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Two-stage lipid induction in the microalga *Tetraselmis striata* CTP4 upon exposure to different abiotic stresses

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ABSTRACT

Tetraselmis striata CTP4 is a euryhaline, robust, fast-growing microalga suitable for wastewater treatment and industrial production. Lipid production was induced through a two-stage cultivation strategy: a 1st stage under standard growth-promoting conditions (100 μmol photons m^{-2} s^{-1}, salinity 36 ppt and 20 °C) to achieve high biomass concentration and a 2nd stage of 6 days for lipid induction by the application of abiotic stresses such as nutrient depletion, high light intensity (200 and 400 μmol photons m^{-2} s^{-1}), high salinity (75 and 100 ppt), and extreme temperatures (5 and 35 °C). Although nutrient depletion always resulted in a decrease in biomass productivity, it had also the highest impact on lipid induction. The highest lipid content (43.2%) and lipid productivity (29.2 mg L^{-1} d^{-1}) were obtained using a combination of nutrient depletion and high light intensity (400 μmol m^{-2} s^{-1}). The fatty acid profile was mainly composed of C16:0 (palmitic), C18:1 (oleic) and C18:2 (linoleic) acids. The low content of unsaturated fatty acids and absence of C18:3 (linolenic) acid render the oil of this microalga suitable for biodiesel production, a renewable source of energy.

1. Introduction

Global energy consumption is steadily increasing due to a rising world's population and higher requirements on energy in modern society. This energy demand is mainly covered by fossil fuels, which accounted for about 84% of the total energy consumed in 2019 [1]. However, the decline in oil reserves as well as political instability and the physical disruption of supply versus demand has a strong effect on oil prices. In 2022, although still far from the historical maximum observed in June 2008, the oil price is rising again boosted by higher demand of richer countries and decreased production of oil requested by investors with intent to keep the prices high as well as because of political instability in Europe. This trend for higher prices for fossil fuels is also driven by the action of environmentalists through the promotion of policies for a lower carbon footprint (Energy Information Administration; https://www.eia.gov/). These events in addition to the growing concerns in environmental pollution by fossil fuels are once more highlighting the need to explore biofuel production aiming at increasing its feasibility.

Microalgae are renewable, sustainable and eco-friendly resources, which have been considered to be the third generation of biofuels due to their ability to accumulate large amounts of compounds such as lipids and carbohydrates that can be processed into biodiesel and bioethanol, respectively [2]. Unlike terrestrial plants, microalgae grow faster, can be cultivated all year around on non-arable land using salt- or wastewaters and represent a biomass rich in high-value compounds (e.g., pigments, omega-3 fatty acids) [3]. Microalgal lipids can be classified as non-polar (tri- [TAG], di- and monoacylglycerols, fatty acids, and sterols) and polar lipids (phospholipids and glycolipids). Non-polar or weakly polar lipids often accumulate in the form of lipid droplets, whereas polar lipids, together with other lipophilic molecules (carotenoids, terpenes, among others), usually form lipid bilayers such as the plasma membrane and endoplasmic reticulum as well as the mitochondrial and plastidial envelopes [4]. Cytoplasmic or plastidial lipid droplets, the latter

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frequently named as plastoglobuli, usually contain high amounts of TAGs consisting of a glycerol moiety esterified with three fatty acids [5].

Despite the described advantages of microalgal biofuels, the high capital (CAPEX) and operational (OPEX) expenditures that the production and downstream processing of microalgal biomass entails render microalgae-based biofuels economically unfeasible [6]. One possible way is the cultivation of lipid-rich strains at large-scale industrial facilities using low-cost cultivation vessels and agricultural, aquacultural or municipal wastewaters as a sustainable source of nutrients or flue gases as carbon source [7]. Another crucial strategy in the biofuel production pipeline should include the reuse of spent biomass (e.g., defatted biomass) for feed purposes or as fertilizer, extraction of high-valuable compounds for nutra- and pharmaceuticals and the optimization of TAG production using robust microalgae able to withstand wide variations of abiotic (e.g., light and temperature) and biotic (e.g., predators and contaminants) parameters [8]. Furthermore, to increase the lipid content in microalgae, different stresses have been applied, including extreme temperatures, light intensities, or high salinities alone or in combination with nitrogen depletion [9–11]. However, microalgae grown under such conditions have relatively low biomass productivity, considering that active microalgal growth and high lipid production are generally mutually exclusive. Nonetheless, two-stage growth systems can be employed to achieve high lipid productivities under which the first and second stages should provide optimal growth conditions resulting in high biomass productivity and stressful conditions to induce the biosynthesis and accumulation of lipids, respectively [12,13].

Another factor that will be essential to develop microalgae-based biofuels is the use of robust, thermotolerant, salt tolerant, fast-growing microalgae able to accumulate large amounts of lipids without collapsing even in the presence of competitors and predators. *Tetraselmis striata* CTP4 is a euryhaline microalga isolated from Ria Formosa Lagoon, Algarve, Portugal [14]. The isolation of this microalga was performed via a high throughput method comprising a pre-cultivation stage and fluorescence activated cell sorting based on BODIPY 505/515 staining of lipid-rich strains. This strain has successfully been cultivated under batch and continuous culture systems using either modified algal growth medium or urban/fish farm wastewater [15]. Moreover, it was able to grow in industrial 35- and 100-m^3 photobioreactors, showing high resilience to different irradiances, temperatures and contaminants [16]. This work reports the optimization of a two-stage growth system with the application of different environmental stressors on *T. striata* to enhance its lipid productivity ($g.L^{-1}d^{-1}$) and therefore increase the economic feasibility and cost-effectiveness of microalgal biodiesel.

2. Methods

2.1. Microalgae growth and experimental design

2.1.1. First stage growth

The chlorophyte *Tetraselmis striata* CTP4 is a euryhaline species which was isolated from the Ria Formosa, Algarve, Portugal [14]. Standard growth conditions were previously optimized and set to 20 °C, 36 ppt of salinity and a continuous photon flux density (PFD) of 100 μmol m^{-2} s^{-1} [14,15]. All experiments were performed in 5-L aerated reactors (0.8 L min^{-1}) containing an initial cell concentration of $2 \times 10^5 \pm 1 \times 10^3$ cells mL^{-1} and placed in specialized growth chambers (Aralab Fitoclima s 600 PL clima plus 400) to control and monitor abiotic growth factors, namely light intensity, temperature and humidity. Cultures were supplemented with 1 mL of modified algal medium (Table 1) for each litre of seawater [14].

2.1.2. Second stage growth

After reaching late exponential phase, the culture was divided between 100-mL glass tubes (80 mL per tube) and exposed to different conditions. A set of cultures was left without any further addition of nutrients (N-) while another was supplemented with 1 mL of the modified algal medium described in Table 1, per litre of culture (N+). Different light intensities (100, 200 and 400 μmol photons m^{-2} s^{-1}), temperature (5, 20 and 35 °C) and salinity (36, 75 and 100 ppt) regimes, were tested in both scenarios. All cultures were subjected to 24h light. Salinity was adjusted by the addition of commercial NaCl to the cultures exposed to 75 and 100 ppt salinity. Experiments were performed in triplicate. At the end of the experiment, cultures were centrifuged at 8000 *g* for 10 min, and the biomass collected and frozen for later fatty acid methyl esters (FAME) profile determination.

Table 1
Modified algal medium composition (x1000 concentrated).

Nutrient	Concentration
$NaNO_3$	2 M
KH_2PO_4	100 mM
$ZnCl_2$	1 mM
$ZnSO_4.H_2O$	1 mM
$MnCl_2.4H_2O$	1 mM
$Na_2MoO_4.2H_2O$	0.1 mM
$CoCl_2.6H_2O$	0.1 mM
$CuSO_4.5H_2O$	0.1 mM
$MgSO_4.7H_2O$	2 mM
$FeCl.6H_2O$	20 mM
EDTA-Na	26.4 mM

2.1.3. Determination of algal growth and productivity calculations

Growth was monitored by cell number counting and dry weight evaluation. Cell counting was performed using a Neubauer chamber and dry weight was obtained by optical density measurement at 750 nm, previously correlated with dry weight measurements (Eq. (1), $r = 0.950$; $p < 0.001$) as described previously by Pereira et al. [14].

$$Dw\ (g.L^{-1}) = \frac{OD_{750}}{9.48 \times 10^{-1}} \quad (1)$$

where Dw - dry weight and OD_{750} - optical density at 750 nm.

Biomass and lipid productivities (eqs. (2) and (3)) were calculated, for the second stage only, between day 0 and the day when the maximum lipid content was observed. For most conditions, that meant a period of 6 days, except for the cultures cultivated at 35 °C (N+ and N-) in which the maximum lipid content was attained on day 4.

$$Biomass\ productivity\ (mg.L^{-1}.d^{-1}) = \frac{Dw_{final} - Dw_{initial}}{\Delta t_{initial-final}} \quad (2)$$

$$Lipid\ productivity (mg.L^{-1}.d^{-1}) = \frac{Lw_{final} - Lw_{initial}}{\Delta t_{initial-final}} \quad (3)$$

where Lw –lipid weight.

2.2. Total lipids determination

The lipid content of cultures was calculated based on a calibration curve (eq. (4), $r = 0.883$; $p < 0.001$) between fluorescence of cultures stained with Nile-red, using a method adapted from Ref. [17], and total lipid content determined by gravimetry after extraction with chloroform as described previously [14]. For the Nile red staining, to 250 μL of diluted algal culture 50 μL of the staining solution (Nile red 1 μM in 25% of DMSO) were added and fluorescence was read in a BioTek Synergy 4 plate reader preheated to 37 °C. Prior to reading at the emission wavelength of 580 nm (excitation at the wavelength of 530 nm), the cultures were mixed for 10 min at 100 rpm.

$$Lw\ (mg.L^{-1}) = \frac{FI_{580}}{9.18 \times 10^{-3}} \quad (4)$$

where FI_{580} – fluorescence intensity at 580 nm (excitation at 530 nm).

2.3. Fatty acid methyl esters profile

The fatty acid methyl esters (FAME) profile of the samples was determined as described previously [18]. Briefly, dry biomass was resuspended in a solution of methanol:acetyl chloride (20:1, v/v) and dispersed by Ultra-Turrax. After addition of hexane, samples were heated for 1h to 90 °C to derivatize the fatty acids. The dried organic phase was then filtered and injected in an Agilent 6890 GC System Network equipped with a DB-5MS column (25 m; 0.250 mm; 0.25 μm) coupled to mass spectrometry (GC-MS) to identify and quantify the FAME. Helium was used as carrier gas at a flow rate of 0.8 mL min^{-1} and the separation of the compounds was achieved by a temperature program starting at 60 °C for 1 min, increase to 120 °C by 30 °C min^{-1}, increase over 26 min to 250 °C by 4 °C min^{-1} and finally reaching 300 °C by 20 °C min^{-1}. For identification and quantification of different compounds, calibration curves of the 37 FAMEs standard mixture (Supelco) were used.

2.4. Evaluation of nitrate concentration

Nitrate concentrations of the medium were determined via cadmium-based nitrate reduction to nitrites and nitrite quantification by the Griess method according to standard methods (4500-NO_3^- E) [19].

2.5. Microscopy

Prior to microscopy with a Zeiss AXIOMAGER Z2 coupled to a coollSNApHQ2 camera and AxioVision software version 4.8 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany), cells were stained using BODIPY 505/515 dye (4,4-difluro-1,3,5,7tetramethyl-4-bora-3a, 4a-diaza-*s*-indacene, Life Technologies Europe BV, Porto, Portugal) as described by Cooper et al. [20]. Samples were vortexed for 1 min with BODIPY solution, at 1 mM final concentration, and incubated in the dark for 10 min. Microscopic images were acquired as described previously [14], namely for transmitted light images differential interference contrast (DIC) was used while for fluorescence the Zeiss 38 He filter set (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) was applied, followed by an image treatment using Image J software (Research Service Branch, NIH, Bethesda, MD).

2.6. Statistics

Lipid content values were compared using ANOVA and Tuckey's post-hoc test with a confidence interval of 95% using Statistica v. 7.0 software. Excel v. 2205 and SigmaPlot v. 14.0 were used for visualization of the graphs.

3. Results and discussion

3.1. Growth and biomass productivity of the two-stage growth system

In the first stage, cultures grew exponentially with a specific growth rate (μ) of 0.34 d^{-1}. Nitrogen was completely depleted from the cultivation medium after 3 days of growth (Fig. 1A). After 11 days of growth, cells entered the stationary phase reaching a cell concentration of 2.7 (±0.1) x10^6 cells mL^{-1} or 0.76 g L^{-1} (Fig. 1A and Fig. S1). The second growth stage lasted for 6 days, in which the cultures were exposed to different stress conditions (light intensity, salinity and temperature) in combination with nutrient repletion (N+) and nutrient depletion (N-) conditions to induce lipid accumulation. Cultures under N+ conditions continued to grow although at a slower rate as compared to the 1st stage reaching a cell concentration of 3.2 (±0.1) x10^6 cells mL^{-1} and a biomass concentration of 1.34 g L^{-1} (Fig. 1A and Fig S1), with a productivity of 74.6 ± 6.5 mg L^{-1} d^{-1} (Table 2), which was significantly

Table 2
Biomass and lipid productivities of *T. striata* CTP4 calculated for the second stage of the two-stage growth system, under Standard Growth Conditions (SGC) and upon the application of different light, salinity and temperature stresses. Results are shown as means ± standard deviation (n = 9). Significant differences between culture conditions of each column (biomass and lipid productivity) are indicated by letters ($p < 0.05$).

Culture conditions	Biomass productivity (mg L^{-1} d^{-1})		Lipid productivity (mg L^{-1} d^{-1})	
	N+	N-	N+	N-
SGC (20 °C, 100 μmol m−2 s−1, 36 ppt)	74.6 ± 6.5^{d}	54.2 ± 4.0^{d}	5.3 ± 0.6^{e}	10.7 ± 0.7^{e}
Light 200 μmol m^{-2} s^{-1}	100.0 ± 7.8^{b}	70.5 ± 0.9^{a}	16.2 ± 3.1^{b}	24.9 ± 1.9^{b}
Light 400 μmol m^{-2} s^{-1}	108.2 ± 5.1^{a}	64.6 ± 1.8^{b}	27.1 ± 0.3^{a}	29.2 ± 3.5^{a}
NaCl 75 ppt	61.8 ± 2.0^{e}	47.2 ± 2.6^{e}	6.1 ± 1.1^{e}	16.2 ± 1.5^{c}
NaCl 100 ppt	54.4 ± 1.9^{f}	43.6 ± 1.3^{f}	2.4 ± 0.3^{f}	8.7 ± 0.4^{f}
Temperature 5 °C	45.2 ± 1.3^{g}	39.4 ± 0.7^{g}	11.5 ± 1.4^{c}	10.9 ± 0.5^{e}
Temperature 35 °C [a]	89.9 ± 0.5^{c}	59.7 ± 1.5^{c}	9.4 ± 1.6^{d}	12.9 ± 0.8^{d}

[a] Productivity at day 4.

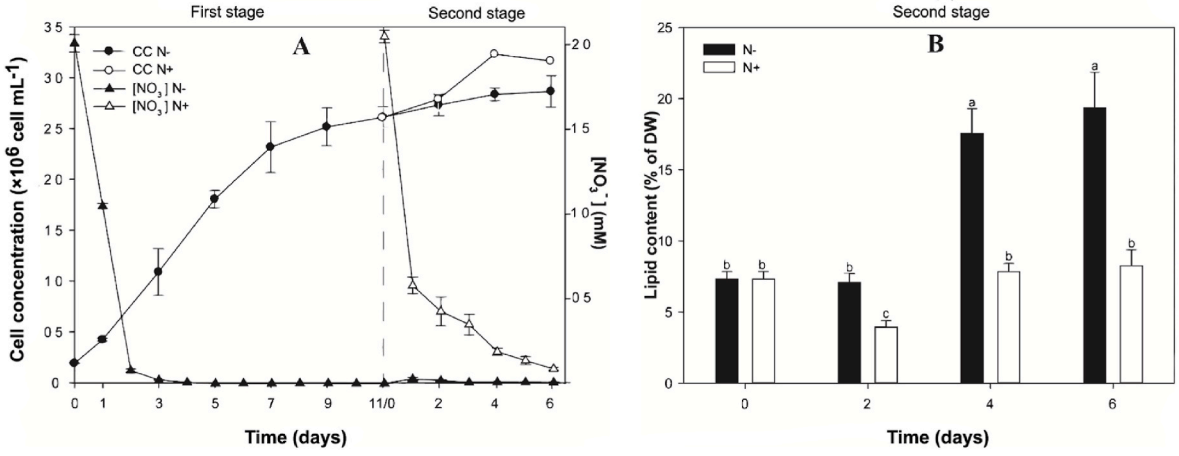

Fig. 1. Cell concentration (CC; circles) and nitrate concentration (triangles) of *T. striata* cultivated at 20 °C and 100 μmol m^{-2} s^{-1} with nutrient depletion (N-) and nutrient supplementation (N+) during the two stages (A). Values shown are means and standard deviation of three replicates. Lipid content of the same cultures during the second growth stage (B). Values represent mean and standard deviation. Bars labelled with different letters are significantly different (n = 9, $p < 0.05$).

higher ($p < 0.05$) than cultures grown under N- conditions, which remained in stationary phase during the second stage.

It is clear from these results that N+ conditions resulted in better biomass productivities. As expected, the lack of nutrients, particularly nitrogen, reduces culture growth probably due to decreased photosynthetic capacity of the cells, as observed by other authors for *Microchloropsis* (*Nannochloropsis) gaditana* under nitrogen starvation [21]. Remarkably, when higher light intensities were applied to the cultures, biomass productivities increased significantly under both N+ and N- conditions as compared to standard growth conditions (Fig. S2A and B in supplemental Material) with the highest productivity of 108.2 ± 5.1 mg L^{-1} d^{-1} under N+ and 400 μmol m^{-2} s^{-1} (Table 2). Moreover, incubation at 35 °C resulted in increased biomass productivities as compared to standard growth conditions reaching 89.9 ± 0.5 mg L^{-1} d^{-1} in N+ cultures (Fig. S3A and B in supplemental Material). Conversely, exposure to salinity stress and low temperatures (5 °C) resulted in lower biomass productivities (45.2–61.8 mg L^{-1} d^{-1}, Fig. S4A and B in supplemental Material). These results are further discussed below.

3.2. Effect of abiotic stress on lipid production

The lipid content and productivity of both N+ and N- cultures was monitored during the second stage (Fig. 1B, Table 2). In N+ cultures, lipid concentration was approximately constant during the 6-day period (3.90 ± 0.47–7.30 ± 0.50% of DW) reaching a lipid productivity of 5.3 ± 0.6 mg L^{-1} d^{-1}. In N- cultures, however, the lipid content and productivity significantly increased throughout the second stage reaching 19.3 ± 2.5% DW and 10.7 ± 0.7 mg L^{-1} d^{-1} at the end of the experiment, respectively (Fig. 1B, Table 2). Hence, nutrient limitation is an efficient environmental factor for lipid accumulation in *Tetraselmis striata* CTP4. Other authors have also reported similar lipid induction in the form of TAG under nitrogen-depleted conditions in different *Tetraselmis* spp. [22–24] as well as other microalgae, namely *Nannochloropsis oculata*, *Chlorella* sp., *Dunaliella* sp., *Desmodesmus* sp. or *Phaeodactylum tricornutum* [25–27]. Under stress conditions, microalgae decrease or maintain the level of structural lipids and increase the mostly energy-dense storage lipids TAG [9,28]. Although the exact influence of competing pathway on lipid biosynthesis in microalgae is unknown, TAG assembly and storage is enhanced by upregulation of anabolic pathways. Lack of nitrogen limits the metabolic processes of amino acid and nucleotide biosynthesis and diverts carbon skeletons to the lipid biosynthetic pathways leading to fatty acid and glycerol biosynthesis [20,29].

3.2.1. Light stress

Lipid contents and productivities increased significantly in cultures grown at the higher light intensities of 200 and 400 μmol m^{-2} s^{-1} as compared to those grown at 100 μmol m^{-2} s^{-1}, under both N- and N+ conditions (Fig. 2A and B, Table 2). However, the highest lipid concentrations and productivities of 43.2 ± 3.2% DW and 29.2 ± 3.5 mg L^{-1} d^{-1} were reached in cultures under nutrient starvation at day 6 exposed to 400 μmol m^{-2} s^{-1}, respectively. On the other hand, cultures grown under N+ conditions reached the respective maximum lipid content and productivity of 22.8% ± 0.7 DW and 27.1 ± 0.3 mg L^{-1} d^{-1} at a light intensity of 400 μmol m^{-2} s^{-1}. These values are similar to those reported previously and obtained for other *Tetraselmis* spp. (27.0–85.5 mg L^{-1} d^{-1}) [14,30]. Although high light has been shown to increase TAG contents in previous reports, the optimal light intensity depends on the microalgal species [9,10]. In a study on *Chlorella* spp., a lipid productivity of 300 mg L^{-1} d^{-1} was obtained at a light intensity of 600 μmol m^{-2} s^{-1} [31]. Similarly, a two-stage approach applied to *Nannochloropsis gaditana* led to a lipid productivity of 51 mg L^{-1} d^{-1} upon nitrogen depletion combined with high light intensity, 950 μmol m^{-2} s^{-1} 6]. A similar strategy increased the TAG content in *Neochloris oleoabundans* by about 30%, reaching 24.36% of DW upon nitrogen depletion and exposure to a light intensity of 200 μmol m^{-2} s^{-1} as compared to a lower light intensity, 50 μmol m^{-2} s^{-1} [32]. Ma et al. [33] observed also higher lipid induction in *Nannochloropsis oculata* exposed to simultaneous high light intensities and decreased nutrient concentration. In *N. oculata*, such increase was accompanied by elevated expression of acetyl-CoA carboxylase and diacylglycerol acyltransferase, and higher levels of NADPH leading to increased production of TAGs. Although the mechanism behind lipid induction in microalgae under high light stress is not yet fully understood, Goold et al. [34] demonstrated that *de novo* fatty acid synthesis occurs in *Chlamydomonas reinhardtii* exposed to saturating light conditions, probably as a means to accommodate extra fluxes of ATP and reducing power produced under increasing irradiance. Thus, it is possible that high light exposure in *Tetraselmis striata* CTP4 has boosted the alga photosynthetic carbon fixation, leading to higher lipid accumulation and increasing lipid productivity.

Generally, microalgae photosynthetic systems become saturated at a relatively high light irradiance; above which the algal photosynthesis may be inhibited [34]. However, in our experiment, photoinhibition was not evident at the maximum light intensity tested, 400 μmol m^{-2} s^{-1}, as the biomass productivity at this light intensity was significantly higher than at 200 μmol m^{-2} s^{-1} ($p < 0.05$; Table 2).

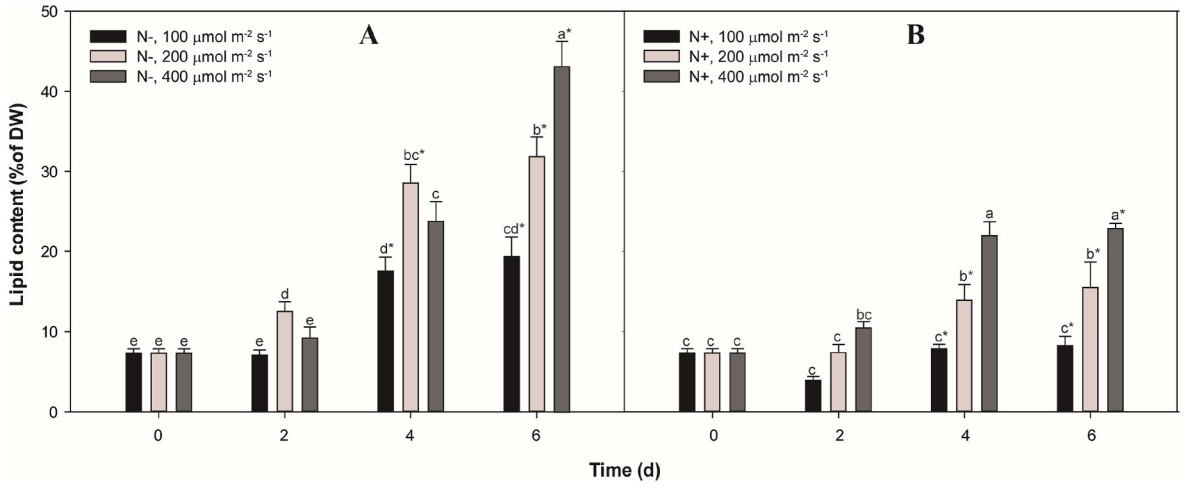

Fig. 2. Lipid content (% DW) of *T. striata* CTP4 cultivated in the two-stage growth with light intensities of 100, 200 and 400 μmol m^{-2} s^{-1} at 20 °C under nutrient depletion (A) and nutrient supplementation (B) conditions, N- and N+, respectively. Bars show means and standard deviation ($n = 3$). Within each treatment, bars labelled with different letters are significantly different ($p < 0.05$).

3.2.2. Salinity stress

Increases in the salinity of the cultivation medium were not effective in inducing lipid production in *T. striata* CTP4, in neither nutrient regime ($p > 0.05$, Fig. 3 A and B). Similar results have been found in other studies on *Tetraselmis suecica,* in which only minor changes in lipid content with changing salinities were observed [35,36]. However, salinity stress is a known lipid inducer for several freshwater microalgal strains as a response to oxidative stress to better survive these stressful conditions [37]. For example, increased salinities of up to 200 mM NaCl in *Acutodesmus dimorphus* led to higher lipid contents, 33.40 ± 2.29% DW compared to 22.24 ± 1.57% DW in the control culture without NaCl [38]. Furthermore, in *Chlamydomonas* sp., the highest lipid productivity, 330 mg L^{-1} d^{-1}, was reached when 2% sea salt was added [39]. On the contrary, *T. striata* CTP4 was isolated near a wastewater discharge in a brackish lagoon (Ria Formosa, Portugal) and has been shown to grow in wastewater with salinities as low as 5 ppt as well as in seawater with a salinity of 35 ppt [14,15]. It is therefore expected that this microalga has developed mechanisms to sustain salinity variations. Several microalgae produce osmolytes like glycerol to cope with excess salt concentrations in a process that competes with TAG assembly, which could explain the lack of lipid induction in *T. striata* CTP4 [40].

3.2.3. Thermal stress

Thermal stress was induced either by lowering the cultivation temperature to 5 °C or raising it to 35 °C, while the control was maintained at 20 °C. The highest lipid contents were observed in cultures grown at 35 °C under nutrient depletion (Fig. 4A). However, unlike other applied stresses, in which lipid contents generally increased over time until day 6, the lipid content and productivity of nutrient depleted *T. striata* CTP4 (N- cultures) grown at 35 °C peaked at day 4 (33.2% ± 3.1 DW and 12.9 ± 0.8 mg L^{-1} d^{-1}) and decreased afterwards. Such decrease may be the result of heat-induced cell damage caused by the disruption of cellular homeostasis or of physiological processes such as photosynthetic activity [41]. However, if a 4-day period is respected, the elevation of the cultivation temperature to 35 °C can be an effective inducer of lipids in this strain. Furthermore, growth at a low temperature (5 °C) for cultures under N+ conditions led to a significant increase in lipid content after 6 days of growth (24.4 ± 2.9%). An elevated lipid content and increased lipid productivity at extreme temperatures, as observed for *T. striata* CTP4, suggests that lipids may have an important function at extreme temperatures probably as storage compounds [42]. Changes in lipid content under both low and high temperature are often a species-dependent factor and literature reports are inconsistent as to what effect temperature has on lipid production. In a study on *Tetraselmis subcordiformis* and *Nannochloropsis oculata* the optimal temperature to achieve high lipid contents was 20 °C (22.25% DW) and 30 °C (24.44% DW), respectively [43]. In *Nannochloropsis salina* the lipid content increased when the culture temperature was raised from 15 to 30 °C [44]. However, in the microalgae *Thalassiosira pseudonana*, *Odontella aurita*, *Nannochloropsis oculata*, *Isochrysis galbana*, *Chromulina ochromonoides*, and *Dunaliella tertiolecta*, higher lipid fluorescence was found when these microalgae were grown at 10 °C in nutrient repleted conditions when compared to cultures grown at the optimum temperature, 20 °C [39]. On the contrary, Roleda et al. [45] did not observe significant differences in lipid contents between cultures grown at two different temperatures under nutrient depleted conditions.

3.3. Microscopic observations

Microscopic observation of the cells stained with BODIPY 505/515 revealed not only the localization but also gave visual proof of the concentration of lipid bodies inside the cells (Fig. 5). The acquired images compare well with the lipid contents obtained previously (Figs. 1–4). For example, highest lipid contents were found under nutrient depleted cultures under high light exposure, while nutrient repleted cultures showed lower lipid contents. This observation can be clearly seen from the micrographs of the single cells representing large amounts of lipid bodies in cells under stress conditions as compared to standard growth conditions (SGC). Furthermore, the images confirm that nutrient depletion had a major impact on lipid induction in *T. striata* CTP4. Interestingly, lipid bodies mostly accumulated at the periphery of the cells when exposed to high light under nutrient repletion, which was not the case for cells under thermal stress where migration towards the outer surface is not evident. Previous studies on *T. striata* CTP4 have revealed that carotenoids accumulate under similar conditions (170 µmol m^{-2} s^{-1}, N+), however, it seemed as they are located in the U-shaped chloroplast rather than in lipid bodies [46]. Therefore, the biosynthesis of this microalgal species is clearly distinctive from other species as *Haematococcus pluvialis* and *Dunaliella salina*, where lipid bodies serve as sink for the accumulation of carotenoids to protect the photosynthetic apparatus from damages caused by high irradiance [5,47]. Nevertheless, excess light can increase the cells photosynthetic activity leading to higher production of ATP and NADPH. These need to be consumed by biosynthetic pathways, among which lipid biosynthetic pathways, which play a major role in restoring the levels of ADP and NADP+ in the cell [9]. Although under excess light, carotenoids are the

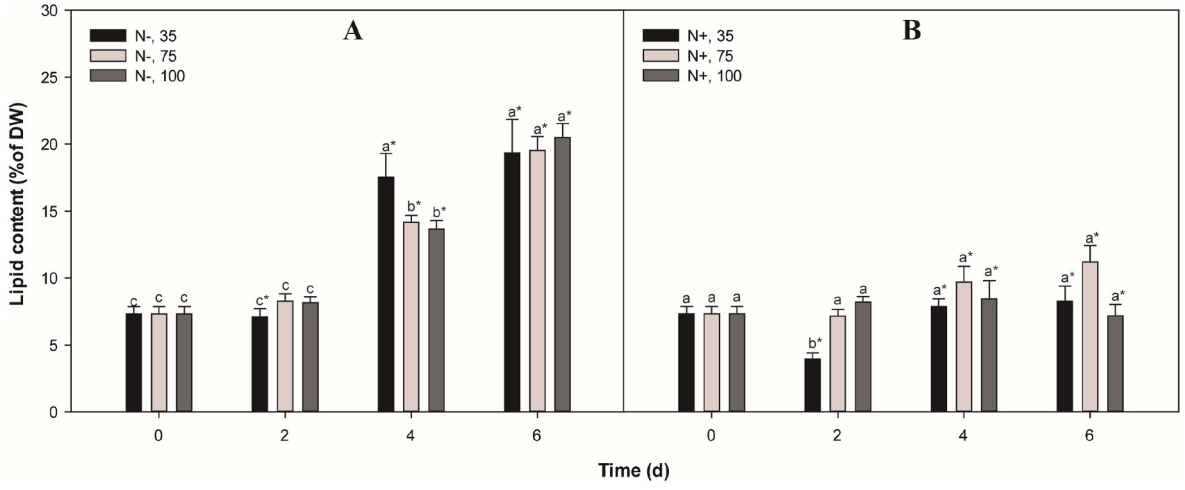

Fig. 3. Lipid content (% DW) of *T. striata* CTP4 cultivated in the two-stage growth with salinities of 35, 75 and 100 ppt at 20 °C and light intensity of 100 µmol m^{-2} s^{-1} using: A) nutrient depletion (N^-); and B) nutrient supplementation (N^+) conditions. Bars show means and standard deviation ($n = 3$). Within each treatment, bars labelled with different letters are significantly different ($p < 0.05$).

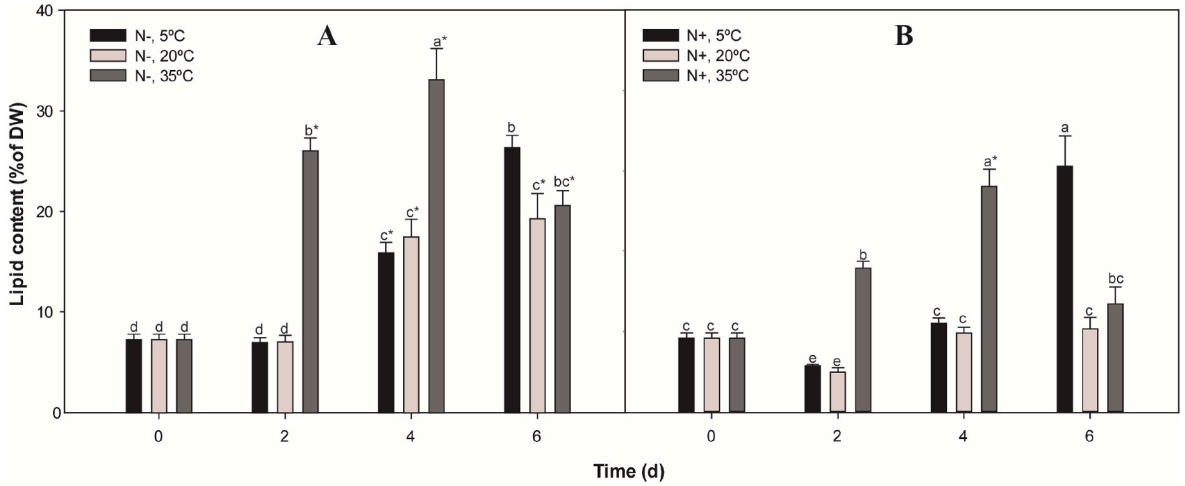

Fig. 4. Lipid content (% DW) of *T. striata* CTP4 cultivated in the two-stage growth with temperatures of 5, 20 and 35 °C and light intensity of 100 μmol m^{-2} s^{-1} using: A) nutrient depletion (N^-); and B) nutrient supplementation (N^+) conditions. Bars show means and standard deviation ($n = 3$). Within each nutrient condition, bars labelled with different letters are significantly different ($p < 0.05$).

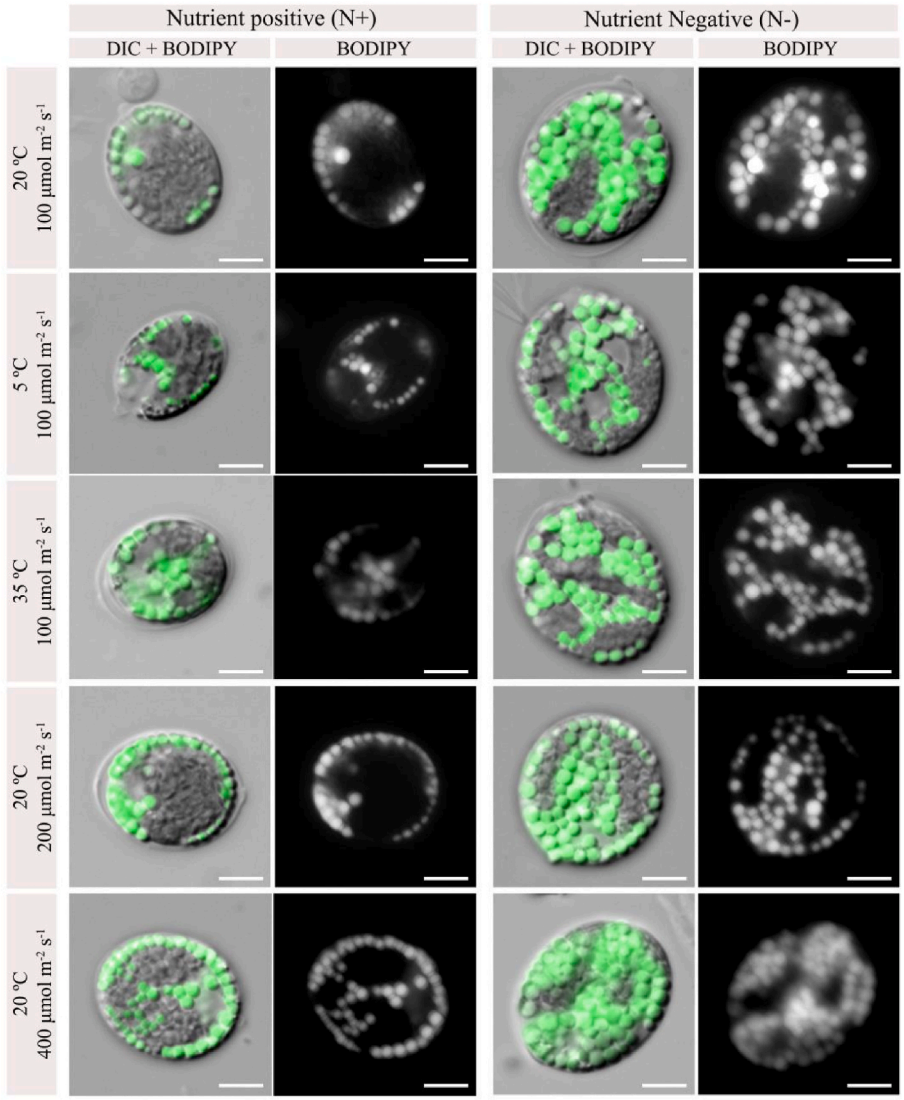

Fig. 5. Fluorescence showing lipid bodies stained with BODIPY in *T. striata* CTP4 grown at different light intensities, temperature and salinity with nutrient supplementation (N^+) or nutrient depletion (N^-). Cells against a grey background correspond to merged DIC and BODIPY fluorescence micrographs. Cells against a black background are micrographs of BODIPY fluorescence alone. Scale bar – 5 μm.

major protectants from photooxidative damage, it appears that in *T. striata* CTP4 lipid biosynthesis might help the cell to reach a more balanced redox state by this conversion of excess light to chemical energy.

3.4. Effect of culture conditions on FAME profile and biodiesel properties

The fatty acid profile was mainly (approximately 80% of TFA) composed of C16:0 (palmitic), C18:1 (oleic) and C18:2 (linoleic) acids, while C16:3 (hexadecatrienoic), C16:2 (hexadecadienoic), C16:1 (palmitoleic), C18:3 (linolenic) and C18:0 (stearic) acids were minor FAs (Table 3). This FA profile is in accordance with previous publications pertaining *T. striata* CTP4 [14,15]. Under standard growth conditions, nutrient repletion promoted a significantly higher accumulation of SFA, mainly palmitic acid, at the expense of PUFA (particularly linoleic acid; $p < 0.05$). A decrease in SFA under nutrient-depleted conditions like what was observed for *T. striata* CTP4 has been observed previously in *Nannochloropsis oculata*; however, no such changes were observed in *Chlorella vulgaris* [48]. Conversely, in *Ankistrodesmus falcatus*, SFA increased under nutrient depletion [49].

A decrease in temperature to 5 °C further increased the degree of FAs saturation of *T. striata* CTP4 under both nutrient repleted (from 47.2 ± 1.5% to 54.6 ± 1.8% SFA) and depleted conditions (from 39.3 ± 1.2% to 49.7 ± 3.4% SFA), while increased temperature did not change the FA profile as compared to standard growth conditions (Table 3). These findings are in accordance with another study on *Nannochloropsis oculata* and *Chlorella vulgaris* where increased temperature led to lower amounts of SFA [48]. However, the opposite trend is often observed in different studies, since higher percentage of unsaturation improves the maintenance of membrane integrity (increasing membrane fluidity) to withstand osmotic forces and nutrient exchange with the environment under lower temperatures [49–51]. Nevertheless, the response of microalgae to low and high growth temperatures is highly species-dependent with no general relationship between temperature and FA unsaturation [52]. Compared to cultures grown at standard growth conditions, *T. striata* CTP4 grown with light intensities of 200 and 400 μmol m^{-2} s^{-1} had a more unsaturated FA profile with significantly higher amounts of the PUFA C16:2 and C18:2 under both nutrient repletion and depletion (Table 3). This is most probably related with the improved growth of this species under higher light intensities, since actively growing cells usually synthesize more membranes, which are mainly composed of unsaturated FAs [9]. On the other hand, SFA and the MUFA C18:1 acid increased accompanied with a decrease in PUFA when *T. striata* CTP4 was cultivated at increased salinities of 10% under nutrient-replete conditions. These results are consistent with those observed for other microalgal species, such as *Chlamydomonas* sp., *Scenedesmus obliquus* and *Desmodesmus abundans* [37,39,53]. Generally, high contents of C18:2 and C18:3 acids in the fatty acid profile of microalgae are considered to be major contributors for a poor oxidative stability of the produced biodiesel. In particular, C18:2 acid, since the unsaturated bond of this FA is closer to the end methyl group, therefore being more oxidizable [54]. In this respect, the increased content of C18:2 acid observed in light induced cultures could be a drawback to the use of *T. striata* CTP4 for biodiesel production as it would require higher amounts of antioxidant supplementation. Nonetheless, the C18:3 content of this species was negligible rendering the oil of this microalga suitable for biodiesel production [55]. Furthermore, the observed increases in C18:1 acid in lipid induced cultures could contribute to improve the cold flow properties of the biodiesel [56].

Overall, *T. striata* CTP4 represents a suitable candidate for high-quality biodiesel production, since the FA profile is mainly composed of C16-18 methyl esters with very low amounts of C18:3 [54,57]. This is further supported by the good properties, e.g., cetane number and oxidation stability of the produced biodiesel within the EN14214 and ASTM D6751 specifications of this strain as shown previously [14].

3.5. Industrial applicability

As we demonstrate in this work, lipid accumulation can be trigged by abiotic stresses, stimulating microalgal metabolism to synthesize and store lipids, counteracting stress-induced damage to the cell [51]. However, scaling up from lab culture settings to an industrial production context is not straight forward. Costs of stress application, the technology needed and especially energy consumption need be considered. The application of high light intensity and thermal stresses can only be performed indoors or in locations with high solar irradiation during specific periods of the year (e.g., summer). In the case of the photo stimulation stress, light permeation into the cultured system reduces exponentially with distance from the light source, therefore requiring the use of photobioreactors with very low light paths as tubular photobioreactors or thin layer cascades [58]. Temperature control is limited by energy costs of and need of equipment to raise or lower the cultivation temperature accordingly [59]. Similar to the imposition of light stress, temperature control requires indoor facilities.

On the other hand, nutrient depletion is an effective, simple and inexpensive way of increasing lipid content that can be easily scalable to

Table 3
FAME profile of *T. striata* CTP4 cultivated under a two-stage growth system combining nutrient starvation (N^-) and supplementation (N^+) with different light intensities, salinity concentrations and temperatures. Values are represented as percentage of total FA (means ± standard deviation, $n = 6$).

FAME	SGC[a]		5 °C		35 °C		200 μmol m^{-2} s^{-1}		400 μmol m^{-2} s^{-1}		NaCl 75 ppt		NaCl ppt	
	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-	N+	N-
C16:0	46.9 ± 1.5	39.4 ± 1.2	54.6 ± 1.8	49.7 ± 3.4	44.0 ± 1.8	39.7 ± 1.2	36.4 ± 2.5	33.7 ± 2.2	33.7 ± 1.5	32.7 ± 0.6	45.2 ± 3.4	42.3 ± 0.2	52.1 ± 1.0	41.0 ± 2.6
C16:1	7.7 ± 1.4	8.3 ± 1.5	2.4 ± 0.8	2.4 ± 0.4	10.1 ± 0.7	10.5 ± 0.5	2.6 ± 0.7	3.3 ± 0.8	3.0 ± 0.2	3 ± 0.4	5.9 ± 1.3	5.6 ± 0.7	6.1 ± 1.8	7.2 ± 1.7
C16:2	4.2 ± 0.6	5.1 ± 0.6	1.5 ± 0.4	1.5 ± 0.7	4.0 ± 0.5	4.5 ± 0.4	8.4 ± 0.9	8.9 ± 0.5	8.5 ± 0.5	8.8 ± 0.3	5.7 ± 0.6	4.8 ± 0.5	2.2 ± 0.5	4.4 ± 0.7
C18:0	0.3 ± 0.1	0.3 ± 0.1	n.d.	n.d.	0.7 ± 0.1	0.7 ± 0.1	n.d.	n.d.	0.3 ± 0.1	n.d.	0.5 ± 4.0	n.d	1.0 ± 0.5	n.d
C18:1	17.1 ± 0.8	16.1 ± 1.0	18.8 ± 0.7	20.3 ± 0.8	15.0 ± 0.9	14.2 ± 0.5	10.8 ± 0.6	11.7 ± 0.6	11.6 ± 0.4	11.2 ± 0.2	15.2 ± 0.8	16.5 ± 0.9	20.2 ± 1.4	15.8 ± 1.2
C18:2	23.8 ± 2.6	31.2 ± 2	22.7 ± 1.8	27.6 ± 1.7	26.8 ± 1.9	30.4 ± 1.1	41.8 ± 1.7	42.4 ± 1.2	42.9 ± 1.0	43.9 ± 0.3	28 ± 3.2	28.1 ± 2.7	19.3 ± 2.4	33.9 ± 4.8
SFA	47.2 ± 1.5	39.3 ± 1.2	54.6 ± 1.8	49.7 ± 3.4	44.7 ± 1.8	40.4 ± 1.2	36.4 ± 2.5	33.7 ± 2.2	34.0 ± 1.5	32.7 ± 0.6	45.5 ± 3.4	43.5 ± 0.2	52.6 ± 1.1	40.1 ± 2.6
MUFA	24.8 ± 1.6	24.4 ± 1.9	21.2 ± 1.1	22.6 ± 0.1	25.0 ± 1.1	24.7 ± 0.7	13.4 ± 0.9	14.9 ± 1.8	14.5 ± 0.6	14.1 ± 0.5	21.1 ± 1.5	22.7 ± 1.2	26 ± 2.3	22.5 ± 2.1
PUFA	28.0 ± 2.7	36.3 ± 2.1	24.1 ± 1.8	29.1 ± 1.8	30.8 ± 2.0	34.9 ± 1.2	50.1 ± 1.9	51.3 ± 1.5	51.5 ± 1.2	52.7 ± 0.4	33.5 ± 3.2	33.8 ± 2.9	21.4 ± 2.5	37.4 ± 4.8

[a] Standard Growth Conditions (20 °C, 100 μmol m^{-2} s^{-1}, salinity 3.6%).

industrial production in closed or open systems [60]. In the same way, salinity modulation proved not to interfere with lipid accumulation on *T. striata* CTP4 triggered by nutrient starvation. This is a condition that can be easily applied at industrial scale to act as chemical barrier to biological contaminants as well [61].

4. Conclusions

Two-stage cultivation using abiotic stressors in the 2nd cultivation stage, e.g., nutrient starvation combined with high light (400 μmol m^{-2} s^{-1}), is an efficient strategy to induce lipids in *Tetraselmis striata* CTP4. Under these conditions, lipid productivity increased 2.7-fold when compared to cultures grown at standard growth conditions (100 μmol m^{-2} s^{-1}). Also, enhanced lipid productivity was accompanied with an increased unsaturation of the fatty acid profile through an increase of linoleic acid in detriment of palmitic acid. Considering that lipid derived biofuels have better compatibility with existing infrastructures and microalgae do not compete for arable land or drinking water, the robustness and lipid productivities along with the well-balanced fatty acid profile observed for *Tetraselmis striata* CTP4 make this species a promising candidate for high-quality biodiesel production. However, as the application of high light stress may require an additional expense in case of using artificial light or a longer time in the photobioreactor appropriate for light-induced stress, an economic evaluation of the costs involved in the production of lipid-rich biomass should be performed to aid the stockholders in reaching a suitable strategy for using this microalga as a feedstock and how that impacts on the final price of biodiesel.

CRediT authorship contribution statement

Ivo Monteiro: Conceptualization, Investigation, Writing – original draft, Writing – review & editing. **Lisa M. Schüler:** Data curation, Writing – review & editing, Validation. **Eunice Santos:** Investigation, Writing – review & editing. **Hugo Pereira:** Conceptualization, Supervision, Writing – review & editing, Validation. **Peter S.C. Schulze:** Software, Writing – review & editing, Validation. **Cláudia Florindo:** Investigation, Data curation. **João Varela:** Supervision, Writing – review & editing, Validation, Funding acquisition. **Luísa Barreira:** Conceptualization, Supervision, Writing – review & editing, Validation, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.renene.2023.03.103.

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