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The Effect of Trophic Modes on Biomass and Lipid Production of Five Microalgal Strains

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Abstract: Five microalgae strains, namely *Isochrysis galbana*, *Microchloropsis gaditana*, *Scenedesmus obliquus*, *Nannochloropsis oculata* and *Tetraselmis suecica*, were selected as potential candidates for polyunsaturated fatty acids' production, evaluating biomass productivity and their capacity to accumulate high lipid contents under different trophic modes. Microalgae strains were cultivated in the presence of 1% glucose using mixotrophic and heterotrophic conditions, while autotrophic cultures served as control experiments. The results demonstrate that *S. obliquus* performed the highest biomass productivity that reached 0.13 and 0.14 $g \cdot L^{-1} \cdot d^{-1}$ under mixotrophic and heterotrophic conditions, respectively. *I. galbana* and *S. obliquus* utilized elevated contents of glucose in mixotrophy, removing 55.9% and 95.6% of the initial concentration of the carbohydrate, respectively, while glucose consumption by the aforementioned strains also remained high under heterotrophic cultivation. The production of lipids was maximal for *I. galbana* in mixotrophy and *S. obliquus* in heterotrophy, performing lipid productivities of 24.85 and 22.77 $mg \cdot L^{-1} \cdot d^{-1}$, respectively. The most abundant saturated acid detected for all microalgae strains evaluated was palmitic acid (C16:0), while oleic and linolenic acids (C18:1n9c/C18:3n3) comprised the most abundant unsaturated fatty acids. *I. galbana* performed the highest linoleic acid (C18:2n6c) content under heterotrophic nutrition, which reached 87.9 $mg \cdot g^{-1}$ of ash-free dry weight. Among the microalgae strains compared, the biomass and lipid production monitored for *I. galbana* and *S. obliquus* confirm that both strains could serve as efficient bioproducers for application in algal biorefineries.

Keywords: polyunsaturated fatty acids; microalgae; mixotrophic; heterotrophic; lipid accumulation; biomass

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1. Introduction

Polyunsaturated fatty acids (PUFAs) comprise bioactive lipids, which are essential for human health since they cannot be synthesized de novo, necessitating a supply via dietary intake [1]. The two main categories of PUFAs constitute omega-3 and omega-6 fatty acids, incorporating alpha-linolenic acid (ALA) and linoleic acid (LA) as principal molecules of each class, respectively. Fatty acids are originally synthesized by microalgae at the base of the marine food chain. Thus, microalgae comprise the main source of specific PUFAs, such as arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), holding multiple applications in the pharmaceutical, nutraceutical and food industries [2]. In addition to microalgae, macroalgae and fungi are additionally considered as potential sources for the synthesis of PUFAs [3,4]. However, microalgae are often preferable for the production of lipids due to significant technical and commercial advantages such as CO_2 mitigation and high growth rates [5].

The manufacture of multiple high-value metabolites from microalgae has been explored as a promising approach for enhancing the economic feasibility of third generation biofuels (produced by microalgae) and other algae-based commodities. Successful pigment

production using microalgae exhibited that the specific microorganisms hold great potential as primary producers for the pharmaceutical, food and agronomical industries [6]. Brennan and Owende [7] studied the production of microalgae biomass as food additive in the health food market, demonstrating the advantages of *Spirullina*, *Chlorella* and other species for human nutrition. Cost-effective production of biofuels from *N. salina* has been demonstrated at a commercial scale [8], while *I. galbana* has been applied for the simultaneous production of docosahexaenoic acid and fucoxanthin, providing valuable insights into the future exploration of microalgae for the integrated production of high-value compounds [9].

Microalgae can be cultivated autotrophically, heterotrophically or mixotrophically, based on their ability to adapt to specific environmental conditions. Although most strains grow autotrophically, autotrophic nutrition is typically hampered by limited biomass production as a result of cellular self-shading, lowering light availability at the end of cultivation [10], resulting in low biomass concentrations and an increased harvesting cost [11]. However, inorganic carbon sources can enhance the photosynthetic activities of specific microalgae species resulting in improved biomass concentration [12]. Heterotrophic nutrition could be alternatively applied, employing organic substrates (e.g., sugars, organic acids) as carbon and energy sources, eliminating the requirement for light provision while supporting the scale-up concerning the reactor size, mixing and the surface to volume ratio of the reactor [13]. The specific trophic mode contributes several advantages, including rapid growth, high production of biomass, increased lipid content, and low harvesting costs, due to the higher cell densities achieved [14]. The aforementioned advantages could potentially offset the limitations of heterotrophic nutrition, which include the high cost of organic carbon sources, contamination, substrate inhibition and the limited number of microalgal species capable for heterotrophic growth [15]. Nevertheless, various microalgae strains can grow mixotrophically, holding the capacity to use either autotrophic or heterotrophic metabolic processes [16]. Mixotrophic algae can photosynthesize as well as assimilate and metabolize organic carbon simultaneously, reducing the dependency on light penetration, which enables the formation of higher cell densities as compared to autotrophy. Moreover, mixotrophic nutrition reduces the impact of biomass loss during dark respiration while using lower amounts of organic substrates, as opposed to heterotrophic growth. An improved performance for PUFA synthesis has been previously demonstrated by several microalgae species under mixotrophic and heterotrophic conditions, as compared to autotrophy, pointing out the opportunities contributed for reducing costs and scale up [15,17]. The results obtained by Liu et al. [18] and Penhaul Smith et al. [19] showed that mixotrophic cultivation promotes both *S. obliquus* and *T. suecica* productivity. The combination of autotrophic and heterotrophic growth therefore offers a highly efficient carbon conversion method leading to enhanced biomass and lipid productivity, which constitute mixotrophy as a promising strategy for microalgae cultivation [14,16].

Low-cost feedstocks and biowaste should be explored to overcome the high carbon cost entailed in mixotrophic and heterotrophic cultivation. Choi and Lee [20] evaluated biomass growth and the lipid content formed by *Neochloris oleabundans*, *Botryococcus braunii* and *Dunaliella* sp. under mixotrophic conditions using different contents of crude glycerol, demonstrating that higher biomass and lipid production could occur, as compared to autotrophic growth. Moreover, Mata et al. [21] exhibited that the use of brewery wastewater for *S. obliquus* growth constitutes a technically feasible approach for biodiesel production, while the same strain has been successfully applied for lipids' accumulation, aiming to produce the biofuel from cheese whey permeate [22]. *S. obliquus*, also cultivated in municipal wastewater, showed improved lipid production and pollutant removal capabilities [23].

The objectives of the current study comprised of (i) assessing the autotrophic, mixotrophic and heterotrophic cultivation of *I. galbana*, *M. gaditana*, *S. obliquus*, *N. oculata* and *T. suecica*, via (ii) evaluation of glucose consumption, biomass and lipid productivity as well as fatty acid composition of the microalgae tested and (iii) identification of the strains that hold the capacity for successful application in biowaste-based algal biorefineries. The strains selected were evaluated for their capacity to form biomass and lipids under

different culture conditions that targeted the production of biomass rich in PUFAs. The screening of strains capable for enhanced PUFA manufacture under mixotrophic and heterotrophic conditions enables the future development of algal biorefinery systems for production of the specific high added-value products from biowaste.

2. Materials and Methods

2.1. Microalgal Strains and Growth Conditions

Four marine algae strains *I. galbana*, *M. gaditana*, *N. oculata* and *T. suecica* as well as one freshwater algae, *S. obliquus*, were obtained from the Culture Collection of Algae and Protozoa (CCAP). All strains used in the present study are listed on Table 1.

Table 1. Overview of the microalgae strains employed in the present study.

Class	Genus/Species	Habitat	Strain
Prymnesiophyceae	*I. galbana*	Marine	CCAP 927/1
Eustigmatophyceae	*M. gaditana*	Marine	CCAP 849/5
Chlorophyceae	*S. obliquus*	Freshwater	CCAP 276/3A
Eustigmatophyceae	*N. oculata*	Marine	CCAP 849/1
Chlorodendrophyceae	*T. suecica*	Marine	CCAP 66/4

The freshwater microalgal strain *S. obliquus* was grown in sterile (autoclaved) Bold's Basal medium (pH 6.8), while the rest of the microalgae employed were cultivated using sterile f/2 media (pH 8). The marine algae media was prepared using natural seawater and distilled water was used in the media applied for freshwater algae cultivation. The composition of f/2 comprised (mg L^{-1}): $NaNO_3$ 75, $NaH_2PO_4{\cdot}2H_2O$ 5.65, NA_2EDTA 4.16, $FeCl_3{\cdot}6H_2O$ 3.15, $CuSO_4{\cdot}5H_2O$ 0.01, $ZnSO_4{\cdot}7H_2O$ 0.022, $CoCl_2{\cdot}6H_2O$ 0.01, $MnCl_2{\cdot}4H_2O$ 0.18, $Na_2MoO_4{\cdot}2H_2O$ 0.006, cyanocobalamin (Vitamin B_{12}) 0.0005, thiamine HCl (Vitamin B_1) 0.1, and biotin 0.0005 [24]. Bold's Basal media was composed of (mg L^{-1}): $NaNO_3$ 250, $MgSO_4{\cdot}7H_2O$ 75, NaCl 25, K_2HPO_4 75, KH_2PO_4 175, $CaCl_2{\cdot}2H_2O$ 25, $ZnSO_4{\cdot}7H_2O$ 8.82, $MnCl_2{\cdot}4H_2O$ 1.44, MoO_3 0.71, $CuSO_4{\cdot}5H_2O$ 1.57, $Co(NO_3)_2{\cdot}6H_2O$ 0.49, H_3BO_3 11.42, EDTA 50, KOH 31, $FeSO_4{\cdot}7H_2O$ 4.98, and 1 μL L^{-1} H_2SO_4 (added as concentrated solution) [24]. Autotrophic growth was performed without addition of organic carbon source, whereas mixotrophic and heterotrophic cultivation was conducted by supplementing the medium with 1% D-glucose. Cultures were performed under batch conditions using 1 L glass bottles with 800 mL working volume under continuous shaking at 100 rpm and room temperature. Autotrophic and mixotrophic cultures were maintained under blue (450 nm) and red (650 nm) light (12 h light followed by 12 h darkness cycle) at 30 μmol s^{-1} m^{-2} of light intensity. The flasks were aerated using sterile air and 350 mL min^{-1} flow rate, in the presence of CO_2 (approximately 5%). All cultures were conducted in duplicate for each strain.

2.2. Analyses

2.2.1. Biomass Optical Density and Ash-Free Dry Weight (AFDW)

A known volume of algal culture was filtered through preweighed, precombusted glass microfiber filters (Whatman GF/C, 47 mm diameter) and washed using 10 mL of 0.01 M HCl for freshwater strains and 0.065 M ammonium formate for saline strains. The filters were then dried at 100 °C for 1 h, cooled under vacuum overnight and weighed. Subsequently, the filters were ashed in a muffle furnace at 450 °C for 5 h, cooled in a vacuum desiccator and weighed to obtain AFDW.

Biomass absorbance was measured using a Jenway 7315 spectrophotometer (Staffordshire, UK) employing wavelength range between 530–750 nm to avoid interference with the absorbance of chlorophyll or other photosynthetic pigments. Thus, the absorbance of *M. gaditana*, *S. obliquus*, *N. oculata* and *T. suecica* were measured at 680, 650, 750 and 530 nm, respectively. Algae cultures were diluted to ensure that the absorbance ranged

between 0 and 1, while the concentration of biomass was determined as AFDW employing a previously established calibration curve.

2.2.2. Glucose Concentration

The content of glucose in mixotrophic and heterotrophic cultures was obtained using the 3,5-dinitrosalicilic acid (DNS) assay [25].

2.2.3. Lipid Extraction and Quantification

Prior to analysis, algae were concentrated by filtering a known volume of culture sample while employing glass microfiber filters. Rapture of the cell wall was achieved using liquid nitrogen and a glass rod. Extraction of total lipid was performed using the Folch method [26] following modifications. In brief, 6 mL of chloroform:methanol solution (2:1, *v*/*v*) was added to the sample, using 200 mg L^{-1} of butylated hydroxytoluene (BHT) as antioxidant, and vortex mixed for 3 min (extractions were performed in duplicate). The mixture was filtered through a Büchner funnel under vacuum and the filtrate was transferred to a glass tube, 3 mL of distilled water was added and the mixture was centrifuged to allow phase separation. The methanol/water layer was removed and the chloroform phase containing the lipids was transferred to a preweighed vial, which was placed under a stream of N_2 gas on a heating plate at 40 °C. Following evaporation, the sample was maintained in a vacuum desiccator overnight prior weighing of the vial.

2.2.4. Analysis of Fatty Acid Composition

Lipid transesterification and analysis of fatty acid composition were performed as described in [27], following modifications. Within the lipid extracts obtained via the previous step (Section 2.2.3) fatty acids were derivatized to fatty acid methyl esters (FAMEs) by adding 1 mL of boron trifluoride in methanol (14%, *w*/*w*) and 200 mg L^{-1} BHT. The sample was vortex mixed and heated at 100 °C for 1 h. Following cooling of the mixture at room temperature, methylesters were extracted by adding 1 mL of hexane aliquots and 2 mL of saturated $NaHCO_3$. The mixture was vortexed for 15 s, both layers were allowed to separate, while the hexane phase was collected and subjected to GC analysis. Analysis of FAMEs was performed using a Shimadzu GC-2014 gas chromatograph (Shimadzu, Milton Keynes, UK) equipped with an AOC-20i liquid autosampler and a flame ionization detector (FID). The samples were analyzed on a DB-5ms capillary column (30 m × 0.25 mm I.D.) with 0.25 μm film thickness (Agilent, Santa Clara, CA, USA). The stationary phase of the column was (5%-phenyl)-methylpolysiloxane. The initial oven temperature was 110 °C (5 min hold time), which was increased to 222 °C at a rate of 4 °C min^{-1}, followed by a further increase to 246 °C at a rate of 2 °C min^{-1}, and a final increase to 270 °C at a rate of 4 °C min^{-1} (maintained for 12 min). The method was sufficient to detect FAMEs with chains between 12–22 carbons in length. Nitrogen was used as the carrier gas at a flow rate of 1.03 mL min^{-1}, and the column head pressure was 100 kPa. The temperature of injector and detector was 250 and 300 °C, respectively, while an injection volume of 1 μL under split-mode was employed. Individual fatty acids were identified by comparing their retention times with those of authentic standards (a chromatogram of fatty acids formed by *S. obliquus* under mixotrophy is shown in Figure S1). All chemicals were obtained from Sigma-Aldrich Company Ltd. (Dorset, UK) and were of analytical grade.

3. Results and Discussion

3.1. Algal Biomass Production under Different Trophic Conditions

Significant research effort conducted over the past few years has documented the great benefits of mixotrophic algae production over autotrophy. The nitrogen and phosphorus requirements of *I. galbana* was studied under different cultivation conditions by Alkhamis and Qin [28], indicating that the depletion rates of both elements in mixotrophic cultivation were two times faster as compared to autotrophy. Moreover, *N. oculata* cultures performed under autotrophic and mixotrophic conditions enhanced the maximum specific growth rate

when the concentration of glucose was increased from 0 to 0.1 g L^{-1} [29]. The growth and lipid content of *S. obliquus* were evaluated under different trophic modes, exhibiting that biomass production was more prominent in mixotrophy as opposed to photoautotrophic conditions [30]. A similar trend was monitored for *M. gaditana* and *T. suecica* [19,31], where mixotrophy enabled an elevated production of lipids and carotenoids, while increasing microalgal productivity. Considering that *I. galbana*, *M. gaditana*, *S. obliquus*, *N. oculata* and *T. suecica* can grow mixotrophically, utilizing organic carbon efficiently, the specific microalgae could serve as candidates for the development of biorefineries designed to produce high added value commodities from biowaste. Therefore, in an attempt to identify efficient bioproducers for the development of algal biorefineries, the aforementioned strains were tested here for biomass and lipid production under mixotrophic and heterotrophic conditions via the use of 1% glucose, while autotrophic cultivation served as the control.

The growth curves of the selected microalgae obtained using autotrophic, mixotrophic and heterotrophic nutrition are depicted in Figure 1, where each experiment was maintained until the beginning of the stationary phase. Comparing the growth of microalgae demonstrated that the different trophic conditions employed resulted in significant variations in biomass production for each strain. *S. obliquus* exhibited the highest AFDW in all trophic modes evaluated, producing 1.26, 3.16 and 2.92 g L^{-1} under autotrophic, mixotrophic and heterotrophic cultivation, respectively. A similar performance was previously reported for *S. obliquus* cultivation using cheese whey permeate, where mixotrophic and heterotrophic nutrition resulted in 3.6 and 2.7 g L^{-1}, respectively [22]. Moreover, the response of *I. galbana*, *M. gaditana* and *T. suecica* in the different trophic modes employed demonstrated that mixotrophy could result in higher biomass production, as opposed to autotrophic and heterotrophic nutrition. However, *N. oculata* achieved an elevated biomass formation under heterotrophic conditions, producing 0.92 g L^{-1}, whereas autotrophic and mixotrophic cultivation accumulated 0.10 and 0.29 g L^{-1}, respectively. Autotrophic nutrition resulted in the lowest biomass productivity for all algae strains evaluated, apart from *M. gaditana* where heterotrophy stimulated the lowest biomass production. Although mixotrophic nutrition achieved the highest biomass titre for all microalgae strains compared, cultivation in mixotrophy was prolonged as compared to heterotrophy. The aforementioned effect has been previously reported, demonstrating that mixotrophic conditions result in higher biomass production as opposed to autotrophic and heterotrophic conditions, given that mixotrophy combines the advantages of both autotrophic and heterotrophic cultivation, such as increased growth rates and lipid production [32].

The productivity of biomass for each strain tested is shown in Table 2, where each value was calculated upon entry into the stationary phase. The highest biomass productivity was achieved under heterotrophic nutrition using *S. obliquus*, which reached 0.14 g·L^{-1}·d^{-1}, while *N. oculata* performed 0.058 g·L^{-1}·d^{-1}. Nevertheless, under autotrophic nutrition *N. oculata* was the least productive microalgae tested, yielding 0.003 g·L^{-1}·d^{-1}. Chiu et al. [33] reported that *N. oculata* could be inhibited when fed with CO_2 contents higher than 5%. Thus, the low biomass productivity obtained in autotrophic cultivation of the specific strain could be potentially attributed to the CO_2 level applied, which could be inhibitory for *N. oculata* cells. The biomass productivity of *I. galbana* was significantly higher in the mixotrophic culture as compared to autotrophy and heterotrophy. Alkhamis and Qin [34] achieved 0.011 and 0.022 g·L^{-1}·d^{-1} biomass productivity for *I. galbana* under autotrophic and mixotrophic conditions, respectively, demonstrating that mixotrophy doubled the productivity. These results are consistent with the current study where biomass productivity was raised from 0.033 g·L^{-1}·d^{-1} in autotrophy to 0.057g·L^{-1}·d^{-1} in mixotrophy, and *T. suecica* has exhibited a similar trend achieving a 2-fold increase in the growth rate obtained in autotrophy with the use of mixotrophic cultivation (0.018 vs. 0.036 g·L^{-1}·d^{-1}, respectively) [35], while a 3-fold increase was monitored in the current work.

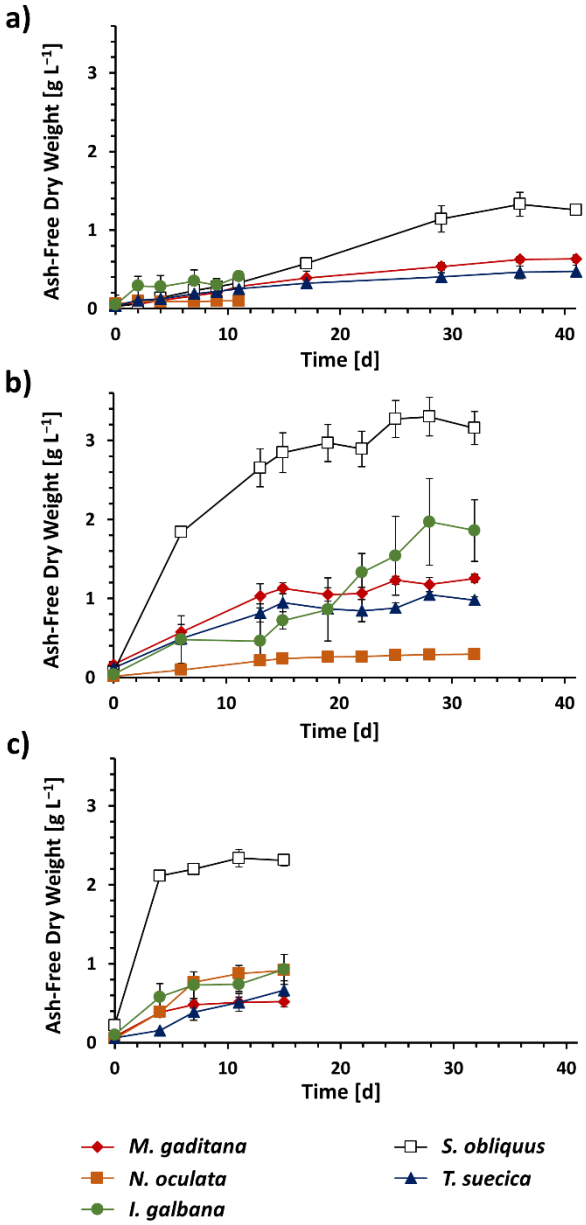

Figure 1. Algal biomass growth achieved using (**a**) autotrophic, (**b**) mixotrophic and (**c**) heterotrophic nutrition. Error bars represent mean values ± standard deviation (n = 6).

Table 2. Biomass productivity (g L^{-1} d^{-1}) of the five microalgae species investigated. Values represent mean values ± standard deviation (n = 6).

Genus/Species	Autotrophic	Mixotrophic	Heterotrophic
I. galbana	0.033 ± 0.0049	0.057 ± 0.011	0.055 ± 0.013
M. gaditana	0.014 ± 0.0011	0.041 ± 0.0031	0.029 ± 0.0041
S. obliquus	0.030 ± 0.0010	0.130 ± 0.0094	0.140 ± 0.0032
N. oculata	0.003 ± 0.0015	0.011 ± 0.0018	0.058 ± 0.0014
T. suecica	0.011 ± 0.0017	0.033 ± 0.0060	0.040 ± 0.0076

The culture medium was enriched with 1% glucose as the organic carbon source in mixotrophy and heterotrophy. The evolution of the glucose concentration in the aforementioned experiments is shown in Figure 2. The results demonstrate that during the time course of mixotrophic and heterotrophic growth, *S. obliquus* and *I. galbana* consumed increased quantities of the carbohydrate. Thus, 95.6% and 55.4% of the initial glucose content

was utilized by *S. obliquus* under mixotrophic and heterotrophic nutrition, respectively, while *I. galbana* used 55.9% of the glucose supplied in mixotrophy and 57% under dark heterotrophic growth conditions. The rest of the three microalgae species evaluated used glucose, employing similar patterns under both types of nutrition and achieved lower consumption rates as compared to *S. obliquus* and *I. galbana*. One-way ANOVA was conducted for all microalgae strains under mixotrophic conditions, demonstrating significant differences in the glucose concentration between 19 and 32 d, apart from *T. suecica* which did not utilize the carbohydrate in mixotrophy. However, there was a statistically significant difference in the glucose concentration between 7 and 15 d under heterotrophic conditions for all microalgae strains ($p < 0.05$), exhibiting that all selected strains could grow in glucose in the absence of light.

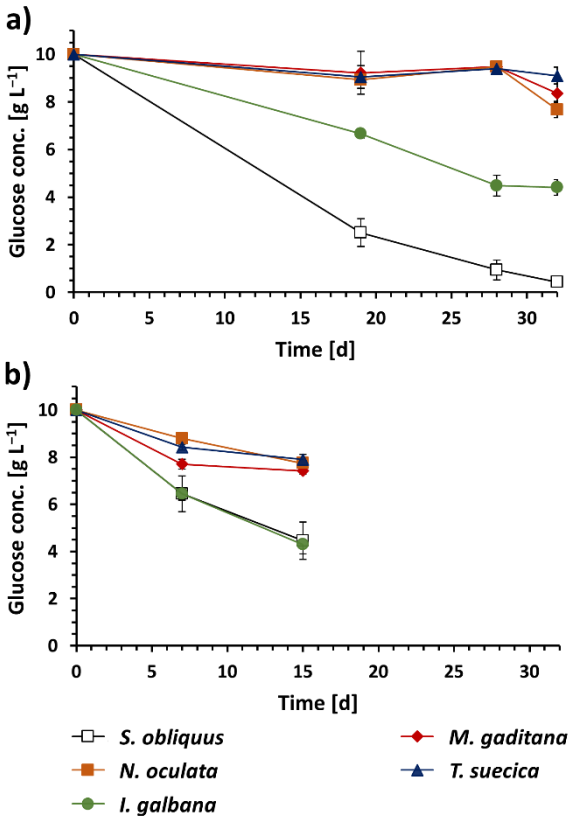

Figure 2. Glucose consumption monitored in (**a**) mixotrophic and (**b**) heterotrophic cultures. Error bars represent mean values ± standard deviation ($n = 6$).

3.2. Lipid Content and Fatty Acid Composition under Autotrophic, Mixotrophic and Heterotrophic Nutrition

Various metabolic pathways employed in microalgae grown under different trophic modes have been studied, given that the supply of different organic substances can stimulate distinct mechanisms for fatty acid synthesis. Msanne et al. [36] explored the growth of *Scenedesmus* sp. under mixotrophic and heterotrophic conditions for wastewater treatment, highlighting the importance of understanding the function of organic carbon metabolism. Glucose could be metabolized through various routes, among which the Pentose Phosphate pathway (PPP), the Embden-Meyerhof-Parnas pathway (EMP) and the Entner-Doudoroff pathway (ED) comprise the most studied mechanisms [37]. The main difference between glucose assimilation in mixotrophic and heterotrophic growth of microalgae constitutes that in the dark, glucose could be commonly metabolized via PPP, while EMP could be more often employed in mixotrophy [12]. The specific effect has been confirmed experimentally by Yang, Hua and Shimizu [38], where although the metabolic flux of *Chlorella pyrenoidosa*

grown in the dark was routed mainly through PPP (90%), while only 10% was directed via EMP, the flux through PPP during illumination was substantially low.

The total lipids produced from the five microalgae strains under the different trophic conditions studied are shown in Figure 3 as percentages of AFDW. Lipid content and composition were determined at 41 d in autotrophy (apart from *I. galbana* and *N. oculata*, which were analyzed at 11 d), while in mixotrophy and heterotrophy the samples processed were withdrawn at 22 and 15 d, respectively. *N. oculata* exhibited the highest lipid accumulation, which reached 44.5% of AFDW in autotrophy and 36.9% of AFDW in mixotrophy. However, the specific strain was the least productive in heterotrophy, accumulating 9.2% of lipids. *T. suecica* generated 28.5% of AFDW as lipids in autotrophy, while mixotrophic and heterotrophic conditions formed 19.3 and 21.2% of lipids, respectively. *M. gaditana* accumulated a higher lipid content under autotrophic conditions (26.5% of AFDW), which was reduced to 19% of AFDW in heterotrophy. However, *S. obliquus* produced more lipids in mixotrophy (21.2% of AFDW) as compared to autotrophic and mixotrophic nutrition which triggered similar lipid production (13.0 and 14.8% of AFDW, respectively). One-way ANOVA was conducted to assess the effect of the different trophic modes on lipid production for each algae strain. The analysis confirmed that significant differences occurred in lipid production between all growth conditions applied in *N. oculata* cultures, while *M. gaditana* exhibited significant differences between mixotrophic and heterotrophic cultivation ($p < 0.05$).

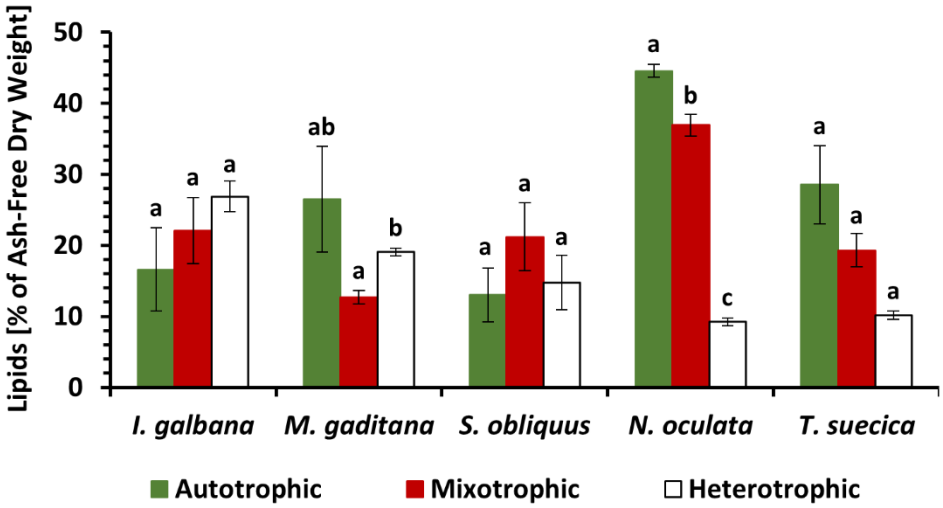

Figure 3. Total lipid content as percentage of ash-free dry weight. Error bars represent mean values ± standard deviation ($n = 2$), while small letters denote significant differences between the mean values obtained ($p < 0.05$).

Ohse et al. [39] studied lipid production from *I. galbana* as well as other strains under autotrophic conditions, demonstrating that the specific microalgae could accumulate up to 19.5% of AFDW as lipids. Moreover, the cultivation of *S. obliquus*, using 10 g L^{-1} of pretreated waste glycerol under mixotrophic conditions, resulted in 24.39% of dry cell weight as lipids [40], which was similar to the content accumulated in the current study under mixotrophic conditions that reached 21.2%. The lipid content of *M. gaditana*, grown autotrophically in different photo-bioreactors, was determined at 24%, employing a photobioreactor of 10 cm width [41], which was slightly lower as compared to the production achieved here (26.5%).

The composition of fatty acids in terms of the different classes and contents entailed in the microalgae studied are given in Table 3, demonstrating that the specific strains exhibited small variations in the type and proportions of fatty acids accumulated. The most abundant saturated acid detected was palmitic acid (C16:0), while oleic and linolenic acids (C18:1n9c/C18:3n3) comprised the most abundant unsaturated fatty acids, which were determined here as a single unit. Among the strains monitored, *I. galbana* performed

the highest LA (C18:2n6c) content under heterotrophic nutrition (87.9 mg·g^{-1} of AFDW), while as opposed to other algae oleic and linolenic acids were maximized under heterotrophic nutrition. Moreover, *I. galbana* accumulated higher contents of palmitic and stearic (C18:0) acids using mixotrophic and heterotrophic conditions. Overall, the content of fatty acids was maximized under heterotrophic nutrition of the specific strain, while autotrophy substantially reduced the production of all fatty acids evaluated. A previous study indicated that *I. galbana* could accumulate up to 9.05 mg·g^{-1} of AFDW as DHA [9], depending on a range of factors, including cultivation conditions (e.g., light intensity, N and P concentration) and the method used for lipids extraction. Moreover, Abirami, Murugesan, and Narender [42] focused on enhancing lipid production from *M. gaditana* by testing different pretreatment methods prior to the lipids' extraction, demonstrating that the specific microalgae could form elevated quantities of EPA (C20:5). *M. gaditana* accumulated the highest content of EPA, as compared to other strains, under autotrophic conditions (8.1 mg·g^{-1} of AFDW), while the production of C18:1n9c/C18:3n3 achieved under autotrophic nutrition was substantial reaching 108.6 mg·g^{-1} of AFDW. Palmitic acid (C16:0) was the most abundant saturated fatty acid accumulated by *S. obliquus*, comprising 61.2 mg·g^{-1} of AFDW under mixotrophic nutrition. The aforementioned strain produced higher fatty acid contents under mixotrophic nutrition as compared to autotrophy and mixotrophy, while the fatty acid composition determined was in agreement to the current literature relevant to mixotrophic cultivation of *S. obliquus* [40]. However, autotrophy was the trophic mode that enabled formation of high quantities of palmitic acid (C16:0) from *N. oculata*, that reached 70.8 mg·g^{-1} of AFDW, while the production of C18:1n9c/C18:3n3 was maximized in mixotrophy (48.3 mg·g^{-1} of AFDW). Park et al. [43] have also reported that *N. oculata* is characterized by the accumulation of high contents of the specific fatty acids in autotrophy (58% C16:0/C18:1n9c/C18:3n3 of total fatty acids) and mixotrophy (65% C16:0/C18:1n9c/C18:3n3 of total fatty acids). *T. suecica* formed high contents of C18:1n9c/C18:3n3 that ranged between 22.3 and 99.6 mg·g^{-1} of AFDW. The specific microalgae strain produced higher amounts of palmitic acid, oleic/linolenic acid and EPA under autotrophy, as compared to mixotrophy, while the content of stearic and linoleic acids was enhanced in mixotrophy.

Table 3. Fatty acid profiles (mg·g^{-1} of AFDW) obtained from microalgae cultivated under different trophic conditions.

	Nutrition	C12:0	C16:0	C16:1n7	C18:0	C18:1n9c/ C18:3n3	C18:2n6c	C20:5n3	C22:6n3
I. galbana	Autotrophic	0.0	18.0	0.0	12.9	14.0	11.4	0.0	0.0
	Mixotrophic	1.3	44.6	2.0	28.5	65.0	38.1	0.5	0.0
	Heterotrophic	0.0	74.0	2.5	61.9	77.4	87.9	1.3	0.4
M. gaditana	Autotrophic	0.0	68.6	1.9	7.9	108.6	9.9	8.1	0.0
	Mixotrophic	7.0	22.9	1.1	8.2	9.1	20.6	0.6	0.0
	Heterotrophic	0.0	63.7	3.5	33.4	66.9	51.1	0.0	0.0
S. obliquus	Autotrophic	0.0	30.8	0.0	10.6	30.3	2.8	0.0	0.0
	Mixotrophic	0.6	61.2	3.6	12.5	57.7	27.5	1.2	0.0
	Heterotrophic	0.4	23.1	0.6	10.9	17.1	10.1	1.1	0.0
N. oculata	Autotrophic	8.0	70.8	0.0	45.3	31.8	24.8	0.0	4.8
	Mixotrophic	0.0	61.3	3.5	23.7	48.3	28.6	2.2	2.3
	Heterotrophic	0.0	31.2	0.5	15.4	28.6	18.3	0.0	0.5
T. suecica	Autotrophic	0.0	66.5	1.1	10.1	99.6	11.7	9.2	0.0
	Mixotrophic	0.6	43.2	2.3	19.9	42.4	22.9	1.0	0.0
	Heterotrophic	0.0	22.6	1.4	11.7	22.3	15.0	1.1	0.0

Further experiments could be conducted to enhance the production of added value products, such as PUFA, avoiding a significant loss of biomass. The concentration of nutrients provided, such as carbon, nitrogen and phosphorus, constitutes a key factor

to enhance the synthesis of bioactive compounds in microalgae cells. In a recent paper by Shen et al. [44], *S. obliquus* grown heterotrophically using glucose showed high lipid accumulation, while the regulation of nitrogen and phosphorus was highly important for the conversion of the carbohydrate to fatty acids. Moreover, the use of different carbon sources could play a vital role on the production of microalgae biomass and optimization of high-value products manufacture [12]. According to Yang et al. [45], the salinity level could increase the lipid content of the freshwater microalgae *Chlorella pyrenoidosa*, constituting salinity as a significant factor for the enhancement of bioactive compounds synthesis, while the effect of light intensity and pH value on the growth and lipid production of *S. obliquus* maximized the formation of lipids [23]. Furthermore, the application of metal nanoparticles in cultures of microalgae such as *S. obliquus* and *T. suecica* can enhance both biomass production and lipid content [46].

PUFAs contribute major beneficial effects on human health, including cytotoxic activity against cancer cells via apoptosis, given that high amounts of EPA/DHA have previously exhibited positive action towards cancer prevention and treatment, mainly in the pancreas, colon, breast and prostate [47]. Moreover, Toelzer et al. [48] demonstrated that the receptor binding domains of the spike (S) glycoprotein of SARS-CoV-2 and MERS-CoV tightly binds LA in three composite binding pockets. Thus, LA prevents the binding of the specific spike protein to the angiotensin-converting enzyme 2 receptor on human cells, preventing the negative effects caused by two highly pathogenic coronaviruses. Based on the high LA content produced by *I. galbana* in the current study, the strain exhibits high potential for application in algal biorefineries. Moreover, a large number of studies support the hypothesis that omega-3 PUFAs and their products can serve as functional molecules capable of limiting inflammation-associated neurologic disorders [49], while imposing beneficial effects on blood pressure and hypertension-related organ complications [50]. The production of essential PUFAs achieved in the current work demonstrates that all microalgae strains tested could serve as potential candidates in biorefineries targeting the production of algal extracts as nutraceuticals.

3.3. Implications for the Development of Algal Biorefineries

Microalgae are known for their capacity to produce a wide range of added value commodities, including lipids, proteins, carbohydrates and secondary metabolites, such as carotenoids. The production of biomass and lipids achieved in the present study by the strains employed has been compared against the relevant literature as shown in Table 4. *I. galbana* and *S. obliquus* were capable of consuming large quantities of the organic content supplemented, exhibiting statistically significant lower glucose levels in mixotrophy and heterotrophy as compared to the respective control culture. *S. obliquus* performed the highest biomass productivity for all trophic conditions tested. Moreover, as far as the production of lipids is concerned, *I. galbana* in mixotrophy and *S. obliquus* in heterotrophy demonstrated the highest lipid productivities that reached 24.85 $mg \cdot L^{-1} \cdot d^{-1}$ and 22.77 $mg \cdot L^{-1} \cdot d^{-1}$, respectively. The aforementioned response of the specific strain shows that *S. obliquus* holds the capacity to perform a high growth rate and tolerance to climatic variations, as well as having a rich protein and PUFAs content, demonstrating that the microalgae could be suitable for application in the cosmetic [51] and food [52] industry. Also, *S. obliquus* has exhibited effective application in wastewater biodegradation [53]. Thus, the biomass and lipid production monitored for *S. obliquus* in this study confirms that the strain could serve as a great candidate for application in algal biorefineries [54]. Moreover, *I. galbana* could be additionally considered as a valuable strain for the development of algal biorefineries, given that the use of the strain's biomass (lipids, proteins, polysaccharides) in a range of applications fulfills a series of green chemistry goals [55]. Therefore, different extracts produced from *I. galbana* hold the potential for application as nutraceuticals and cosmetics. Although *N. oculata* did not consume glucose as an organic carbon source, the specific strain holds the capacity to grow (together with *I. galbana*) on oilfield produced water effluent of high salinity, achieving significant oil and COD removal [56]. Therefore,

N. oculata could be useful in wastewater treatment applications. Previous research on *M. gaditana* has documented its capacity to accumulate high lipid contents, especially valuable omega-3 fatty acids, as well as to grow in outdoor photobioreactors and raceway ponds, performing elevated biomass production [57,58]. However, although increased concentrations of EPA were monitored in *M. gaditana* autotrophic cultivation, the strain could not remove substantial quantities of glucose. *T. suecica* could serve as an important strain for wastewater treatment, based on the capacity of the microalgae to grow using media supplemented with 75% stickwater and the use of elevated quantities of N and P [35]. The results obtained here highlight that between the microalgae evaluated, the most promising strains for application on algal biorefineries comprised of *I. galbana* and *S. obliquus*. Thus, future work should focus in optimizing the use of the bioproducers identified for the production of added-value commodities from biowaste.

Table 4. Overview of biomass and lipid production from the microalgae of the current study.

Genus/ Species	Nutrition	Organic Carbon/ Nitrogen Source	Biomass Production	Lipid Production	Reference
S. obliquus	Mixotrophic	LFAH15-WG10	0.248 $g \cdot L^{-1} \cdot d^{-1}$	59.7 $mg \cdot L^{-1} \cdot d^{-1}$	[40]
S. obliquus	Autotrophic Mixotrophic Heterotrophic	- 40% Cheese whey 40% Cheese whey	0.15 $g \cdot L^{-1} \cdot d^{-1}$ 0.28 $g \cdot L^{-1} \cdot d^{-1}$ 0.21 $g \cdot L^{-1} \cdot d^{-1}$	36.9 $mg \cdot L^{-1} \cdot d^{-1}$ 37.8 $mg \cdot L^{-1} \cdot d^{-1}$ -	[22]
Nannochloropsis sp.	Autotrophic Mixotrophic Heterotrophic	- glucose glucose	0.016 $g \cdot L^{-1} \cdot d^{-1}$ 0.044 $g \cdot L^{-1} \cdot d^{-1}$ 0.013 $g \cdot L^{-1} \cdot d^{-1}$	30 $mg \cdot L^{-1} \cdot d^{-1}$ 1200 $mg \cdot L^{-1} \cdot d^{-1}$ 40 $mg \cdot L^{-1} \cdot d^{-1}$	[59]
N. oculata	Autotrophic Mixotrophic	- Yeast	0.387 $g \cdot L^{-1} \cdot d^{-1}$ 1.011 $g \cdot L^{-1} \cdot d^{-1}$	37.9 $mg \cdot L^{-1} \cdot d^{-1}$ 109.2 $mg \cdot L^{-1} \cdot d^{-1}$	[43]
I. galbana	Autotrophic Mixotrophic Heterotrophic	- Glycerol Glycerol	0.011 $g \cdot L^{-1} \cdot d^{-1}$ 0.022 $g \cdot L^{-1} \cdot d^{-1}$ Inhibited	-	[34]
T. suecica	Autotrophic Mixotrophic	- Yeast extract:glucose 4:1	0.93 × 10^6 cell mL^{-1} 2.97 × 10^6 cell mL^{-1}	2.3 $mg \cdot L^{-1} \cdot d^{-1}$ 9.3 $mg \cdot L^{-1} \cdot d^{-1}$	[60]
I. galbana	Autotrophic Mixotrophic Heterotrophic	- Glucose Glucose	0.033 $g \cdot L^{-1} \cdot d^{-1}$ 0.057 $g \cdot L^{-1} \cdot d^{-1}$ 0.055 $g \cdot L^{-1} \cdot d^{-1}$	6.18 $mg \cdot L^{-1} \cdot d^{-1}$ 24.85 $mg \cdot L^{-1} \cdot d^{-1}$ 16.67 $mg \cdot L^{-1} \cdot d^{-1}$	Present study
M. gaditana	Autotrophic Mixotrophic Heterotrophic	- Glucose Glucose	0.014 $g \cdot L^{-1} \cdot d^{-1}$ 0.041 $g \cdot L^{-1} \cdot d^{-1}$ 0.029 $g \cdot L^{-1} \cdot d^{-1}$	4.07 $mg \cdot L^{-1} \cdot d^{-1}$ 7.82 $mg \cdot L^{-1} \cdot d^{-1}$ 6.6 $mg \cdot L^{-1} \cdot d^{-1}$	Present study
S. obliquus	Autotrophic Mixotrophic Heterotrophic	- Glucose Glucose	0.030 $g \cdot L^{-1} \cdot d^{-1}$ 0.13 $g \cdot L^{-1} \cdot d^{-1}$ 0.14 $g \cdot L^{-1} \cdot d^{-1}$	4 $mg \cdot L^{-1} \cdot d^{-1}$ 21.97 $mg \cdot L^{-1} \cdot d^{-1}$ 22.77 $mg \cdot L^{-1} \cdot d^{-1}$	Present study
N. oculata	Autotrophic Mixotrophic Heterotrophic	- Glucose Glucose	0.0020 $g \cdot L^{-1} \cdot d^{-1}$ 0.011 $g \cdot L^{-1} \cdot d^{-1}$ 0.058 $g \cdot L^{-1} \cdot d^{-1}$	2.04 $mg \cdot L^{-1} \cdot d^{-1}$ 6 $mg \cdot L^{-1} \cdot d^{-1}$ 5.67 $mg \cdot L^{-1} \cdot d^{-1}$	Present study
T. suecica	Autotrophic Mixotrophic Heterotrophic	- Glucose Glucose	0.011 $g \cdot L^{-1} \cdot d^{-1}$ 0.033 $g \cdot L^{-1} \cdot d^{-1}$ 0.074 $g \cdot L^{-1} \cdot d^{-1}$	3.27 $mg \cdot L^{-1} \cdot d^{-1}$ 6 $mg \cdot L^{-1} \cdot d^{-1}$ 9.33 $mg \cdot L^{-1} \cdot d^{-1}$	Present study

4. Conclusions

In this work, five important algal strains were successfully compared for their capacity to produce biomass and lipids under auto-, mixo- and heterotrophic conditions. The results exhibited that *S. obliquus* in heterotrophy performed the highest biomass productivity (0.14 $g \cdot L^{-1} \cdot d^{-1}$), while *I. galbana* achieved the most prominent lipid productivity, which reached 24.85 $mg \cdot L^{-1} \cdot d^{-1}$ using mixotrophic nutrition. The screening of the algae investi-

gated employing different trophic modes highlighted that, based on the biomass and lipid production achieved as well as the capacity of *I. galbana* and *S. obliquus* to form PUFAs in the presence of organic carbon, both microalgae comprise efficient biocatalysts for their potential application in microalgal biorefinery systems.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w14020240/s1, Figure S1: Gas chromatography chromatogram of fatty acids formed by *S. obliquus* under mixotrophy. Specific fatty acids are depicted on different chromatographic peaks.

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References

1. Punia, S.; Sandhu, K.S.; Siroha, A.K.; Dhull, S.B. Omega 3-metabolism, absorption, bioavailability and health benefits–A review. *PharmaNutrition* **2019**, *10*, 100162. [CrossRef]
2. Lu, Q.; Li, H.; Xiao, Y.; Liu, H. A state-of-the-art review on the synthetic mechanisms, production technologies, and practical application of polyunsaturated fatty acids from microalgae. *Algal Res.* **2021**, *55*, 102281. [CrossRef]
3. Kothri, M.; Mavrommati, M.; Elazzazy, A.M.; Baeshen, M.N.; Moussa, T.A.A.; Aggelis, G. Microbial sources of polyunsaturated fatty acids (PUFAs) and the prospect of organic residues and wastes as growth media for PUFA-producing microorganisms. *FEMS Microbiol. Lett.* **2020**, *367*, 1–11. [CrossRef] [PubMed]
4. Cvitković, D.; Dragović-Uzelac, V.; Dobrinčić, A.; Čož-Rakovac, R.; Balbino, S. The effect of solvent and extraction method on the recovery of lipid fraction from Adriatic Sea macroalgae. *Algal Res.* **2021**, *56*, 102291. [CrossRef]
5. Suganya, T.; Varman, M.; Masjuki, H.H.; Renganathan, S. Macroalgae and microalgae as a potential source for commercial applications along with biofuels production: A biorefinery approach. *Renew. Sustain. Energy Rev.* **2016**, *55*, 909–941. [CrossRef]
6. Sosa-Hernández, J.E.; Romero-Castillo, K.D.; Parra-Arroyo, L.; Aguilar-Aguila-Isaías, M.A.; García-Reyes, I.E.; Ahmed, I.; Parra-Saldivar, R.; Bilal, M.; Iqbal, H.M.N. Mexican microalgae biodiversity and state-of-the-art extraction strategies to meet sustainable circular economy challenges: High-value compounds and their applied perspectives. *Mar. Drugs* **2019**, *17*, 174. [CrossRef]
7. Brennan, L.; Owende, P. Biofuels from microalgae-A review of technologies for production, processing, and extractions of biofuels and co-products. *Renew. Sustain. Energy Rev.* **2010**, *14*, 557–577. [CrossRef]
8. Silva, C.; Soliman, E.; Cameron, G.; Fabiano, L.A.; Seider, W.D.; Dunlop, E.H.; Coaldrake, A.K. Commercial-scale biodiesel production from algae. *Ind. Eng. Chem. Res.* **2014**, *53*, 5311–5324. [CrossRef]
9. Sun, Z.; Wang, X.; Liu, J. Screening of *Isochrysis* strains for simultaneous production of docosahexaenoic acid and fucoxanthin. *Algal Res.* **2019**, *41*, 101545. [CrossRef]
10. Patel, A.K.; Choi, Y.Y.; Sim, S.J. Emerging prospects of mixotrophic microalgae: Way forward to sustainable bioprocess for environmental remediation and cost-effective biofuels. *Bioresour. Technol.* **2020**, *300*, 122741. [CrossRef]
11. Valdovinos-García, E.M.; Barajas-Fernández, J.; de los Ángeles Olán-Acosta, M.; Petriz-Prieto, M.A.; Guzmán-López, A.; Bravo-Sánchez, M.G. Techno-Economic Study of CO_2 Capture of a Thermoelectric Plant Using Microalgae (*Chlorella vulgaris*) for Production of Feedstock for Bioenergy. *Energies* **2020**, *13*, 413. [CrossRef]
12. Ma, X.; Mi, Y.; Zhao, C.; Wei, Q. A comprehensive review on carbon source effect of microalgae lipid accumulation for biofuel production. *Sci. Total Environ.* **2021**, *806*, 151387. [CrossRef] [PubMed]
13. Barros, A.; Pereira, H.; Campos, J.; Marques, A.; Varela, J.; Silva, J. Heterotrophy as a tool to overcome the long and costly autotrophic scale-up process for large scale production of microalgae. *Sci. Rep.* **2019**, *9*, 13935. [CrossRef] [PubMed]
14. Zili, F.; Bouzidi, N.; Ammar, J.; Zakhama, W.; Ghoul, M.; Sayadi, S.; Ben Ouada, H. Mixotrophic cultivation promotes growth, lipid productivity, and PUFA production of a thermophilic Chlorophyta strain related to the genus *Graesiella*. *J. Appl. Phycol.* **2017**, *29*, 35–43. [CrossRef]
15. Jareonsin, S.; Pumas, C. Advantages of Heterotrophic microalgae as a host for phytochemicals production. *Front. Bioeng. Biotechnol.* **2021**, *9*, 628597. [CrossRef] [PubMed]
16. Sim, S.J.; Joun, J.; Hong, M.E.; Patel, A.K. Split mixotrophy: A novel cultivation strategy to enhance the mixotrophic biomass and lipid yields of *Chlorella protothecoides*. *Bioresour. Technol.* **2019**, *291*, 121820. [CrossRef]

17. López, G.; Yate, C.; Ramos, F.A.; Cala, M.P.; Restrepo, S.; Baena, S. Production of polyunsaturated fatty acids and lipids from autotrophic, mixotrophic and heterotrophic cultivation of *Galdieria* sp. strain USBA-GBX-832. *Sci. Rep.* **2019**, *9*, 10791. [CrossRef] [PubMed]
18. Liu, J.; Yin, J.; Ge, Y.; Han, H.; Liu, M.; Gao, F. Improved lipid productivity of *Scenedesmus obliquus* with high nutrient removal efficiency by mixotrophic cultivation in actual municipal wastewater. *Chemosphere* **2021**, *285*, 131475. [CrossRef]
19. Penhaul Smith, J.K.; Hughes, A.D.; McEvoy, L.; Thornton, B.; Day, J.G. The carbon partitioning of glucose and DIC in mixotrophic, heterotrophic and photoautotrophic cultures of *Tetraselmis suecica*. *Biotechnol. Lett.* **2021**, *43*, 729–743. [CrossRef]
20. Choi, H.J.; Lee, S.M. Biomass and oil content of microalgae under mixotrophic conditions. *Environ. Eng. Res.* **2015**, *20*, 25–32. [CrossRef]
21. Mata, T.M.; Melo, A.C.; Meireles, S.; Mendes, A.M.; Martins, A.A.; Caetano, N.S. Potential of microalgae *Scenedesmus obliquus* grown in brewery wastewater for biodiesel production. *Chem. Eng. Trans.* **2013**, *32*, 901–906. [CrossRef]
22. Girard, J.M.; Roy, M.L.; Hafsa, M.B.; Gagnon, J.; Faucheux, N.; Heitz, M.; Tremblay, R.; Deschênes, J.S. Mixotrophic cultivation of green microalgae *Scenedesmus obliquus* on cheese whey permeate for biodiesel production. *Algal Res.* **2014**, *5*, 241–248. [CrossRef]
23. Han, W.; Jin, W.; Li, Z.; Wei, Y.; He, Z.; Chen, C.; Qin, C.; Chen, Y.; Tu, R.; Zhou, X. Cultivation of microalgae for lipid production using municipal wastewater. *Process Saf. Environ. Prot.* **2021**, *155*, 155–165. [CrossRef]
24. Culture Collection of Algae & Protozoa. Available online: https://www.ccap.ac.uk/index.php/media-recipes/ (accessed on 1 September 2020).
25. Miller, G.L. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Anal. Chem.* **1959**, *31*, 426–428. [CrossRef]
26. Folch, J.; Lees, M.; Sloane Stanley, G. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **1957**, *226*, 497–509. [CrossRef]
27. Araujo, P.; Nguyen, T.T.; Frøyland, L.; Wang, J.; Kang, J.X. Evaluation of a rapid method for the quantitative analysis of fatty acids in various matrices. *J. Chromatogr. A* **2008**, *1212*, 106–113. [CrossRef]
28. Alkhamis, Y.; Qin, J.G. Comparison of N and P requirements of *Isochrysis galbana* under phototrophic and mixotrophic conditions. *J. Appl. Phycol.* **2015**, *27*, 2231–2238. [CrossRef]
29. Pagnanelli, F.; Altimari, P.; Trabucco, F.; Toro, L. Mixotrophic growth of *Chlorella vulgaris* and *Nannochloropsis oculata*: Interaction between glucose and nitrate. *J. Chem. Technol. Biotechnol.* **2014**, *89*, 652–661. [CrossRef]
30. Song, Y.; Wang, X.; Cui, H.; Ji, C.; Xue, J.; Jia, X.; Ma, R.; Li, R. Enhancing growth and oil accumulation of a palmitoleic acid–rich *Scenedesmus obliquus* in mixotrophic cultivation with acetate and its potential for ammonium-containing wastewater purification and biodiesel production. *J. Environ. Manage.* **2021**, *297*, 113273. [CrossRef]
31. Menegol, T.; Romero-Villegas, G.I.; López-Rodríguez, M.; Navarro-López, E.; López-Rosales, L.; Chisti, Y.; Cerón-García, M.C.; Molina-Grima, E. Mixotrophic production of polyunsaturated fatty acids and carotenoids by the microalga *Nannochloropsis gaditana*. *J. Appl. Phycol.* **2019**, *31*, 2823–2832. [CrossRef]
32. Moon, M.; Kim, C.W.; Park, W.K.; Yoo, G.; Choi, Y.E.; Yang, J.W. Mixotrophic growth with acetate or volatile fatty acids maximizes growth and lipid production in *Chlamydomonas reinhardtii*. *Algal Res.* **2013**, *2*, 352–357. [CrossRef]
33. Chiu, S.Y.; Kao, C.Y.; Tsai, M.T.; Ong, S.C.; Chen, C.H.; Lin, C.S. Lipid accumulation and CO_2 utilization of *Nannochloropsis oculata* in response to CO_2 aeration. *Bioresour. Technol.* **2009**, *100*, 833–838. [CrossRef]
34. Alkhamis, Y.; Qin, J.G. Cultivation of *Isochrysis galbana* in phototrophic, heterotrophic, and mixotrophic conditions. *Biomed Res. Int.* **2013**, *2013*, 983465. [CrossRef]
35. Shekarabi, S.P.H.; Mehrgan, M.S. Cultivation of *Tetraselmis suecica* using a fishmeal factory effluent: Effect on the growth, biochemical composition, and nutrient removal. *J. Appl. Phycol.* **2021**, *33*, 1949–1959. [CrossRef]
36. Msanne, J.; Polle, J.; Starkenburg, S. An assessment of heterotrophy and mixotrophy in *Scenedesmus* and its utilization in wastewater treatment. *Algal Res.* **2020**, *48*, 101911. [CrossRef]
37. Onay, M. Bioethanol production via different saccharification strategies from *H. tetrachotoma ME03* grown at various concentrations of municipal wastewater in a flat-photobioreactor. *Fuel* **2019**, *239*, 1315–1323. [CrossRef]
38. Yang, C.; Hua, Q.; Shimizu, K. Energetics and carbon metabolism during growth of microalgal cells under photoautotrophic, mixotrophic and cyclic light-autotrophic/dark-heterotrophic conditions. *Biochem. Eng. J.* **2000**, *6*, 87–102. [CrossRef]
39. Ohse, S.; Bianchini Derner, R.; Ávila Ozório, R.; Gordo Corrêa, R.; Badiale Furlong, E.; Roberto Cunha, P.C. Lipid content and fatty acid profiles in ten species of microalgae. *Idesia (Arica)* **2015**, *33*, 93–101. [CrossRef]
40. Abomohra, A.E.F.; Eladel, H.; El-Esawi, M.; Wang, S.; Wang, Q.; He, Z.; Feng, Y.; Shang, H.; Hanelt, D. Effect of lipid-free microalgal biomass and waste glycerol on growth and lipid production of *Scenedesmus obliquus*: Innovative waste recycling for extraordinary lipid production. *Bioresour. Technol.* **2018**, *249*, 992–999. [CrossRef]
41. Camacho-Rodríguez, J.; González-Céspedes, A.M.; Cerón-García, M.C.; Fernández-Sevilla, J.M.; Acién-Fernández, F.G.; Molina-Grima, E. A quantitative study of eicosapentaenoic acid (EPA) production by *Nannochloropsis gaditana* for aquaculture as a function of dilution rate, temperature and average irradiance. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 2429–2440. [CrossRef]
42. Abirami, S.; Murugesan, S.; Narender, S. Effect of various pretreatment methods prior to extraction of omega 3 fatty acids from *Nannochloropsis gaditana*. *Int. J. Appl. Res.* **2016**, *2*, 81–85.
43. Park, S.J.; Choi, Y.E.; Kim, E.J.; Park, W.K.; Kim, C.W.; Yang, J.W. Serial optimization of biomass production using microalga *Nannochloris oculata* and corresponding lipid biosynthesis. *Bioprocess Biosyst. Eng.* **2012**, *35*, 3–9. [CrossRef]

44. Shen, X.F.; Gao, L.J.; Zhou, S.B.; Huang, J.L.; Wu, C.Z.; Qin, Q.W.; Zeng, R.J. High fatty acid productivity from *Scenedesmus obliquus* in heterotrophic cultivation with glucose and soybean processing wastewater via nitrogen and phosphorus regulation. *Sci. Total Environ.* **2020**, *708*, 134596. [CrossRef]
45. Yang, Z.; Gao, F.; Liu, J.; Yang, J.; Liu, M.; Ge, Y. Improving sedimentation and lipid production of microalgae in the photobioreactor using saline wastewater. *Bioresour. Technol.* **2021**, 126392. [CrossRef]
46. Dev Sarkar, R.; Singh, H.B.; Chandra Kalita, M. Enhanced lipid accumulation in microalgae through nanoparticle-mediated approach, for biodiesel production: A mini-review. *Heliyon* **2021**, *7*, e08057. [CrossRef]
47. Dierge, E.; Larondelle, Y.; Feron, O. Cancer diets for cancer patients: Lessons from mouse studies and new insights from the study of fatty acid metabolism in tumors. *Biochimie* **2020**, *178*, 56–68. [CrossRef]
48. Toelzer, C.; Gupta, K.; Yadav, S.K.N.; Borucu, U.; Davidson, A.D.; Kavanagh Williamson, M.; Shoemark, D.K.; Garzoni, F.; Staufer, O.; Milligan, R.; et al. Free fatty acid binding pocket in the locked structure of SARS-CoV-2 spike protein. *Science* **2020**, *3255*, eabd3255. [CrossRef]
49. Layé, S.; Nadjar, A.; Joffre, C.; Bazinet, R.P. Anti-inflammatory effects of omega-3 fatty acids in the brain: Physiological mechanisms and relevance to pharmacology. *Pharmacol. Rev.* **2018**, *70*, 12–38. [CrossRef]
50. Guo, X.F.; Li, K.L.; Li, J.M.; Li, D. Effects of EPA and DHA on blood pressure and inflammatory factors: A meta-analysis of randomized controlled trials. *Crit. Rev. Food Sci. Nutr.* **2019**, *59*, 3380–3393. [CrossRef]
51. Cengiz Sahin, S. *Scenedesmus obliquus*: A potential natural source for cosmetic industry. *Int. J. Second. Metab.* **2019**, *6*, 129–136. [CrossRef]
52. da Silva, M.E.T.; Correa, K.d.P.; Martins, M.A.; da Matta, S.L.P.; Martino, H.S.D.; Coimbra, J.S.d.R. Food safety, hypolipidemic and hypoglycemic activities, and in vivo protein quality of microalga *Scenedesmus obliquus* in Wistar rats. *J. Funct. Foods* **2020**, *65*, 103711. [CrossRef]
53. Dayana Priyadharshini, S.; Suresh Babu, P.; Manikandan, S.; Subbaiya, R.; Govarthanan, M.; Karmegam, N. Phycoremediation of wastewater for pollutant removal: A green approach to environmental protection and long-term remediation. *Environ. Pollut.* **2021**, *290*, 117989. [CrossRef]
54. Patnaik, R.; Mallick, N. Utilization of *Scenedesmus obliquus* biomass as feedstock for biodiesel and other industrially important co-products: An integrated paradigm for microalgal biorefinery. *Algal Res.* **2015**, *12*, 328–336. [CrossRef]
55. Gilbert-López, B.; Mendiola, J.A.; Fontecha, J.; Van Den Broek, L.A.M.; Sijtsma, L.; Cifuentes, A.; Herrero, M.; Ibáñez, E. Downstream processing of *Isochrysis galbana*: A step towards microalgal biorefinery. *Green Chem.* **2015**, *17*, 4599–4609. [CrossRef]
56. Ammar, S.H.; Khadim, H.J.; Mohamed, A.I. Cultivation of *Nannochloropsis oculata* and *Isochrysis galbana* microalgae in produced water for bioremediation and biomass production. *Environ. Technol. Innov.* **2018**, *10*, 132–142. [CrossRef]
57. Chukhutsina, V.U.; Fristedt, R.; Morosinotto, T.; Croce, R. Photoprotection strategies of the alga *Nannochloropsis gaditana*. *Biochim. Biophys. Acta-Bioenerg.* **2017**, *1858*, 544–552. [CrossRef] [PubMed]
58. Ajjawi, I.; Verruto, J.; Aqui, M.; Soriaga, L.B.; Coppersmith, J.; Kwok, K.; Peach, L.; Orchard, E.; Kalb, R.; Xu, W.; et al. Lipid production in *Nannochloropsis gaditana* is doubled by decreasing expression of a single transcriptional regulator. *Nat. Biotechnol.* **2017**, *35*, 647–652. [CrossRef] [PubMed]
59. Cheirsilp, B.; Torpee, S. Enhanced growth and lipid production of microalgae under mixotrophic culture condition: Effect of light intensity, glucose concentration and fed-batch cultivation. *Bioresour. Technol.* **2012**, *110*, 510–516. [CrossRef] [PubMed]
60. Cid, A.; Abalde, J.; Herrero, C. High yield mixotrophic cultures of the marine microalga *Tetraselmis suecica* (Kylin) Butcher (Prasinophyceae). *J. Appl. Phycol.* **1992**, *4*, 31–37. [CrossRef]