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High-value compound induction by flashing light in *Diacronema lutheri* and *Tetraselmis striata* CTP4

Lisa M. Schüler [a,b], Juline M. Walter [c,d], Hidehiko Kato [d], Hirono Suzuki [d], Christopher J. Hulatt [d], Ralf Rautenberger [e], Sofia Navalho [a], Benjamin Schmid [f], João Varela [a,b], Viswanath Kiron [d], Peter S.C. Schulze [a,d,*]

[a] GreenCoLab – Associação Oceano Verde, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[b] Centre of Marine Sciences (CCMAR), University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal
[c] Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway
[d] Nord University, Faculty of Biosciences and Aquaculture, Bodø, Norway
[e] Norwegian Institute of Bioeconomy Research (NIBIO), Division of Biotechnology and Plant Health, P.O. Box 115, NO-1431 Ås, Norway
[f] Necton S.A., Belamandil s/n, 8700-152 Olhão, Portugal

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ABSTRACT

Phototrophic microalgae use light to produce biomass and high-value compounds, such as pigments and polyunsaturated fatty acids (PUFA), for food and feed. These biomolecules can be induced by flashing light during the final growth stage. We tested different exposure times (1–6 days) of flashing light ($f = 0.5$, 5, 50 Hz; duty cycle = 0.05) on biomass, pigment and fatty acid productivity in *Diacronema lutheri* and *Tetraselmis striata*. A three-day exposure to low-frequency (5 Hz) flashing light successfully increased the production of fucoxanthin, diatoxanthin, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in *D. lutheri* up to 4.6-fold and of lutein, zeaxanthin and EPA in *T. striata* up to 1.3-fold compared to that of continuous light. Biomass productivity declined 2-fold for *D. lutheri* and remained similar for *T. striata* compared to that of continuous light. Thus, short-term treatments of flashing light may be promising for industrial algal production to increase biomass value.

1. Introduction

Microalgae are a natural and sustainable source of high-value compounds such as carotenoids and polyunsaturated fatty acids (PUFA), which find their applications as high-end products in the food, feed, nutra-, cosme- and pharmaceutical industries (Barkia et al., 2019). Despite the increasing interest in these microalgae-derived natural compounds, large-scale production of microalgae under photoautotrophic conditions is still challenging due to low biomass and compound productivities and high energy demands concerning production and downstream processes (Silva et al., 2020).

Under photoautotrophic conditions, the major factor that influences the productivity of microalgal biomass and of target compounds is light, which is usually supplied by the sun in eco-friendly production approaches. However, in dense cultures, light becomes limiting to the cells due to reduced light penetration and self-shading effects resulting into decreased biomass productivities. Alternatively, algae could be cultivated heterotrophically in the dark, which has been shown to lead to biomass concentrations 10 times higher as compared to those of microalgae grown photoautotrophically, decreasing production costs significantly (Barros et al., 2019). However, only a limited number of microalgal species is able to grow heterotrophically and biomass quality decreases due to lower pigment contents (Hu et al., 2018). On the other hand, two-stage cultivation strategies are common procedures to induce carotenoid and lipid contents in microalgae, where in a first step biomass is produced under growth promoting conditions and in the second stage pigment or lipid production is induced by the application of different environmental stressors, such as high salinities, high light or nutrient depletion (Liyanaarachchi et al., 2021). For example, accumulation of carotenoids and lipids can be achieved in *Tetraselmis* sp. under nutrient starvation in combination with high light (Dammak et al., 2021), while the same compounds are induced by salt and high-light stress in *Chlorella vulgaris* (Ali et al., 2021). However, these induction procedures often take several days or weeks, during which the

* Corresponding author at: GreenCoLab – Associação Oceano Verde, University of Algarve, Campus de Gambelas, 8005-139 Faro, Portugal.
E-mail address: peterschulze@greencolab.com (P.S.C. Schulze).

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cultures do not grow and sometimes even lose biomass due to cell respiration. Alternatively, recent studies showed that using flashing (or pulsed-) light, being the periodic emission of light- and dark periods, can trigger the induction and improve the production of xanthophylls, chlorophylls and PUFA in the chlorophytes *Koliella antarctica* and *Tetraselmis chui* as well as the eustigmatophyte *Nannochloropsis gaditana* (Lima et al., 2021). Strikingly, unlike nutrient starvation and salinity stress, the flashing light may still drive photosynthetic light reactions, allowing a further increase in biomass in the culture. Lastly, because light reactions in photosynthetic organisms take place quickly to avoid photodamage (Schulze et al., 2017b), time needed to induce pigments and fatty acids may be significantly shorter (e.g., hours or few days) compared to traditional two-stage induction processes such as nutrient starvation that can last for several days or weeks (Ali et al., 2021; Dammak et al., 2021).

The chlorophyte *Tetraselmis striata* has been shown to be a very robust strain with the possibility to grow in wastewaters and in industrial scale photobioreactors (Pereira et al., 2018; Schulze et al., 2017a). Furthermore, the biochemical profile is rich in lipids containing eicosapentaenoic acid (EPA) and the carotenoids lutein and β-carotene, which are important compounds for nutraceutical applications (Schüler et al., 2021). On the other hand, the haptophyte *Diacronema lutheri* (formerly known as *Pavlova lutheri*) is less studied for industrial production, although being promising strains for aquafeeds and human nutrition due to high contents of EPA and docosahexaenoic acid (DHA) and pigments such as fucoxanthin, diatoxanthin or β-carotene (Guihéneuf and Stengel, 2017; Tibbetts et al., 2019; Kanamoto et al., 2021).

Given the importance of these two marine obligate photoautotrophic species, *D. lutheri* and *T. striata* CTP4, the present study aims to stimulate biomass, pigment and fatty acid productivities by investigating the optimal frequency and exposure time of flashing light in a two-stage cultivation approach. To the best of our knowledge, this is the first report to investigate different exposure times of flashing light on *D. lutheri* and *T. striata* CTP4 to accumulate high value xanthophylls and omega-3 fatty acids.

2. Materials and methods

2.1. Experimental setup

Diacronema lutheri NIVA-4/92 (*Pavlova lutheri*) was obtained from the Norwegian Culture Collection of Algae (NORCCA) and *Tetraselmis striata* CTP4 was isolated from the Algarve coast, Portugal (Pereira et al., 2016). In the first growth stage, *D. lutheri* and *T. striata* were grown in 250 mL tissue culture flasks (Falcon Scientific, Seaton Delaval, UK, light paths: 3.7 cm) containing 200 mL of seawater (35 ppt) enriched with a modified F-medium (5.3 mM $NaNO_3$, 0.22 mM $NaH_2PO_4.H_2O$, 35 µM $FeCl_3.6H_2O$, 35 µM $Na_2EDTA.2H_2O$, 0.12 µM $CuSO_4.5H_2O$, 0.078 µM $Na_2MoO_4.2H_2O$, 0.23 µM $ZnSO_4.7H_2O$, 0.126 µM $CoCl_2.6H_2O$ and 2.73 µM $MnCl_2.4H_2O$, pH of 7.2) for the respective six and nine days at I_a = 300 µmol s^{-1} m^{-2} under continuous light (CL) as described previously (Lima et al., 2021). In the second stage, 25 mL of cultures were shifted to smaller 30-mL tissue culture flasks (light paths: 2.0 cm) and exposed to either CL (control) or flashing light (FL) with frequencies of 0.5, 5 or 50 Hz. The employed duty cycle (*DC*) was 0.05, while the average light (I_a) and flash intensity (I_l) were 300 and 6000 µmol s^{-1} m^{-2}, respectively. Under FL 0.5 the light period (t_l) was 0.1 s and the dark period (t_d) was 1.9 s, whereas under FL 5 t_l was 0.01 s and t_d was 0.09 s and under FL 50 Hz t_l was 0.02 s and t_d was 0.08 s. The light source was composed of an array of 36 warm-white LEDs (MHD-G, 2700 K, 12.6 W, Opulent Americas, Raleigh, US) and mounted on an actively cooled aluminum heat sink as described previously (Lima et al., 2021). Mixing of the cultures was achieved by aeration with humidified and 0.2 µm-filtered air enriched with CO_2 at a flow rate of 160 mL min^{-1}.

The growth of the cultures was monitored by daily optical density measurements at 540 nm (OD_{540}) and dry weight (DW) determination. For each microalga, linear correlations between OD_{540} and DW were obtained with significance of $r^2 \geq 0.9$, $p < 0.05$. The biomass productivity for each alga culture and cultivation approach was calculated by the following equation:

$$P = \left(DW_{dayX} - DW_{day0}\right)/X \quad (1)$$

with X being the number of days of the experiment.

Biomass, pigment, and fatty acid productivity were determined throughout the experiment using replicates from four separately grown cultures for each treatment. Biomass samples for biochemical analysis were taken by centrifugation (5000*g*, 5 min) at four different time points, namely at the beginning of the 2nd-stage and after 1, 3 and 6 days of FL exposure. Afterwards, biomass was washed (0.5 M ammonium bicarbonate), freeze-dried and stored at −80 °C until further analysis.

2.2. Pigment analysis

Chlorophylls and carotenoids were extracted and analyzed as described previously (Schüler et al., 2021). Briefly, biomass was disrupted in methanol by bead beating (Retsch Mixer Mill 400). The methanol extract was separated from the algae pellet via centrifugation and the pellet was re-extracted until both the pellet and the supernatant became colorless.

Chlorophyll *a* (C_a), *b* (C_b) and total carotenoids (C_{car}) were determined spectrophotometrically according to the following equations as described in Lichtenthaler and Wellburn (1983):

$$C_a = 15.65\, A_{666} - 7.34\, A_{653} \quad (2)$$

$$C_b = 27.05\, A_{653} - 11.21\, A_{666} \quad (3)$$

$$C_{car} = (1000\, A_{470} - 2.86\, C_a - 129.2\, C_b)/221 \quad (4)$$

Individual carotenoids were identified and quantified by HPLC using calibration curves of the pigments lutein, β-carotene, violaxanthin, neoxanthin, fucoxanthin, diatoxanthin and diadinoxanthin as described previously (Schüler et al., 2020a). To this end, carotenoid extracts were injected in a Dionex 580 HPLC System (DIONEX Corporation, Sunnyvale, US) equipped with a photodiode-array detector and the column oven was set to 20 °C. Compounds were separated using a LiChroCART RP-18 (5 µm, 250 × 4 mm, LiChrospher, Merck KGaA, Germany) column and a solvent gradient at constant flow rate of 1 ml min^{-1} consisting of acetonitrile:water (9:1; v/v) as solvent A and ethyl acetate as solvent B. The solvent program was set as follows: (i) 0–16 min, 0–60 % B; (ii) 16–30 min, 60 % B; (iii) 30–32 min 100 % B and (iv) 32–35 min 100 % A.

2.3. Fatty acid analysis

Fatty acids were extracted from microalgal biomass and analyzed by gas chromatography (GC) coupled with a flame ionization detector (FID) as described in Lima et al. (2021). Briefly, freeze-dried microalgal biomass resuspended in a mixture of chloroform:methanol (2:2.5 v/v) containing an internal standard (Tripentadecanoin, C15:0) was disrupted by bead milling (Precellys Evolution, Bertin technologies, Montigny-le-Bretonneux, France). After the addition of tris-buffer, phase separation was achieved by mixing and centrifugation. The lower chloroform-phase containing the lipids was transferred to a new glass tube and evaporated under a gentle nitrogen flow. Transesterification of the lipids was performed using methanol containing 5 % H_2SO_4 at 70 °C for 3 h. Hexane and distilled water were added and mixed well to allow phase separation. Afterwards, fatty acid methyl esters (FAMEs) in the hexane phase were analyzed by GC-FID equipped with a CP-Wax 52 CB column (Agilent, Santa Clara, US) using split-less mode.

2.4. Data analysis

Data were tested for normality using the Shapiro-Wilk test (XLStat software, Vers. 2016.02.27444, Addinsoft, USA). ANOVA and two-sided Dunnett's tests were performed for comparison of means of treatments with the beginning of the treatment (day 0) with a confidence interval of 95 %.

3. Results and discussion

3.1. Growth

The growth of the haptophyte *Diacronema lutheri* (*Pavlova lutheri*) and the chlorophyte *Tetraselmis striata* was studied under three flashing light regimes (FL 0.5, 5 and 50 Hz) and compared to continuous light (CL) in a two-stage setup (Fig. 1a, b). In the first stage, *D. lutheri* and *T. striata* cells were grown under CL for nine and six days, respectively, followed by an induction stage, where FL was applied for six days. The FL employed in this study had a duty cycle (*DC*) of 0.05, an average light intensity (I_a) of 300 µmol s^{-1} m^{-2} and a flash intensity (I_l) of 6000 µmol s^{-1} m^{-2}.

Both microalgae *D. lutheri* and *T. striata* displayed the highest biomass concentrations (5.72 ± 0.44 and 5.77 ± 0.22 g L^{-1}, respectively) and productivities (0.35 ± 0.03 and 0.43 ± 0.02 g L^{-1} d^{-1}, respectively) under CL at the end of the experiment (Fig. 1, Table 1). The FL (f = 0.5, 5 and 50 Hz; FL 0.5, 5 and FL 50, respectively) treatments in the second growth stage applied to cultures of *D. lutheri* significantly affected growth, leading to prolonged lag phases (Fig. 1) and significantly lower biomass productivities (Table 1). Cultures grown under FL 50 with a biomass productivity of 0.25 ± 0.04 g L^{-1} d^{-1} adapted to the changing conditions upon three days after the onset of the second stage, reaching a biomass concentration of 4.18 ± 0.58 g L^{-1} at the end of the experiment. Under FL 5, the cells became adapted to the new growth conditions only after four days of treatment leading to a biomass concentration of 3.35 ± 0.48 g L^{-1} at the end of the experiment. However, the lowest frequency, FL 0.5, led to a significant growth inhibition resulting into the lowest biomass concentration and productivity (2.84 ± 0.34 g L^{-1} and 0.17 ± 0.02 g L^{-1} d^{-1}, respectively) compared to those of other growth conditions. Interestingly, FL 5 and FL 50 treatments applied to *T. striata* cultures did not significantly affect biomass concentrations leading to only slightly lower productivities (average: 0.40 ± 0.02 g L^{-1} d^{-1}) than those of cells under CL (0.43 ± 0.02 g L^{-1} d^{-1}) (Fig. 1, Table 1). Nevertheless, similar to *D. lutheri*, low frequency (0.5 Hz) FL significantly inhibited growth of *T. striata*, reaching a biomass concentration and productivity of 3.97 ± 0.50 and 0.28 ± 0.04 g L^{-1} d^{-1}, respectively (Fig. 1, Table 1).

Flashing light is sensed by the two microalgae differently, since all treatments (FL 0.5, 5 and 50) affected growth of *D. lutheri* but only FL 0.5-led to growth inhibition in *T. striata* cultures. These strain-specific growth behaviors have been linked previously to the cell size and architecture, proposing that larger cells deal better with low-frequency FL than smaller cells (Lima et al., 2021). This assumption might be related to the possibility that larger cells are less prone to photoinhibition (Key et al., 2010), and thus, might adapt better to the long-lasting high-light

Table 1
Biomass productivities (g L^{-1} d^{-1}) of *Diacronema lutheri* and *Tetraselmis striata* during two-stage cultivation. The tested flashing light frequencies during the 2nd stage were 0.5 (FL 0.5), 5 (FL 5) and 50 (FL 50) Hz at a duty cycle of 0.05; continuous light served as control. Different letters represent significant differences between productivities for a given alga and cultivation treatment. Values are shown as means ± SD (n = 4).

Treatments		*Diacronema lutheri* [g L^{-1} d^{-1}]	*Tetraselmis striata* [g L^{-1} d^{-1}]
Continuous light		0.35 ± 0.03^{a}	0.43 ± 0.02^{a}
Flashing light	50 Hz	0.25 ± 0.04^{b}	0.39 ± 0.02^{b}
	5 Hz	0.20 ± 0.03^{c}	0.41 ± 0.02^{a,b}
	0.5 Hz	0.17 ± 0.02^{d}	0.28 ± 0.04^{c}

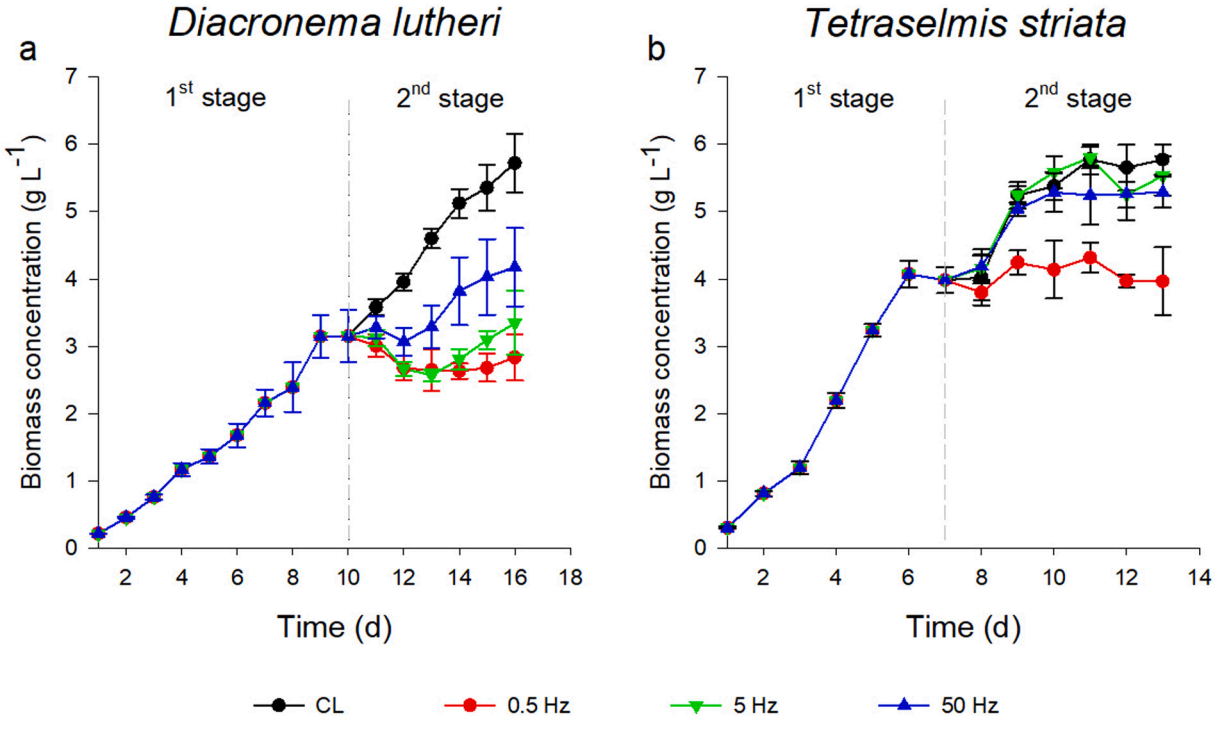

Fig. 1. Growth curves of *Diacronema lutheri* (a) and *Tetraselmis striata* CTP4 (b) in a two-stage cultivation approach. Vertical lines indicate the start of the second stage, at which cultures were exposed to flashing light with a duty cycle of 0.05 and frequencies of 0.5, 5, 50 Hz and compared to continuous light (CL). The average light intensity in all treatments was I_a = 300 µmol s^{-1} m^{-2} and the flash intensity was I_l = 6000 µmol s^{-1} m^{-2}. Data points at each day are displayed as mean ± SD (n = 4).

intensities of low-frequency FL (e.g., FL 0.5 and 5). Indeed, the spherical-shaped *T. striata* cells (15–30 μm) are larger than cells of *D. lutheri* (4–6 μm) and may confirm the aforementioned assumption (Hulatt et al., 2021; Pereira et al., 2016).

3.2. Biochemical composition

3.2.1. Pigments

The highest carotenoid productivities in *D. lutheri* and *T. striata* were achieved under FL 5 treatment, yielding after three days of exposure values of 1.85 ± 0.23 and 1.04 ± 0.10 mg L^{-1} d^{-1}, respectively (Figs. 2d and 3f, respectively). Concerning the individual carotenoids in *D. lutheri*, six days of treatment using either low-frequency FL of 0.5 or 5 Hz led to enhanced fucoxanthin productivities, reaching a maximum of 0.33 ± 0.06 mg L^{-1} d^{-1}, which was 1.2-, 1.3- and 1.6-fold higher as compared to those of cells under CL or FL 50 and those of cells on day 0, respectively (Fig. 2a). Moreover, after the same exposure time using FL 0.5 an over 4-fold increase in diatoxanthin productivity (1.0 mg L^{-1} d^{-1}) as compared to that of microalgae under CL and cells on day 0 was observed (Fig. 2c). Notably, already after three days of exposure to FL 5, a significant increase in diatoxanthin productivity (0.77 ± 0.11 mg L^{-1} d^{-1}) was achieved compared to that of cells under other treatments. Such improvements in diatoxanthin are highly relevant for biotechnological applications in the cosme- and nutraceutical market as due to its recent classification as bioactive and chemo preventive agent (Pistelli et al., 2021). Conversely, the β-carotene productivity was significantly enhanced when cells were grown under CL in the 2nd stage for three and six days instead of low-frequency FL of 0.5 and 5 Hz. In general, when compared to day 0, β-carotene productivities decreased during the second stage in *D. lutheri* among all treatments except for cells under CL (Fig. 2b).

In *T. striata*, the productivity of the major carotenoid lutein showed a 1.3-fold increase as compared to cells exposed to CL after three and six days of FL 5 treatment, reaching maximum values of 0.66 ± 0.07 and 0.70 ± 0.02 mg L^{-1} d^{-1}, respectively (Fig. 3a). Furthermore, microalgae under the same FL 5 treatment applied for six days showed significantly higher zeaxanthin productivity of 0.014 ± 0.001 mg L^{-1} d^{-1} than cultures under other treatments. Moreover, cells exposed to FL 5 for one and three days presented the highest violaxanthin productivities, which were significantly increased as compared to those of cells on day 0. However, exposure to FL for six days led to a decrease in violaxanthin productivities among cultures under all conditions down to levels observed on day 0. The best treatment for high productivity for neoxanthin and β-carotene was FL 50 for one or three days, reaching 0.12 ± 0.01 and 0.23 ± 0.04 mg L^{-1} d^{-1}, respectively. Six days of exposure led to decreased productivities of both pigments although this decrease was not significant when FL 5 was applied (0.10 ± 0.01 and 0.20 ± 0.01 mg L^{-1} d^{-1}).

Concerning chlorophyll *a* productivities in *D. lutheri*, the FL 5 treatment for three days led to a 4-fold increase as compared to CL, reaching 1.60 ± 0.19 mg L^{-1} d^{-1} (Fig. 2e). However, cultures under this treatment were not significantly different from those under FL 0.5 (1.29 ±

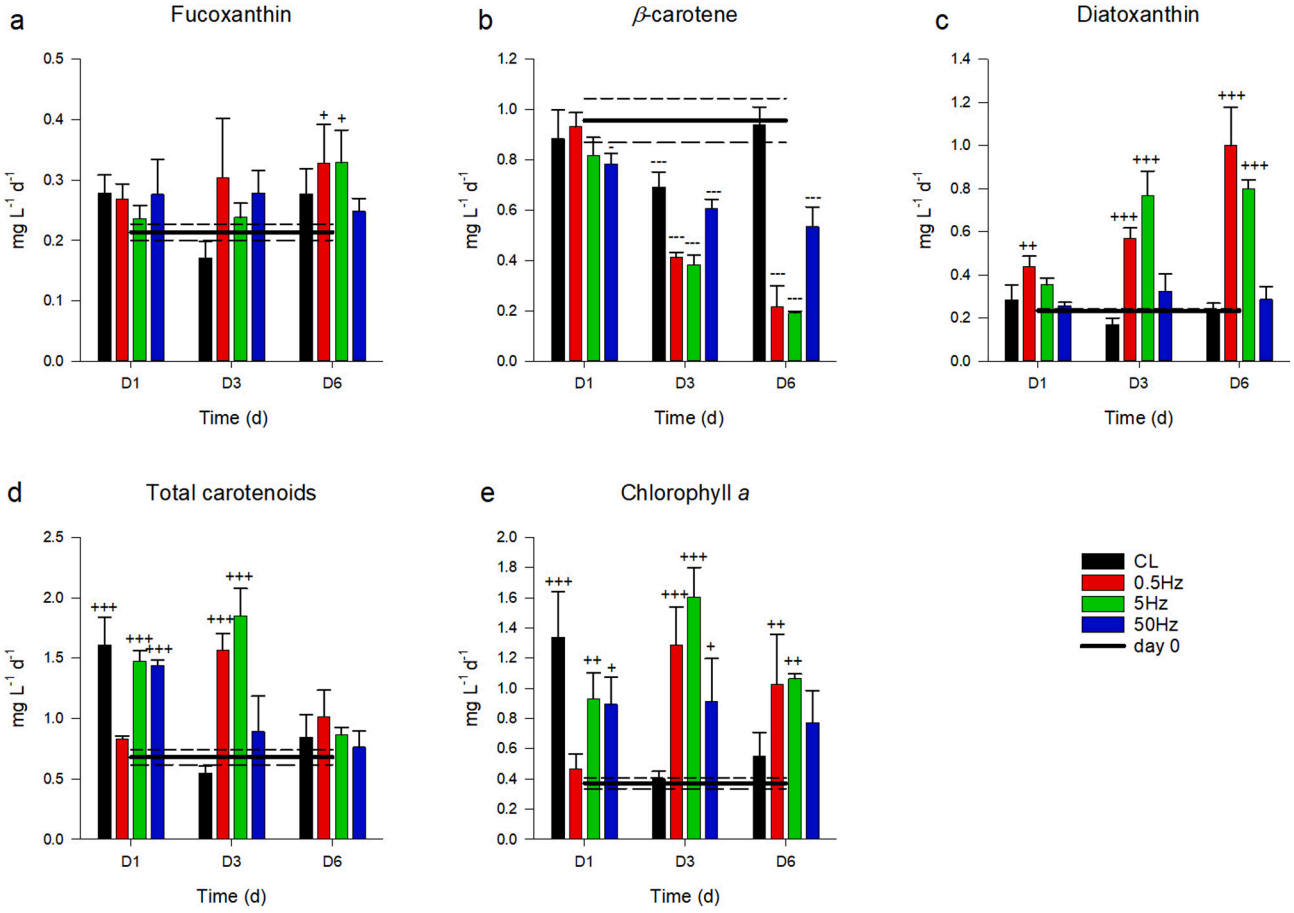

Fig. 2. Pigment productivity of *Diacronema lutheri* exposed to flashing light (FL 0.5, 5 and 50 Hz) with a duty cycle of 0.05 or continuous light (CL) with an averaged light intensity of 300 μmol s^{-1} m^{-2}. Productivities of fucoxanthin (a), β-carotene (b), diatoxanthin (c), total carotenoids (d) and chlorophyll *a* (e) are expressed as mg pigments per L per day (mean ± SD, $n = 4$). The solid line indicates pigment productivities upon the application of the FL treatment (day 0). Significantly higher or lower productivities (Dunnett's test) compared to those on day 0 are represented by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$) plus or minus symbol, respectively.

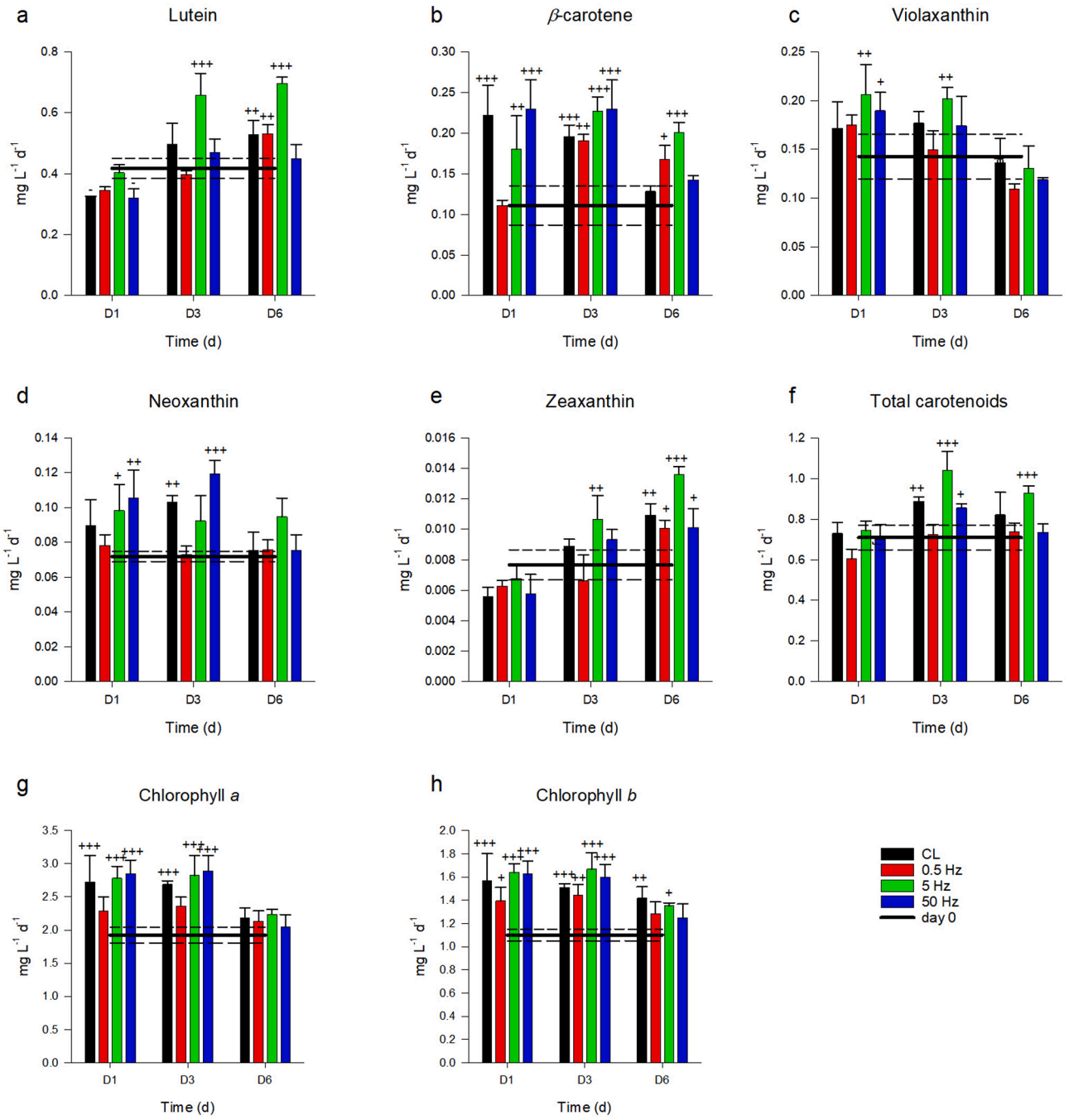

Fig. 3. Pigment productivities of *Tetraselmis striata* CTP4 exposed to flashing light (FL 0.5, 5 and 50 Hz) with a duty cycle of 0.05 or continuous light (CL) with an averaged light intensity of 300 μmol s^{-1} m^{-2}. Productivities of lutein (a), *β*-carotene (b), violaxanthin (c), neoxanthin (d), zeaxanthin (e), total carotenoids (f), chlorophyll *a* (g) and chlorophyll *b* (h) are expressed as mg pigments per L per day (mean ± SD, $n = 4$). The solid line indicates pigment productivities upon the application of the FL treatment (day 0). Significantly higher or lower productivities (Dunnett's test) compared to those on day 0 are represented by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$) plus or minus symbols, respectively.

0.25 mg L^{-1} d^{-1}) and CL upon one day of exposure (1.34 ± 0.30 mg L^{-1} d^{-1}). On the other hand, chlorophyll *a* productivities in *T. striata* did not show significant differences between FL 5, 50 and CL cells after exposure for one and three days, reaching 2.89 ± 0.23 mg L^{-1} d^{-1}, while FL 0.5 led to a significantly lower chlorophyll *a* productivity (Fig. 3g). Notably, after 6 days of the second growth stage, chlorophyll *a* productivities decreased among cells under all treatments down to levels of cultures on day 0. Highest chlorophyll *b* productivities were achieved under exposure to FL 5 for three days (1.67 ± 0.14 mg L^{-1} d^{-1}), however, without significant difference among all treatments and exposure times with exception to six-day FL treatments, which led to decreased productivities (Fig. 3h).

Similar to our results, induction of pigments and productivity improvements under low-frequency FL (e.g., $f < 30$ Hz) were found in *Chlamydomonas reinhardtii* ($f = 0.00138$–1 Hz, $DC = 0.5$) (Takache et al., 2015), *Haematococcus pluvialis* (f = 25–200 Hz, DC = 0.17–0.77; f = 3.49 Hz, $DC = 0.47$ and f = 25–200 Hz, $DC = 0.17$–0.67) (Katsuda et al., 2006, 2008; Kim et al., 2006) and *Tetraselmis chui* (Lima et al., 2021). As pigments function in light harvesting and photoprotection of the photosynthetic machinery, they are important for the cells to adapt to

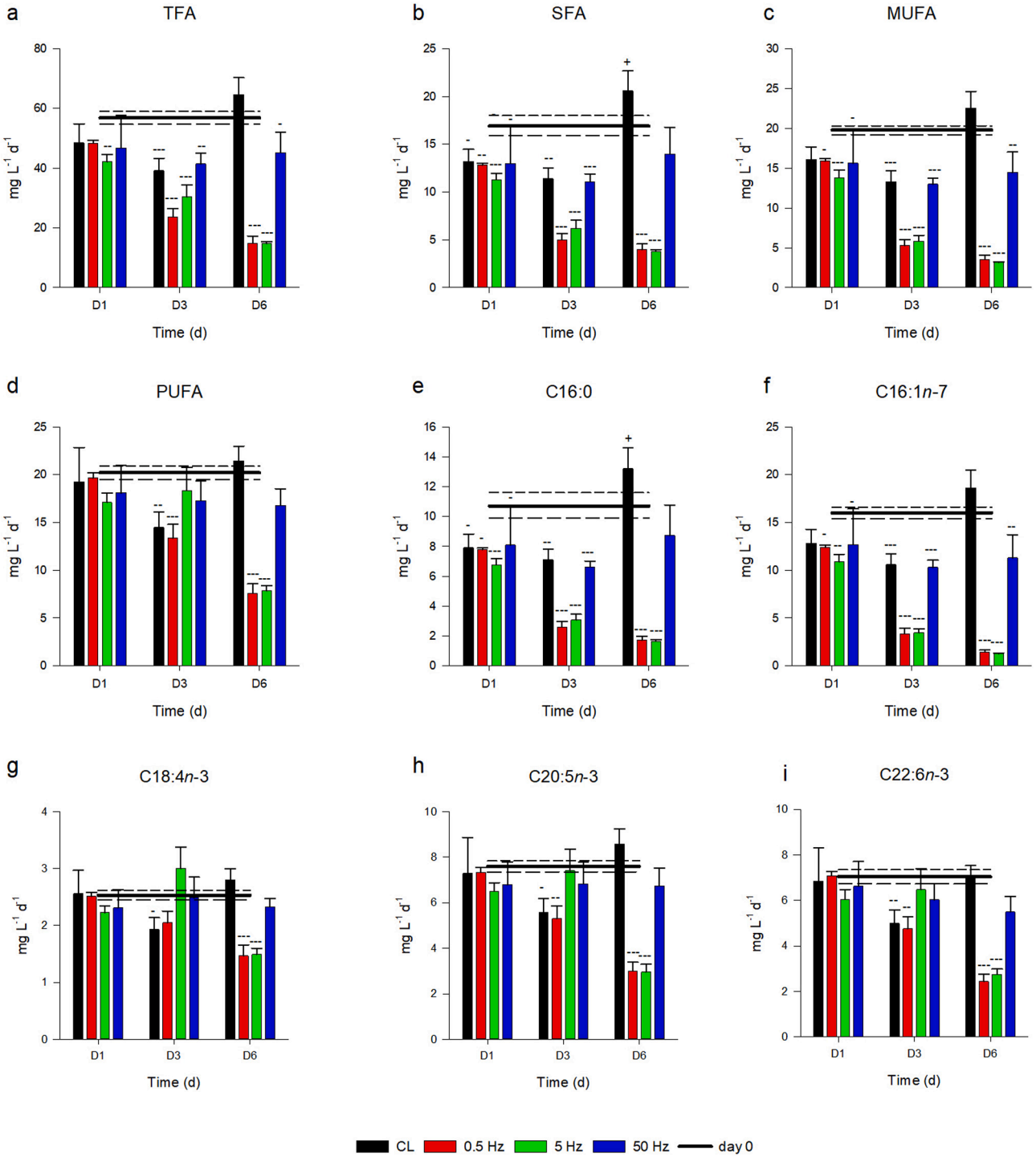

Fig. 4. Fatty acids productivity of *Diacronema lutheri* exposed to flashing light (FL 0.5, 5 and 50 Hz) with a duty cycle of 0.05 or continuous light (CL). Total fatty acids (TFA) (a), saturated fatty acids (SFA) (b), monounsaturated fatty acids (MUFA) (c), polyunsaturated fatty acids (PUFA) (d) palmitic (C16:0) (e), palmitoleic (16:1*n*-7) (f), stearidonic (18:4*n*-3) (g), eicosapentaenoic (20:5*n*-3) (h) and docosahexaenoic (22:6*n*-3) (i) acids are expressed as mg fatty acids per L per day (mean ± SD, $n = 4$). The solid line indicates pigment productivities upon the application of the FL treatment (day 0). Significantly higher or lower productivities (Dunnett's test) compared to those on day 0 are represented by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$) plus or minus symbols, respectively.

changing light intensities. Particularly, the xanthophyll cycles, known as the violaxanthin (Vx) cycle in chlorophytes and the diadinoxanthin (Ddx) cycle in haptophytes or diatoms, are important regulators of light adaptation (Jahns et al., 2009; Lohr and Wilhelm, 1999). Under high light stress, the biosynthesis shifts towards zeaxanthin or diatoxanthin in the respective Vx- and Ddx cycles to protect the photosynthetic apparatus from photoinhibition or even damage (Jahns et al., 2009; Lohr and Wilhelm, 1999). Likewise in our previous study, FL with low frequencies has been already linked to responses to exposure to high-light flash intensities of, e.g., 6000 µmol s^{-1} m^{-2} during a period of $t_l = 0.001$ or 0.01 s (e.g., $f = 5$, 50 Hz, $DC = 0.05$, $I_a = 300$ µmol s^{-1} m^{-2}). Such adaptation of cells to high-light conditions could be confirmed in the present study, because up to 5.3-fold increased intracellular levels of the photoprotective zeaxanthin in *T. striata* and diatoxanthin in *D. lutheri* under

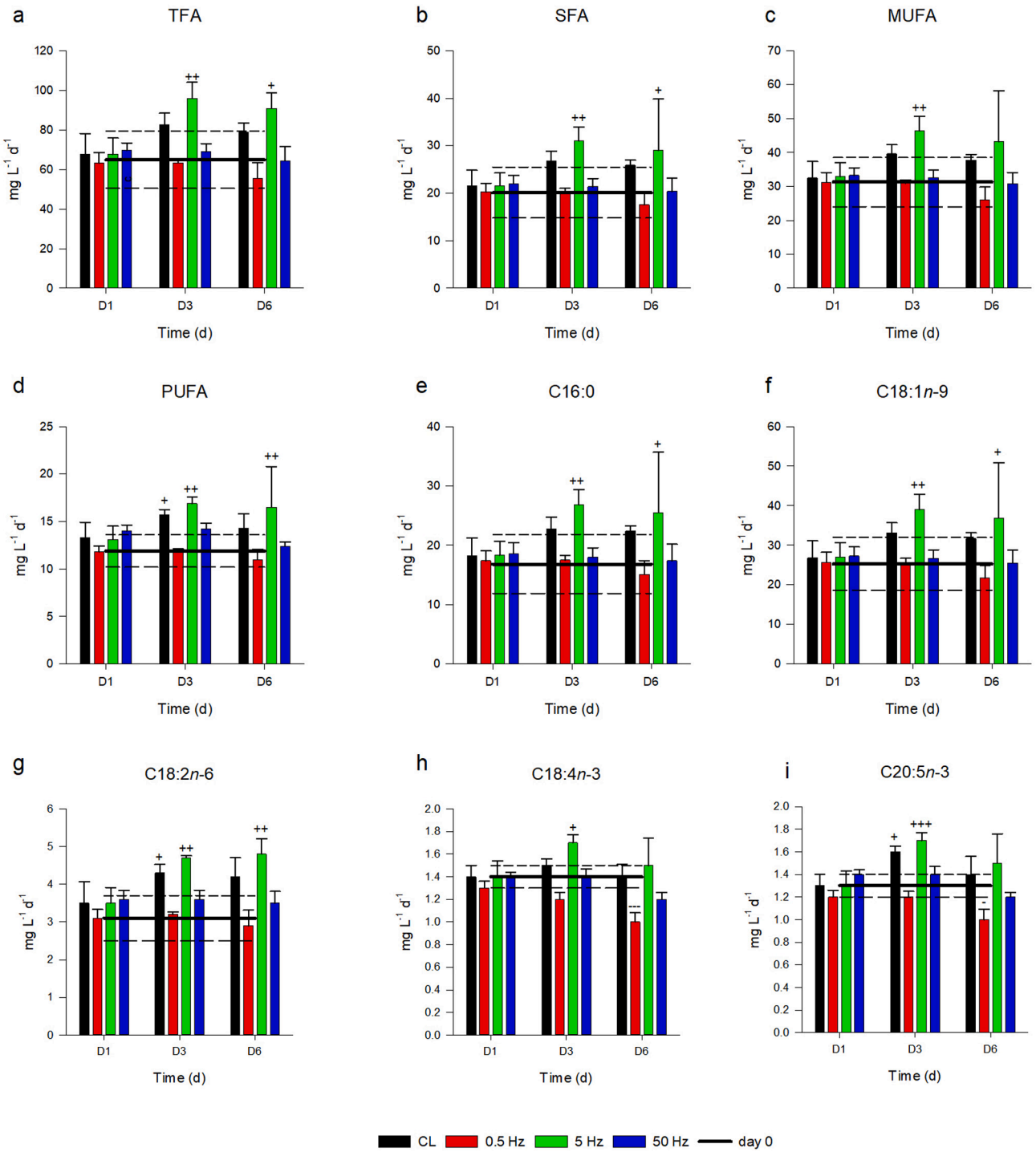

Fig. 5. Fatty acids productivity of *Tetraselmis striata* exposed to flashing light (FL 0.5, 5 and 50 Hz) with a duty cycle of 0.05 or continuous light (CL). Total fatty acids (TFA) (a), saturated fatty acids (SFA) (b), monounsaturated fatty acids (MUFA) (c), polyunsaturated fatty acids (PUFA) (d) palmitic (C16:0) (e), oleic (C18:1*n*-9) (f), linoleic (18:2*n*-6) (g), stearidonic (18:4n-3) (h) and eicosapentaenoic (20:5n-3) (i) acids are expressed as mg fatty acids per L per day (mean ± SD, $n = 4$). The solid line indicates pigment productivities upon the application of the FL treatment (day 0). Significantly higher or lower productivities (Dunnett's test) compared to those on day 0 are represented by one ($p < 0.05$), two ($p < 0.01$) or three ($p < 0.001$) plus or minus symbols, respectively.

low-frequency FL (0.5 and 5 Hz) were found (Table S1).

Moreover, in *T. striata*, the increased levels of lutein accompanied by decreased levels of *β*-carotene have been observed previously under high light intensities (Schüler et al., 2020b). Although both lutein and *β*-carotene are important pigments for light harvesting and photoprotection, in *T. striata*, lutein seemed to be more responsive to exposure to high light. Concerning *D. lutheri*, *β*-carotene contents have been found decreased by over 70 % while fucoxanthin and chlorophyll *a* contents increased 2.3- and 4.2-fold, respectively, under low-frequency flashing light for six days (Table S1). However, in a previous study on *D. lutheri*, both *β*-carotene and fucoxanthin contents have been found to decrease under high light intensities (200 μmol m^{-2} s^{-1}) (Guihéneuf and Stengel, 2017). Moreover, fucoxanthin and chlorophyll *a* have been found interdependent in diatoms and other haptophytes, which can be usually found to be increased under low light conditions (40–100 μmol m^{-2} s^{-1}) (Gao et al., 2020; Li et al., 2019; McClure et al., 2018). Therefore, low-

frequency FL applied in this study might have stimulated the accumulation of target compounds in a similar way to that of cells under low light, which agrees well with the previous assumption described in Lima et al. (2021).

3.2.2. Fatty acids

The fatty acid profile of *D. lutheri* was mainly composed of myristic (C14:0), palmitic (C16:0), palmitoleic (16:1*n*-7), stearidonic (SDA, 18:4*n*-3), eicosapentaenoic (EPA, 20:5*n*-3) and docosahexaenoic (DHA,22:6*n*-3) acids, which compares well with previously reported profiles of this species (Guedes et al., 2010; Guihéneuf and Stengel, 2017). The highest total fatty acid (TFA) productivities in *D. lutheri* were found under CL after six days of exposure leading to 64.6 ± 5.68 mg L^{-1} d^{-1} (Fig. 4a). This treatment led to the highest productivity of saturated fatty acids (SFA), mainly containing myristic (C14:0) and palmitic (C16:0) acids (Fig. 4b, e). Furthermore, the monounsaturated fatty acids (MUFA) increased, leading to productivities of 18.6 ± 1.86 mg L^{-1} d^{-1} in palmitoleic acid, 16:1*n*-7 (Fig. 4f). These high productivities of SFA and MUFA are most probably related with the exposure to high light intensity (300 µmol s^{-1} m^{-2}) under continuous light, whose productivities have been found increased previously under high light (200 µmol s^{-1} m^{-2}) as compared to those of *D. lutheri* cells (40 µmol s^{-1} m^{-2}) under low light (Guihéneuf and Stengel, 2017). However, all fatty acid productivities decreased significantly when cells were exposed to FL with the most significant decreases after six days to low-frequency FL of 0.5 and 5 Hz. These lower values might be related with the low biomass productivity under these conditions (Fig. 1a). Interestingly, when the FL 5 treatment was applied for only three days, the productivities of the PUFAs SDA (C18:4*n*-3, 3.00 ± 0.38 mg L^{-1} d^{-1}), EPA (C20:5*n*-3, 7.41 ± 0.94 mg L^{-1} d^{-1}) and DHA (C22:6*n*-3, 6.47 ± 0.91 mg L^{-1} d^{-1}) increased 1.5-, 1.3 and 1.3-fold as compared to those of CL cells, respectively (Fig. 4g, h, i). Using this treatment, intracellular contents of SDA, EPA and DHA reached, respectively, 10.8 ± 0.21, 26.6 ± 0.65 and 23.2 ± 0.22 mg g^{-1} DW (Table S1), which matched previously reported EPA concentrations in *D. lutheri* grown under low light conditions (60 µmol s^{-1} m^{-2}, light:dark 12:12 photoperiod) (Gu et al., 2022). Increased EPA contents under low light conditions have been found also in other microalgal species, such as *Nannochloropsis* sp. and *Phaeodactylum tricornutum*, which has been related to increased thylakoid membrane synthesis under low light to improve photosynthetic efficiency (Conceição et al., 2020; Ma et al., 2016; Mitra et al., 2015). On the other hand, high light intensities have led to increased DHA contents in *D. lutheri* (340 µmol s^{-1} m^{-2}) as well as in other microalgae such as *Tisochrysis lutea* (150–460 µmol s^{-1} m^{-2}) and *Rhodomonas* sp. (600 µmol s^{-1} m^{-2}) (Gao et al., 2021; Guihéneuf et al., 2009; Oostlander et al., 2020; Premaratne et al., 2021; Tzovenis et al., 1997). The accumulation of DHA might be related with the antioxidant activity of this fatty acid, which may prevent photooxidation reactions induced by high irradiance (Tzovenis et al., 1997).

Concerning *T. striata*, the fatty acid profile was mainly composed of palmitic (C16:0), oleic (C18:1*n*-9) and linoleic (18:2*n*-6) acid, which accounted for over 70 % of TFA. Furthermore, minor quantities of SDA (18:4*n*-3) and EPA (20:5*n*-3) were found. This profile has been reported previously for this strain (Schüler et al., 2021; Schulze et al., 2017a) and compares well with other *Tetraselmis* species (Adarme-Vega et al., 2014; Dammak et al., 2021). The highest TFA productivity was found in cells under low-frequency FL of 5 Hz after three days of exposure, reaching 95.9 ± 8.1 mg L^{-1} d^{-1}, which corresponds to a 1.2-fold and 1.5-fold increase as compared to cells under CL and day-0 microalgal cultures, respectively (Fig. 5a). Notably, cells under this treatment maintained these high TFA productivities even after six days of exposure. A significant productivity increase ($p < 0.05$ and $p < 0.01$) in SFA, MUFA and PUFA, due to the increase in the respective fatty acids C16:0, C18:1*n*-9 and C18:2*n*-6, was found both after three and six days of FL 5 treatment (Fig. 5e–g), whereas a significant increase ($p < 0.05$ and $p < 0.001$) in productivity of the PUFAs SDA and EPA was only observed after three days of exposure to FL 5 (Fig. 5h–i). Nevertheless, the highest EPA and SDA contents of 4.10 ± 0.72 and 4.07 ± 0.66 mg g^{-1} DW were achieved by this treatment after six days, respectively (Table S1). This EPA content was higher than contents previously reported for this species, although lower SDA contents were found in this study when compared to results obtained elsewhere (Schüler et al., 2021). Similar increased PUFA productivities under FL 5 have been reported previously for *T. chui* and other microalgae, such as *N. gaditana* and *K. antarctica* (Lima et al., 2021). The increased SFA productivities in *T. striata* may be connected with the stress response to intense light flashes (6000 µmol s^{-1} m^{-2}), since *Tetraselmis* microalgae accumulate lipids mainly composed of SFA under stress conditions (Farahin et al., 2021; Schüler et al., 2021). Furthermore, SFA have been found increased in other microalgal species under high light intensities, such as *Chlorella vulgaris* (300 µmol s^{-1} m^{-2}), *Tetradesmus obliquus* (300 µmol s^{-1} m^{-2}), and *Microchloropsis salina* (850 µmol s^{-1} m^{-2}) (Khoeyi et al., 2012; Nzayisenga et al., 2020; Van Wagenen et al., 2012). Nevertheless, optimal growth conditions, strategies of light acclimatization and biosynthesis of FAs is highly strain-specific (Alishah Aratboni et al., 2019).

4. Conclusions

An algal cultivation strategy of *Diacronema lutheri* and *Tetraselmis striata* using in a first stage growth stimulating light and in the second stage low-frequency flashing light (0.5, 5 Hz) successfully produced not only typical high-light induced compounds such as zeaxanthin, lutein, diatoxanthin or DHA but also the low-light typical compounds EPA or chlorophyll. Therefore, microalgae may sense low frequency FL as both high- and low light conditions. Algae or even land plant producing companies could apply low-frequency flashing light to improve biomass quality and enhance high-value compound productivities by up to four times as compared to using only continuous light.

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CRediT authorship contribution statement

LS drafted the manuscript and analyzed the pigments. JW assisted in the implementation of the experiments and contributed to discussion and critical revision of the article for important intellectual content. HK, HS and CH analyzed fatty acids and assisted in the implementation of the experiments. RR assisted in the implementation of the experiments and sampling. SN and BS revised the manuscript and designed the graphical abstract. JV, VK contributed to the experimental design, conceived the projects funding this work and provided the administrative support for the research project. PS contributed to the experimental design, carried out the experiment and drafted the manuscript. The authors agree with the authorship of this work and give final approval of the version to be submitted and any revised version.

Declaration of competing interest

The authors declare no competing financial interests.

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