Santos, T.F.; Maia, I.B.; Pinheiro, F.; Marques, J.; Jacinto, R.; Schulze, P.S.C.; Pereira, H.; et al. Carotenoid biosynthetic gene expression, pigment and n-3 fatty acid contents in carotenoid-rich *Tetraselmis striata* CTP4 strains under heat stress combined with high light. *Bioresour. Technol.* **2021**, *337*, 125385. [CrossRef]
58. Tsolcha, O.N.; Tekerlekopoulou, A.G.; Akratos, C.S.; Aggelis, G.; Genitsaris, S.; Moustaka-Gouni, M.; Vayenas, D.V. Biotreatment of raisin and winery wastewaters and simultaneous biodiesel production using a *Leptolyngbya*-based microbial consortium. *J. Clean. Prod.* **2017**, *148*, 185–193. [CrossRef]
59. Fakhri, M.; Sanudi; Arifin, N.B.; Ekawati, A.W.; Yuniarti, A.; Hariati, A.M. Effect of photoperiod regimes on growth, biomass and pigment content of *Nannochloropsis* sp. BJ17. *Asian J. Microbiol. Biotechnol. Environ. Sci.* **2017**, *19*, 263–267.
60. Chauton, M.S.; Winge, P.; Brembu, T.; Vadstein, O.; Bones, A.M. Gene regulation of carbon fixation, storage, and utilization in the diatom *Phaeodactylum tricornutum* acclimated to light/dark cycles. *Plant Physiol.* **2013**, *161*, 1034–1048. [CrossRef]
61. Olaizola, M.; Duerr, E.O.; Freeman, D.W. Effect of CO_2 enhancement in an outdoor algal production system using *Tetraselmis*. *J. Appl. Phycol.* **1991**, *3*, 363–366. [CrossRef]
62. Tahir, A.; Rukminasari, N.; Yaqin, K.; Lukman, M. Increasing CO_2 concentration impact upon nutrient absorption and removal efficiency of supra intensive shrimp pond wastewater by marine microalgae *Tetraselmis chui*. *Int. J. Phytoremediation* **2020**, *23*, 64–71. [CrossRef] [PubMed]
63. Bondioli, P.; Della Bella, L.; Rivolta, G.; Chini Zittelli, G.; Bassi, N.; Rodolfi, L.; Casini, D.; Prussi, M.; David, C.; Tredici, M.R. Oil production by the marine microalgae *Nannochloropsis* sp. F&M-M24 and *Tetraselmis suecica* F&M-M33. *Bioresour. Technol.* **2012**, *114*, 567–572. [PubMed]
64. Kandasamy, L.C.; Neves, M.A.; Demura, M.; Nakajima, M. The effects of total dissolved carbon dioxide on the growth rate, biochemical composition, and biomass productivity of nonaxenic microalgal polyculture. *Sustainability* **2021**, *13*, 2267. [CrossRef]
65. 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]
66. Qi, M.; Yao, C.; Sun, B.; Cao, X.; Fei, Q.; Liang, B.; Ran, W.; Xiang, Q.; Zhang, Y.; Lan, X. Application of an in situ CO_2-bicarbonate system under nitrogen depletion to improve photosynthetic biomass and starch production and regulate amylose accumulation in a marine green microalga *Tetraselmis subcordiformis*. *Biotechnol. Biofuels* **2019**, *12*, 1–21. [CrossRef]
67. White, D.A.; Pagarette, A.; Rooks, P.; Ali, S.T. The effect of sodium bicarbonate supplementation on growth and biochemical composition of marine microalgae cultures. *J. Appl. Phycol.* **2013**, *25*, 153–165. [CrossRef]
68. Patil, L.; Kaliwal, B. Effect of CO_2 Concentration on Growth and Biochemical Composition of Newly Isolated Indigenous Microalga *Scenedesmus bajacalifornicus* BBKLP-07. *Appl. Biochem. Biotechnol.* **2017**, *182*, 335–348. [CrossRef]

69. Darwish, R.; Gedi, M.A.; Akepach, P.; Assaye, H.; Zaky, A.S.; Gray, D.A. *Chlamydomonas reinhardtii* is a potential food supplement with the capacity to outperform *Chlorella* and *Spirulina*. *Appl. Sci.* **2020**, *10*, 6736. [CrossRef]
70. Almutairi, A.W.; Toulibah, H.E. Effect of Salinity and pH on Fatty Acid Profile of The Green Algae *Tetraselmis suecica*. *J. Pet. Environ. Biotechnol.* **2017**, *8*, 1–6. [CrossRef]
71. Hu, Q.; Sommerfeld, M.; Jarvis, E.; Ghirardi, M.; Posewitz, M.; Seibert, M. Microalgal triacylglycerols as feedstocks for biofuel production: Perspectives and advances. *Plant J.* **2008**, *54*, 621–639. [CrossRef]
72. Mandotra, S.P.; Kumar, M.; Suseela, M.R.; Nayaka, S.; Ramteke, P.W. Evaluation of fatty acid profile and biodiesel properties of microalga *Scenedesmus* abundans under the influence of phosphorus, pH and light intensities. *Bioresour. Technol.* **2016**, *201*, 222–229. [CrossRef]
73. Sakarika, M.; Kornaros, M. Effect of pH on growth and lipid accumulation kinetics of the microalga *Chlorella vulgaris* grown heterotrophically under sulfur limitation. *Bioresour. Technol.* **2016**, *219*, 694–701. [CrossRef]
74. Brindhadevi, K.; Mathimani, T.; Renec, E.R.; Shanmugamd, S.; Lan Chie, N.T.; Pugazhendhie, A. Impact of cultivation conditions on the biomass and lipid in microalgae with an emphasis on biodiesel. *Fuel* **2021**, *284*, 119058. [CrossRef]
75. Schüler, L.M.; Schulze, P.S.C.; Pereira, H.; Barreira, I.; León, R.; Varela, J. Trends and strategies to enhance triacylglycerols and high-value compounds in microalgae. *Algal Res.* **2017**, *25*, 263–273. [CrossRef]
76. Wang, Y.; He, B.; Sun, Z.; Chen, Y.F. Chemically enhanced lipid production from microalgae under low sub-optimal temperature. *Algal Res.* **2016**, *16*, 20–27. [CrossRef]
77. Mai, T.D.; Lee-Chang, K.J.; Jameson, I.D.; Hoang, T.; Cai, N.B.A.; Pham, H.Q. Fatty acid profiles of selected microalgae used as live feeds for shrimp postlarvae in Vietnam. *Aquac. J.* **2021**, *1*, 26–38. [CrossRef]
78. Chua, E.T.; Dal'Molin, C.; Thomas-Hall, S.; Netzel, M.E.; Netzel, G.; Schenk, P.M. Cold and dark treatments induce omega-3 fatty acid and carotenoid production in *Nannochloropsis oceanica*. *Algal Res.* **2020**, *51*, 102059. [CrossRef]
79. Lim, K.C.; Zaleha, K. Effect of photoperiod on the cellular fatty acid composition of three tropical marine microalgae. *Malays. J. Appl. Biol.* **2013**, *42*, 41–49.
80. Ma, C.; Zhang, Y.; Ho, S.; Xing, D.; Ren, N.; Liu, B. Cell growth and lipid accumulation of a microalgal mutant *Scenedesmus* sp. Z-4 by combining light/dark cycle with temperature variation. *Biotechnol. Biofuels* **2017**, *10*, 260–272. [CrossRef]
81. Vendruscolo, R.G.; Fagundes, M.B.; Maroneze, M.M.; do Nascimento, T.C.; de Menezes, C.R.; Barin, J.S.; Zepka, L.Q.; Jacob-Lopes, E.; Wagner, R. *Scenedesmus obliquus* metabolomics: Effect of photoperiods and cell growth phases. *Bioprocess Biosyst. Eng.* **2019**, *42*, 727–739. [CrossRef]
82. Srirangan, S.; Sauer, M.L.; Howard, B.; Dvora, M.; Dums, J.; Backman, P.; Sederoff, H. Interaction of temperature and photoperiod increases growth and oil content in the marine microalgae *Dunaliella viridis*. *PLoS ONE* **2015**, *10*, e0127562. [CrossRef]
83. De Jesus, P.C.C.; Mendes, M.A.; Perpétuo, E.A.; Basso, T.O.; do Nascimento, C.A.O. Extracellular carotenoid production and fatty acids profile of *Parachlorella kessleri* under increased CO_2 concentrations. *J. Biotechnol.* **2011**, *329*, 151–159. [CrossRef]
84. Farhadian, O.; Yusoff, F.; Mohamed, S. Nutritional values of *Apocyclops dengizicus* (Copepoda: Cyclopoida) fed *Chaetocerous calcitrans* and *Tetraselmis tetrathele*. *Aquac. Res.* **2008**, *40*, 74–82. [CrossRef]
85. Xiang, Q.; Wei, X.; Yang, Z.; Xie, T.; Zhang, Y.; Li, D.; Pan, X.; Liu, X.; Zhang, X.; Yao, C. Acclimation to a broad range of nitrate strength on a euryhaline marine microalga *Tetraselmis subcordiformis* for photosynthetic nitrate removal and high-quality biomass production. *Sci. Total Environ.* **2021**, *781*, 146687. [CrossRef]
86. De Oliveira, M.; Monteiro, M.; Robbs, P.; Leite, S. Growth and Chemical Composition of *Spirulina maxima* and *Spirulina platensis* Biomass at Different Temperatures. *Aquac. Int.* **1999**, *7*, 261–275. [CrossRef]
87. Schüler, L.; Gangadhar, K.; Duarte, P.; Placines, C.; Molina-Márquez, A.; Léon-Bañares, R.; Sousa, V.; Varela, J.; Barreira, L. Improvement of carotenoid extraction from a recently isolated, robust microalga, *Tetraselmis* sp. CTP4 (chlorophyta). *Bioprocess Biosyst. Eng.* **2020**, *43*, 785–796. [CrossRef]
88. Giannelli, L.; Yamada, H.; Katsuda, T.; Yamaji, H. Effects of temperature on the astaxanthin productivity and light harvesting characteristics of the green alga *Haematococcus pluvialis*. *J. Biosci. Bioeng.* **2015**, *119*, 345–350. [CrossRef]
89. Domínguez-Bocanegra, A.R.; Guerrero Legarreta, I.; Martinez Jeronimo, F.; Tomasini Campocosio, A. Influence of environmental and nutritional factors in the production of astaxanthin from *Haematococcus pluvialis*. *Bioresour. Technol.* **2004**, *92*, 209–214. [CrossRef]
90. Olaizola, M. Commercial production of astaxanthin from *Haematococcus pluvialis* using 25,000 Liter photobioreactors. *J. Appl. Phycol.* **2000**, *12*, 499–506. [CrossRef]
91. do Nascimento, T.; Nass, P.; Fernandes, A.; Vieira, K.; Wagner, R.; Jacob-Lopes, E.; Zepka, L. Exploratory data of the microalgae compounds for food purposes. *Data Br.* **2020**, *29*, 105182. [CrossRef] [PubMed]
92. Vendruscolo, R.; Fernandes, A.; Fagundes, M.; Zepka, L.; de Menezes, C.; Jacob–Lopes, E.; Wagner, R. Development of a new method for simultaneous extraction of chlorophylls and carotenoids from microalgal biomass. *J. Appl. Phycol.* **2021**, *33*, 1987–1997. [CrossRef]