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Flashing light emitting diodes (LEDs) induce proteins, polyunsaturated fatty acids and pigments in three microalgae

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ABSTRACT

As the periodic emission of light pulses by light emitting diodes (LEDs) is known to stimulate growth or induce high value biocompounds in microalgae, this flashing light regime was tested on growth and biochemical composition of the microalgae *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui*. At low flashing light frequencies (e.g., 5 and 50 Hz, Duty cycle = 0.05), a strain-dependent growth inhibition and an accumulation of protein, polyunsaturated fatty acids, chlorophyll or carotenoids (lutein, β-carotene, violaxanthin and neoxanthin) was observed. In addition, a 4-day application of low-frequency flashing light to concentrated cultures increased productivities of eicosapentaenoic acid (EPA) and specific carotenoids up to three-fold compared to continuous or high frequency flashing light (500 Hz, Duty cycle = 0.05). Therefore, applying low-frequency flashing light as finishing step in industrial production can increase protein, polyunsaturated fatty acids or pigment contents in biomass, leading to high-value algal products.

1. Introduction

Microalgae are currently exploited by the nutraceutical and aquafeed sectors, and they have emerged as novel bioresources for the production of bioenergy, food and feed, and as supporter of wastewater bioremediation (Richmond and Hu, 2013).

Light sources, both natural and artificial, are used for the photoautotrophic microalgal production (Blanken et al., 2013). However, production systems employing artificial light run into high capital and operational expenditures, which are incurred upon the acquisition of lamps and electric energy consumption, respectively (Blanken et al., 2013). Light emitting diodes (LEDs) have become the currently preferred artificial light source in most phototrophic cultivations because of their durability (lifetime >25-50,000 h) and lack of toxic elements such as mercury, unlike fluorescent lamps. LEDs have also the advantage of being fast-responding diodes emitting nearly mono- or multichromatic light at desired wavelengths, thus being ideal for studies on the light requirements of microalgae for growth and light-dependent induction of specific target metabolites. Phototrophic cultivations benefit from high power conversion efficiencies of LEDs, transforming more than 40–50 % of electrical energy into light that can be effectively utilised by phototrophs for photosynthesis, e.g., photosynthetic active radiation, PAR; (Pattison et al., 2020).

Furthermore, the fast response time of LEDs allows the emission of flashing- or pulsed light, which is the periodic supply of high-intense light flashes alternated by dark periods. Similar to the optimal supply of light with specific wavelengths, flashing light (FL) can be tailored to induce biomolecules of interest, including pigments (Katsuda et al.,

Abbreviations: PAR, photosynthetic active radiation; FL, flashing light; CL, continuous light; DC, duty cycle.

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2008; Kim et al., 2006). However, microalgae respond differently to FL in terms of growth and biochemical composition, while growth was not always improved compared to continuous light (Schulze et al., 2020; Yoshioka et al., 2012). In addition, the prevailing culture growth stage at the time of harvesting is a major factor affecting intracellular biochemical profile, including protein, carbohydrate, lipid or pigment contents in any microalga. This factor was often not considered in statistical analysis of most studies testing effects of environmental factors on algal cultures (Rebolloso Fuentes et al., 2000).

The present study examined the effect of FL on growth and intracellular protein, carbohydrate, lipid, fatty acid and pigment contents of the eustigmatophyte *Nannochloropsis gaditana*, and the chlorophytes *Koliella antarctica* and *Tetraselmis chui*. Although FL conditions may effectively stimulate the biosynthesis of target molecules, they can inhibit microalgal growth (Schulze et al., 2017). Hence, we additionally employed a two-stage cultivation approach: in the first stage growth-stimulating light was applied until the mid-exponential phase, while in the second stage FL was employed to induce the bioaccumulation of compounds.

2. Materials and methods

2.1. Experimental setup

Nannochloropsis gaditana (CCAP 849/5 Scottish Association for Marine Science, Oban, Scotland), *Tetraselmis chui* and *Koliella antarctica* (SAG 1.96, SAG 2030, Culture Collection of Algae at Göttingen University, Germany) were employed in the experiments.

N. gaditana was cultivated at 20 °C, while *T. chui* and *K. antarctica* were maintained at 15 °C. The growth medium contained seawater from the North Atlantic shoreline of Bodø (Norway, 35 ppt) enriched with a modified F-medium consisting of 5.3 mM $NaNO_3$, 0.22 mM $NaH_2PO_4H_2O$, 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 $MnCl_2$ $4H_2O$ with a pH of 7.2.

Tissue culture flasks (Falcon Scientific, Seaton Delaval, UK) with a total volume of 250 mL and 30 mL (light paths: 3.7 and 2.0 cm, respectively) were used for 200- and 25-mL cultures for one-stage or two-stage cultivation systems, respectively. The cultures were mixed by aeration with humidified and 0.2 µm-filtered air enriched with CO_2 (1 % v/v) at a flow rate of 100 mL min^{-1}, resulting in an averaged pH of 7.8. All algae were cultivated during the one-stage batch cultivation for 13 days (biological replicates: $n = 3$). For the two-stage approach, algae were cultivated for six days until reaching the exponential growth phase in a tubular bubble-column reactor with a light path of 3.5 cm (Schulze et al., 2019) using continuous light with a light intensity of I_a= 300 µmol s^{-1} m^{-2} in the PAR range, being defined as the first stage. After six days, the culture was transferred into the 30-mL culture flasks, which were exposed for four days to the FL treatments, being defined as the second stage. Continuous light was used as a control treatment employing the same average light intensity used under FL. Three flashing light (FL) (5, 50 and 500 Hz) and continuous light (CL) were employed for the cultivation of the microalgae in both cultivation approaches; the average light intensity (I_a) was 300 µmol s^{-1} m^{-2} and the flash intensity (I_l) was 6000 µmol s^{-1} m^{-2}, while the duty cycle (DC) employed was 0.05. Under FL 5, the light period (t_l) was 10 ms and the dark period (t_d) was 190 ms; under FL 50 Hz t_l was 1 ms and t_d was 19 ms and under FL 500, the t_l was 0.1 ms and t_d was 0.19 ms.

Optical density at 540 nm (OD_{540}) was measured daily (technical replicates: $n = 4$) for each algal culture using 96-well plates (Tecan Sunrise A-5082, Männedorf, Switzerland). To determine the biomass concentration in the culture in grams of dry weight (DW) per liter, a known volume of algal suspension was filtered using glass fiber filters (pore size ø = 0.7 µm), washed twice with 10 mL ammonium bicarbonate (0.5 M) and dried at 105 °C for 24 h. The dry weight was determined gravimetrically. Significant linear correlations between OD_{540} and dry weight were obtained for each microalga ($r^2 \geq 0.9$, $p < 0.05$).

Culture samples for biochemical analysis were taken at the end of experiments. The harvested cultures were centrifuged (5000 *g*, 5 min), washed (0.5 M ammonium bicarbonate), freeze-dried and stored at −80 °C until further analysis. Furthermore, a known amount of wet biomass was stored at -20 °C for spectrophotometric quantification of total carotenoids and chlorophylls.

2.2. Light source

An array of 36 warm-white LEDs (MHD-G, 2700 K, 12.6 W, Opulent Americas, Raleigh, US; emission spectrum provided in Table S1), mounted on an actively cooled aluminum heat sink, with a total length, height and width of 300, 75 and 40 cm, respectively, was used as light source. The applied light conditions were continuous light and FL with a duty cycle of 0.05 with the frequencies of 5, 50 and 500 Hz (see Table S1 for definition of FL conditions). FL regimes were adjusted by PWM-OCX (RMCybernetics Ltd, Alsager, UK) pulse width modulators (PWMs) powered by bench EA-PS 2084-05B (EA Elektro-Automatik) power supply units. The pulse signal was provided by a TG4001 (TTi, Huntingdon, UK) function generator or directly through the PWMs. For the continuous light control, the LEDs were directly connected to the power supply units. The voltages and currents supplied to the LEDs were regulated to adjust the target I_a of 300 µmol s^{-1} m^{-2} and compensate switching and working losses at the LEDs and PWMs. The supplied light intensity (i.e., PAR) was measured for 1 min at the same position as the flasks (SPQA 5234 spherical quantum sensor connected to a data logger LI-1500, Li-Cor, Lincoln, USA) and averaged over time.

2.3. Analytical methods

2.3.1. Determination of total lipid content

Extraction of lipids followed the Bligh and Dyer (1959) method as previously described (Pereira et al., 2011). Briefly, 10–30 mg of freeze-dried microalga samples were weighed and transferred to glass tubes (V = 15 mL). Afterwards, 0.8 mL of distilled water was added to soften the samples and kept for 20 min. Subsequently, 2 mL methanol and 1 mL chloroform were added and homogenised with a disperser IKA T18 Ultra Turrax (IKA®-Werke GmbH & Co. KG, Staufen, Germany) in an ice bath for 60 s. Then, 1 mL of chloroform was added, and samples were homogenised for 30 s, followed by the addition of 1 mL of distilled water and a 30 s-homogenization. Phase separation was performed by centrifuging at 2000 g for 10 min. Subsequently, 1 mL of the organic phase was transferred into a new pre-weighed tube. The chloroform was evaporated at 60 °C in a dry bath overnight and cooled down to room temperature in a desiccator. Finally, the remaining lipids in the tube were determined gravimetrically.

2.3.2. Determination of protein content

Protein contents of the algal biomass was determined with a Bio-Rad DCTM Protein Assay (Bio-Rad Ltd., Hemel Hempstead, UK). Water-soluble proteins from freeze-dried biomass were extracted by re-suspending ~7 mg freeze-dried biomass in 4 mL NaOH (1 M) and bead milled (0.1 mm) using three cycles of 60 s (Precellys Evolution, Bertin technologies, Montigny-le-Bretonneux, France). The samples containing the glass beads were centrifuged (2000 *g*, 10 min, 20 °C) and the supernatants were transferred into clean vials. The water-soluble proteins contained in the supernatant were measured according to Bio-Rad DC Protein Assay manual, at a wavelength of 750 nm (Dr3900, Hach Lange, Salford Quays, UK). An elemental analysis of the biomass for carbon (C), hydrogen (H) and nitrogen (N) was conducted (Vario EL iii, Elementar Analysensysteme GmbH, Langenselbold, Germany) to confirm the protein contents obtained by the Bio-Rad DCTM Protein Assay by multiplying the nitrogen content in the biomass (%N) by 4.78.

2.3.3. Determination of total carbohydrate content

Total carbohydrate content was determined according to Trevelyan et al. (1952). Briefly, 10 mg of freeze-dried biomass were suspended in 3 mL HCl 37 % (v/v) and hydrolysed in a water bath for 1 h at 100 °C. Subsequently, 4 mL of a fresh anthrone solution (Sigma-Aldrich, Norway, 2 mg mL-1 in 99 % H_2SO_4) were added to 1 mL of sample extract. The absorbance of each sample was read at 620 nm (Dr3900, Hach Lange, Salford Quays, UK). Aliquots of different glucose concentrations (0.02-0.1 mg L^{-1}) were prepared and processed in the same way as microalgal extracts, to obtain a calibration curve.

2.3.4. Fatty acid analysis

Fatty acids were analysed according to earlier studies (Schulze et al., 2019) with some modification. Briefly, freeze-dried microalgal biomass (6 mg) was extracted with 4 mL of a chloroform:methanol solution (2:2.5 v/v) containing an internal standard (tripentadecanoin, C15:0). Cells were disrupted by bead milling with 0.1 mm glass beads (Precellys Evolution, Bertin technologies, Montigny-le-Bretonneux, France). A tris-buffer (2.5 mL) (6 g L^{-1} Tris, 58 g L^{-1} NaCl) was added to the samples prior to mixing and centrifugation (2000 *g*) to separate the phases. The lower chloroform-phase containing the lipids was transferred into a new glass tube and evaporated under a gentle nitrogen flow to prevent fatty acid oxidation. Transesterification of the lipids was done with a methanol solution (3 mL) containing 5 % H_2SO_4 (3 h at 70 °C). Hexane (3 mL) was added and subsequently mixed for 15 min in an orbital shaker. Then, fatty acid methyl esters (FAMEs) in the hexane phase were analysed using gas chromatography equipped with a Flame Ionisation Detector and a CP-Wax 52 CB column (Agilent, Santa Clara, US) using split-less mode. To identify and quantify the most common FAMEs, an external 37-component standard (Supelco, Bellefonte, US) was used.

2.3.5. Spectrometric pigment analysis

For the chlorophyll and total carotenoid extraction, biomass was disrupted in methanol by bead milling with 0.1 mm glass beads for three cycles of 20 s at 6000 rpm (Precellys Evolution, Bertin technologies, Montigny-le-Bretonneux, France) and subsequently kept in ice and darkness for 2 h. The methanol extract was separated from the algal pellet via centrifugation and 1-mL aliquot of the supernatant was transferred into a micro-cuvette (1 mL, Polystyrene cuvettes VWR, Norway) and the spectrum from 400 to 700 nm was recorded by means of a Uviline 9400 (Schott, Mainz, Germany) spectrophotometer. All analyses were done under dimmed light. Chlorophyll *a* (C_a) and *b* (C_b) and total carotenoids (C_{carot}) were determined according to the method described by Lichtenthaler and Wellburn (1983) by applying the OD measurements at 666, 653 and 470 nm (A_{666}, A_{653}, A_{470}) from the methanol extracts to equations **1**, **2** and **3**:

$$C_a = 15.65\ A_{666}\text{-}7.34\ A_{653} \quad (1)$$

$$C_b = 27.05\ A_{653}\text{-}11.21\ A_{666} \quad (2)$$

$$C_{carot} = (1000\ A_{470}\text{-}2.86\ C_a\text{-}129.2\ C_b)/221 \quad (3)$$

Because species belonging to the *Nannochloropsis* genus do not contain chlorophyll *b*, a modified formula according to Henriques et al. (2007) was used (equations **4** and **5**):

$$C_a = 15.65\ A_{666} \quad (4)$$

$$C_{carot} = (1000\ A_{470}\text{-}44.76\ A_{666})/221 \quad (5)$$

Results obtained from Eqs. 1–5 in $\mu g_{pigments}\ mL^{-1}$ were divided by the sample concentration (1 gDW L^{-1}) to obtain the pigment concentration in terms of algal biomass ($\mu g_{pigment}\ gDW^{-1}$).

2.3.6. HPLC pigment analysis

Single carotenoids (lutein, β-carotene, violaxanthin and neoxanthin) were analysed according to Schüler et al. (2020). Briefly, ~3 mg of freeze-dried biomass was disrupted together with 50 µL deionised water, 3 mL acetone and 0.7 g glass beads (0.1 mm) by vortex mixing for 2 min (Vortex Mixer, Stuart, UK). The extraction with acetone was repeated twice. The samples were evaporated and resuspended in methanol prior to HPLC injection. Carotenoid extracts were analysed with a Dionex 580 HPLC System (DIONEX Corporation, Sunnyvale, US) equipped with a photodiode-array detector and column oven set to 20 °C. Separation of the compounds was achieved using a LiChroCART RP-18 (5 µm, 250 × 4 mm, LiChrospher, Merck KGaA, Germany) column with a mobile phase consisting of acetonitrile:water (9:1; v/v) as solvent A and ethyl acetate as solvent B and a constant flow of 1 mL min-1. The gradient program applied was: (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. Method adapted from Couso et al. (2012). The injection volume was 100 µL; chromatograms were recorded at 450 nm. Quantification of lutein, β-carotene, violaxanthin and neoxanthin were performed through calibration curves of pigment standards (Sigma-Aldrich, Sintra, Portugal).

2.4. Data analysis

Growth parameters were estimated according to Ruiz et al. (2013) and are detailed in Table S1 (supplementary material).

ANCOVA analysis were performed to detect differences in intracellular biochemical contents among treatments, strains and cultivation approaches (one- or two-stage) while considering the co-variate biomass concentration at the time point of harvesting (X_t in g DW L^{-1}) as indicator for the prevailing growth stage (Schulze et al., 2019). Pearson's correlations (*r*) were used to quantify biomass growth-stage effects. A significance level (α) of 0.05 was used for all tests. Normality of the response variables was tested using the Shapiro-Wilk test. ANOVA was used to detect differences in productivity data using the explanatory variables strain and treatment.

Biomass productivities or biochemical contents data were normalised to the control treatment (continuous light). Biomass concentration data were log10-transformed to meet the assumption of linearity and homogeneity of variance. The Type III sum of squares analysis was considered and the output *F*-values together with *p*-values were used to describe the impact of treatment, biomass concentration, strain or cultivation approach on the response variables (biomass productivity, protein, carbohydrates, lipids, fatty acids or pigments). The adjusted means with 95 % confidence interval from Tukey's post hoc tests were used to illustrate the differences among the treatments identified by the ANCOVA and ANOVA analysis in the figures. Notably, the adjusted means represent the differences among treatments while taking into account effects by different species, cultivation approaches or biomass concentration effects at the timepoint of harvesting on the response variables (e.g., lipids, proteins, carbohydrates or pigments).

3. Results and discussion

3.1. Growth

Microalgal cultures that were grown under the highest flashing light (FL) frequency ($f = 500$ Hz; FL 500) reached similar growth as those under continuous light (CL) in the one-stage setup (Fig. 1a, c and e) and the two-stage setup (Fig. 1b, d and f). On the other hand, lower frequencies ($f = 5$ and 50 Hz; FL 5 and FL 50, respectively) often inhibited growth (Fig. 1; Table 1). For example, all tested strains exposed to FL 5 and FL 50 displayed in the one-stage cultivation approach lower biomass productivity and a prolonged lag phase compared to FL 500 and CL. Relative to cultures exposed to CL, the biomass productivity of *T. chui* cultures exposed to FL 50 was reduced by 30 %, while *N. gaditana* and *K. antarctica* displayed a 2-fold decrease (Fig. 1, Table 1).

In the two-stage cultivation approach, all strains exposed to FL 5 consistently presented lower biomass productivities compared to control cultures under CL. The FL 50 treatment decreased biomass productivity

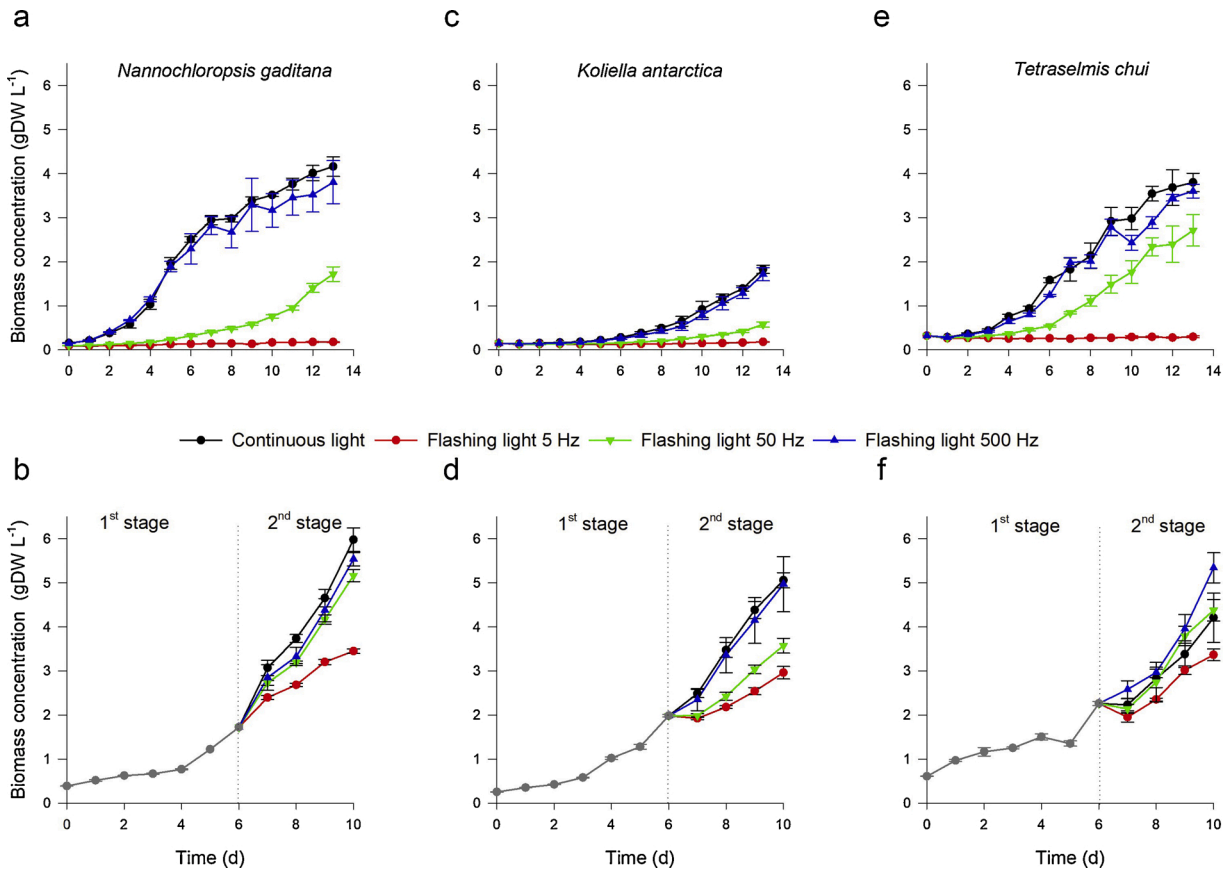

Fig. 1. Biomass (dry weight; DW) evolution of *Nannochloropsis gaditana* (a, b), *Koliella antarctica* (c, d) and *Tetraselmis chui* (e, f). In the one- and two-stage cultivation approaches the microalgae were exposed to flashing light with a duty cycle of 0.05 and frequencies of 5, 50, 500 Hz and continuous light (control). 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 shown as mean ± SD ($n = 3$).

Table 1
Biomass productivities (g dry weight (DW) L^{-1} d^{-1}) of *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui* exposed to flashing light. The tested frequencies of the flashing light were 5 (FL 5), 50 (FL 50) and 500 (FL 500) Hz, at a duty cycle of 0.05. The microalgae grown under continuous light (CL) served as the control in both the one- or two-stage cultivation approaches. Productivities that do not share the same letter for a given alga and cultivation approach are significantly different from each other. The values are means ± standard deviation, $n = 3$.

Condition		*Nannochloropsis gaditana* [g DW L^{-1} d^{-1}]	*Koliella antarctica* [g DW L^{-1} d^{-1}]	*Tetraselmis chui* [g DW L^{-1} d^{-1}]
One-stage cultivation	CL	0.361 ± 0.003^{a}	0.117 ± 0.021^{a}	0.255 ± 0.022^{a}
	FL 5	0.008 ± 0.000^{c}	0.042 ± 0.025^{b}	0.000 ± 0.000^{c}
	FL 50	0.085 ± 0.009^{b}	0.032 ± 0.004^{b}	0.183 ± 0.031^{b}
	FL 500	0.329 ± 0.046^{a}	0.106 ± 0.013^{a}	0.246 ± 0.024^{a}
Two-stage cultivation	CL	0.686 ± 0.056^{a}	0.588 ± 0.024^{a}	0.510 ± 0.029^{a}
	FL 5	0.431 ± 0.012^{b}	0.340 ± 0.012^{b}	0.393 ± 0.002^{b}
	FL 50	0.602 ± 0.016^{a}	0.407 ± 0.023^{b}	0.531 ± 0.029^{a}
	FL 500	0.653 ± 0.035^{a}	0.636 ± 0.069^{a}	0.523 ± 0.032^{a}

of *K. antarctica* by 30 % compared to that of cells under CL (0.41 vs. 0.59 g L^{-1} d^{-1}, $p < 0.05$), whereas *N. gaditana* and *T. chui* cultures were not significantly affected (Table 1). Notably, all strains cultured using the two-stage approach and exposed to FL 5 reached considerably higher biomass productivity (0.34-0.43 g L^{-1} d^{-1}) as compared to cultures cultivated under the one-stage mode (<0.05 g L^{-1} d^{-1}). Likewise, all microalgae showed better growth under the two-stage mode under non-inhibiting FL 500 and CL conditions. Such results can be attributed to the shorter light path of the cultivation flasks used during the second stage of the two-stage cultivation approach compared to the one-stage cultivation approach (2.0 cm vs. 3.7 cm). As explained by the Beer-Lambert law (Lee, 1999), microalgal cultures grow better as the light path decreases, because the light can be better delivered to single cells in dense cultures, increasing productivity and maximal concentration of biomass.

Previous studies conducted in algae, e.g., *Dunaliella salina*, *Nannochloropsis salina* (Combe et al., 2015; Simionato et al., 2013a), indicated that if the FL frequency is high, the photosynthetic apparatus cannot distinguish between single FL pulses and CL, a condition referred to as the "flashing light effect" (Schulze et al., 2017). In the present study, we could not detect a significant growth enhancement in any of the tested strains and only similar productivities were observed for FL 500 and CL treatments. However, our results indicate strain-specific responses to the one-stage cultivation. For example, *T. chui* coped better with low-frequency FL (e.g., FL 50) as compared to *K. antarctica* or *N. gaditana* (Fig. 1, Table 1).

In regard to the cell size and architecture, *T. chui* cells have an oval-shape (13 × 5 × 4 µm) (Bottino et al., 1978) and an estimated volume of about 260 μm^3, making them larger than the cells of the other strains

tested. Specifically, *N. gaditana* is round-shaped, with a diameter of 2−4 μm (Rocha et al., 2003) and a volume of 40 μm^3, and *K. antarctica* has a cylindrical shape with an average size of 7.5 × 2.5 μm (La Rocca et al., 2009) and a volume of 60 μm^3. Considering that light absorbing characteristics of the cell will determine the light available for photosynthesis (Dubinsky et al., 1986), larger cells may withstand better low-frequency FL conditions than smaller cells, because they are less susceptible to photoinhibition (Key et al., 2010), and cope better with long-lasting high-light intensities applied at low FL frequencies (e.g., FL 5).

Under two-stage cultivation conditions, cells were less inhibited by the exposure to low-frequency FL as compared to those grown in the one-stage approach (Fig. 1, Table 1). Notably, the one-stage cultures started at a low initial biomass concentration (0.1 g L^{-1}) and all cells received intense light flashes with the full applied average (I_a) and instantaneous (I_l) light intensities of 300 and 6000 μmol s^{-1} m^{-2}, respectively ($DC = 0.05$). Our study showed that the combination of short duty cycles, low frequencies and high average light intensities is extremely inhibiting for microalgal cultures. As previously mentioned by Xue et al. (2011) and described by the model of Jishi et al. (2015), a decreasing I_a improved light-use efficiency by microalgal cells under FL. Considering that cells exposed to low-frequency FL grew better using a two-stage approach, a lower I_a might have been perceived by cells. Indeed, a higher biomass concentration prior to the application of the FL regime (≈2 g DW L^{-1}) most probably caused a better distribution of light to a higher number of cells in the culture, thus limiting photoinhibition and improving growth as compared to cultures with lower cell counts used in the single stage approach.

3.2. Biochemical composition

3.2.1. Proteins, carbohydrates and lipids

The effect of FL on protein, carbohydrate and total lipid contents were strain-dependent (Fig. 2; Table S1). However, compared to the light treatment effects, these biocompounds were often more significant affected by the co-variable biomass concentrations in the culture as being an indicator for the prevailing growth stage (such as lag, exponential and stationary phases; data provided in Table S1). For example, the protein content in *N. gaditana* was mostly affected by the growth stage ($F = 175.7$, $r_{DW/prot} = -0.96$, $p < 0.01$) but did not differ among the treatments ($F = 1.9$, $p > 0.05$; Fig. 2a; Table S1). Conversely, protein contents in *K. antarctica* were not affected by the growth stage ($F = 0.9$, $p > 0.05$) but differed among the light treatments ($F = 9.1$, $p < 0.01$), being highest in FL 5 and FL 50-treated cultures. Protein contents in *T. chui* were affected by biomass concentration and treatment ($F = 84.5$ and 13.3, respectively; $r_{DW/prot} = -0.84$, $p < 0.01$) with significantly higher values under FL 5 and FL 50 treatments. The carbohydrates (Fig. 2b) in *N. gaditana* and *K. antarctica* were mostly affected by the growth stage ($F = 5.3$ and 6.1, $r_{DW/carb} = -0.61$ and 0.82, respectively; $p < 0.05$) but not by any light treatment ($F < 1$, $p > 0.05$). On the other hand, carbohydrates in *T. chui* were not affected by the growth stage but mainly by the treatments, with 40 % higher contents under CL compared to FL 5 treated cultures ($p < 0.05$). The total lipids in *N. gaditana* were significantly affected by the growth stage ($F = 21.5$, $r_{DW/lip} = 0.78$, $p < 0.01$) and to a lesser extent by the light conditions ($F = 3.7$, $p < 0.05$), where low-frequency FL scored the lowest values (Fig. 2c). Lipids in *K. antarctica* and *T. chui* were neither affected by the growth stage nor by any light treatment ($p > 0.05$).

Generally, the overall protein, carbohydrate and total lipid composition matched those previously reported for *Nannochloropsis*, *Koliella* and *Tetraselmis* strains (Dinesh Kumar et al., 2018; Hulatt et al., 2017; Suzuki et al., 2019). For example, among the tested strains, protein contents were highest in *N. gaditana* (12–49 %) and *K. antarctica* (8–47 %) cultures, while *T. chui* showed significantly lower contents (6–30 %; Table S1). Total carbohydrates were lowest in *N. gaditana* (7–14 %, $p < 0.01$) compared to *K. antarctica* (10–41 %) and *T. chui* (15–45 %). Lastly, total lipids were highest in *N. gaditana* (23–43 %), followed by *K. antarctica* (11–23 %) and *T. chui* (8–17 %, $p < 0.05$).

Previously published studies that examined the effect of FL on the protein, carbohydrate and lipid contents in microalgae reported only minor effects for cultures exposed to low-frequency FL (e.g., $f = 0.5$–5 Hz, $DC = 0.5$, for *Chlamydomonas reinhardtii*; or $f = 51{,}015$ Hz, $DC = 0.5$ for *Scenedesmus obliquus*) (Gris et al., 2014; Kim et al., 2014). In the present study, effects of FL on these compounds seemed to be species dependent. For example, *K. antarctica* exposed to FL 50 doubled its protein contents (from 15 to > 30 %) when compared to cultures under CL (Fig. 2a).

Low solar irradiance at extreme latitudes makes polar strains more responsive to light intensity, which is also connected to dense packaging of pigments and the associated binding proteins. Furthermore, polar microalgae seem to have more ribosomal proteins to counteract cold stress (Lyon and Mock, 2014; Toseland et al., 2013). Thus, high protein levels found in *K. antarctica* cells exposed to low-frequency FL may be explained by their natural adaptation to low temperatures and a more dynamic response to low-light. Unlike *K. antarctica*, the tested *T. chui* strain may not be a true psychrotroph (Schulze et al., 2019), and naturally occurring *T. chui* contains only low amounts of proteins, which could explain the minor effects of FL 5 and 50 treatments on protein contents. However, future work should be carried out to investigate if similar trends also could be observed in other polar strains.

The genera *Nannochloropsis* and *Tetraselmis* are known for their high lipid and carbohydrate contents, respectively, which decrease with increasing protein contents under low-light conditions (Michels et al., 2014; Solovchenko et al., 2011). Such typical low-light adaptation patterns were also found in our tested *N. gaditana* and *T. chui* cultures exposed to FL 5 and FL 50 (Fig. 2), a similar result as reported for other microalgae exposed to low-frequency FL (Grobbelaar et al., 1996). At higher frequencies, protein, carbohydrate and lipid levels of the strains

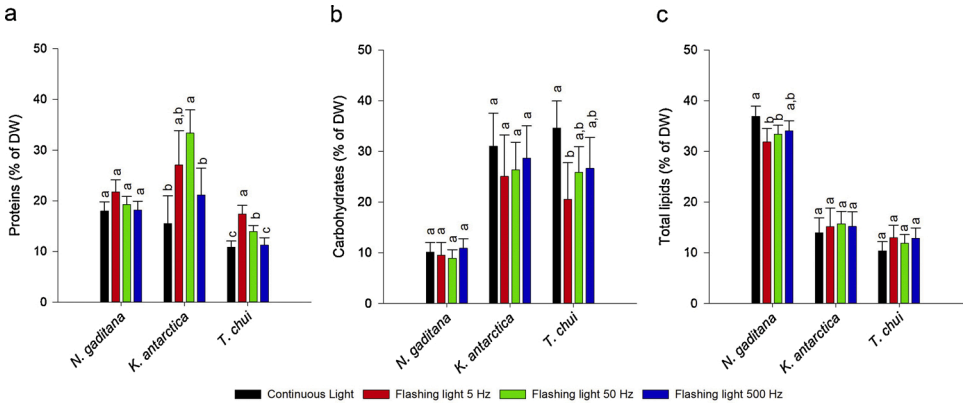

Fig. 2. Biochemical composition of *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui*. The microalgae were exposed to flashing light with a duty cycle of 0.05 or continuous light. Total proteins (a), carbohydrates (b) and lipids (c) are expressed as % in dry weight (DW); shown are the adjusted means ± 95 % confidence interval obtained by Tukey's post hoc test (ANCOVA) from data of the one- and two-stage cultivation ($n = 3$). Treatments that do not share the same letter are significantly different from each other. Original data and statistics about the effects of biomass concentration on proteins, carbohydrates and lipids are available in the supplementary material (Table S1).

were similar to those exposed to CL, indicating no inhibition by high-frequency FL as previously reported (e.g., $f > 100$ Hz) (Yoshioka et al., 2012). In terms of productivity in the two-stage cultivation system (provided in Table S1 supplementary material), the most promising protein producers were *N. gaditana* and *K. antarctica* (average: 75.4 ± 4.3 and 77.8 ± 17.1 mg L^{-1} d^{-1}, respectively). *T. chui* and *K. antarctica* were carbohydrate producers (average: 119.8 ± 49.1 and 137.7 ± 14.2 mg L^{-1} d^{-1}, respectively), while *N. gaditana produced most efficiently*lipids (average: 136.7 ± 46.2 mg L^{-1} d^{-1}). Notably, protein productivities were on average 1.1–1.3 times higher for all microalgae when cultivated under flashing light (FL 5 and FL 50) compared to CL, while carbohydrate and lipid productivity decreased by 20–50 % (Table S1).

Besides the effects of FL, the growth stage effects were considered in the ANCOVA. Protein levels correlated negatively with biomass concentration in *N. gaditana* and *T. chui* ($r = -0.96$ and 0.85, $p < 0.01$). This trend can also be described as a growth stage-dependent protein drop and a strain-specific accumulation of intracellular carbohydrates or lipids at late growth stages (Brown et al., 1996). A primary lipid-accumulating trend could be confirmed for *N. gaditana* from a positive correlation between biomass concentrations and lipid contents ($r = 0.78$, $p < 0.01$), but a negative correlation with carbohydrate contents ($r = -0.61$, $p < 0.05$; Table S1). Indeed, *Nannochloropsis*, is a well-known oleaginous genus, accumulating up to 60 % lipids in dry weight (Rodolfi et al., 2009). In contrast, a carbohydrate accumulating trait can be confirmed for *K. antarctica* cultures; from the positive correlation between biomass and total carbohydrates ($r = 0.8$; $p < 0.05$), while no correlation was found between biomass and lipids ($r = -0.2$, $p > 0.05$). Notably, the present cultivation time may have been too short to observe a lipid build-up as reported earlier for *K. antarctica* cultures reaching late growth stages or subjected to nutrient deprivation (Suzuki et al., 2019). Carbohydrate or lipid accumulating abilities could not be concluded for *T. chui*, as no significant correlation was found between the biomass and these molecules ($p > 0.05$). Nevertheless, a carbohydrate accumulating trend has been previously reported for *Tetraselmis* at the onset of nitrogen depletion, while further nitrogen starvation has led to a lipid accumulation (Gifuni et al., 2018). However, such conditions may not have prevailed in the present experimental setup for *T. chui*, explaining the insignificant correlations.

Taken together, our results suggest that processes regulating the biosynthesis of proteins, carbohydrates and lipids depend on both: the growth stage and the FL conditions. The low-frequency FL treatments may cause a partial low-light response in algae, being evident from the higher protein content in *K. antarctica* and *T. chui* and low content of carbohydrate and lipids in *T. chui* and *N. gaditana*, respectively (Grobbelaar et al., 1996; Yarnold et al., 2016).

3.2.2. Fatty acids

The total fatty acid (TFA; Fig. 3a) content of the tested strains did not differ under any FL treatment compared to exposure to CL. Concerning saturated fatty acids (SFA; Fig. 3b), *N. gaditana* and *K. antarctica* had similar SFA values under the FL and CL treatments, whereas *T. chui* contained less SFA under the FL 50 treatment. Interestingly, the monounsaturated fatty acid (MUFA; Fig. 3c) fraction tended to be lower in the three microalgae under FL 5 and FL 50 compared to those under FL 500 and CL. On the other hand, higher polyunsaturated fatty acid (PUFA; Fig. 3d) contents were accumulated in all species exposed under FL 5 and FL 50 compared to FL 500 and CL. Among all strains, *N. gaditana* showed the highest productivities of fatty acids (104.3 mg TFA, 16.5 mg SFA, 66.1 mg MUFA and 19.7 mg L^{-1} d^{-1}; Table S1 in supplementary material), confirming that this oleaginous species presented the highest lipid contents among the microalgae tested (Fig. 2c).

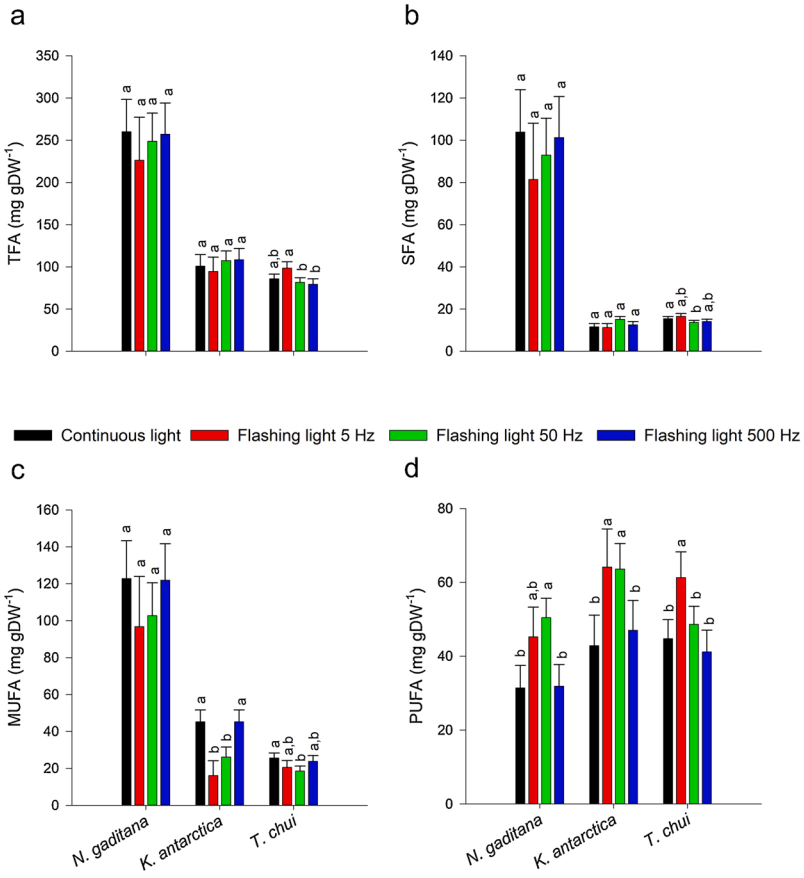

Fig. 3. Major fatty acid classes for *Nannochloropsis gaditana*, *Koliella antarctica* and *Tetraselmis chui*. The microalgae were exposed to flashing light with a duty cycle of 0.05 and continuous light. Contents of total fatty acids, TFA (a), saturated fatty acids, SFA (b), monounsaturated fatty acids, MUFA (c) and polyunsaturated fatty acids, PUFA (d) in dry weight (mg g DW^{-1}) are given as adjusted means ± 95 % confidence interval obtained by Tukey's post hoc test (ANCOVA) from data of the one- and two-stage cultivation ($n = 3$). Treatments that do not share the same letter are significantly different from each other. Original data and statistics about the effects of biomass concentration on major fatty acid classes are available in the supplementary material (Table S1).

The major fatty acids in *N. gaditana* were C16:0 (palmitic acid, 9–40 %), C16:1*n*-7 (palmitoleic acid, 23–40 %) and C20:5*n*-3 (eicosapentaenoic acid; EPA, 6–41 %). In *K. antarctica,* C16:0 (8–13 %), C18:1*n*-9 (oleic acid; 9–43 %), C18:2*n*-6 (linoleic acid; 3–13 %), C18:3*n*-3 (α-linolenic acid; ALA, 10–34 %) and C20:5*n*-3 (4–14 %) were the most abundant fatty acids. Regarding the fatty acid profile of *T. chui,* this microalga mostly had C16:0 (6–16 %), C16:4*n*-3 (hexadecatetraenoic acid; 9–16 %), C18:1*n*-9 (10–23 %), C18:3*n*-3 (11–20 %) and C20:5*n*-3 (9–13 %). The fatty acid profiles obtained are in accordance with those previously reported for the genera *Nannochloropsis* (Hulatt et al., 2017), *Koliella* (Suzuki et al., 2019) and *Tetraselmis* (Lang et al., 2011). The major PUFA (strain-specific, Fig. 4) usually increased under low-frequency FL at the expense of MUFA. For example, *N. gaditana* accumulated more C20:5*n*-3 under FL 5 and FL 50, whereas the MUFA, C16:1*n*-7 and 18:1*n*-9, tended to decrease (Fig. 4a). However, when exposed to FL 500, this microalga showed a fatty acid profile comparable to cells under CL. Similarly, *K. antarctica* increased the major PUFA, C18:3*n*-3 and C20:5*n*-3, at the expense of the MUFA C18:1*n*-9 under FL 5 and 50, compared to CL and FL 500 (Fig. 4b). Lastly, *T. chui* had higher amounts of C16:4*n*-3, C18:3*n*-3 and C18:4*n*-3 (stearidonic acid) and lower C18:1*n*-9 contents upon exposure to FL 5 and FL 50, whereas no effect on C20:5*n*-3 was observed (Fig. 4c, Table S1). Similar species-specific fatty acid shifts under FL have been reported previously. For example, *Chlamydomonas reinhardtii* was not affected by FL ($f = 0.05-5$ Hz, $DC = 0.5$) (Kim et al., 2014), while *Isochrysis galbana* accumulated more phospholipids and docosahexaenoic acid (DHA) when exposed to blue flashing LEDs (e.g., $f = 10$ KHz, $DC = 0.5$) (Yoshioka et al., 2012). Notably, productivities of C20:5*n*-3 increased 1.4-1.9-fold in FL 5 and FL 50 treated *N. gaditana* and *K. antarctica* cultures, while C18:4*n*-3 contents in *T. chui* treated under the same FL regimes was 1.4 times higher compared to cells under CL (Table S1).

Interestingly, we observed a significant correlation between fatty acids and biomass concentration (supplementary material). For example, biomass concentration of *N. gaditana* correlated positively with its TFA, SFA and MUFA ($r = 0.8$ to 0.9, $p < 0.05$). On the other hand, in the case of *T. chui* the corresponding values were negatively correlated ($r = -0.8$ to -0.9, $p < 0.01$). Furthermore, the PUFA contents in all microalgae correlated negatively with their biomass concentration ($r = -0.5$ to -0.8, $p = 0.3$ to 0.001; Table S1). In *N. gaditana,* the major PUFA C20:5*n*-3 decreased with increasing biomass concentration ($r = -0.9$, $p < 0.01$), a stronger effect ($F = 48.9$) compared to the effects of FLl ($F = 5.7$, $p < 0.01$; Fig. 4a). Considering *K. antarctica*, the major PUFA C18:3*n*-3 and C20:5*n*-3 tended to decrease with increasing biomass concentration ($r = -0.8$ to -0.6, $p < 0.5$), an effect that was not as strong ($F = 0.6–8.9$) as that of the FL frequency (Fig. 4b; $F = 9.2–21.5$). In *T. chui*, a correlation between biomass concentration and C18:3*n*-3 ($r = 0.7$, $p < 0.01$) and C18:4*n*-3 ($r = -0.9$, $p < 0.01$) levels was stronger ($F = 26–78$) as compared to that under different FL regimes ($F = 9.2–9.6$; Fig. 4c). Notably, strong effects of the prevailing growth stage on major fatty acids have previously been reported for these genera (Suzuki et al., 2019) and where indeed important when statistically evaluating the effects of FL or any other environmental parameter on microalgae. Compared to the slower growing *K. antarctica*, the fatty acid profiles of *N. gaditana* and *T. chui* (Table S1) were usually more affected by the biomass concentration than the FL treatment. These different responses may be linked to a higher biomass productivity and faster transition from one growth stage to another (e.g., lag, exponential and stationary

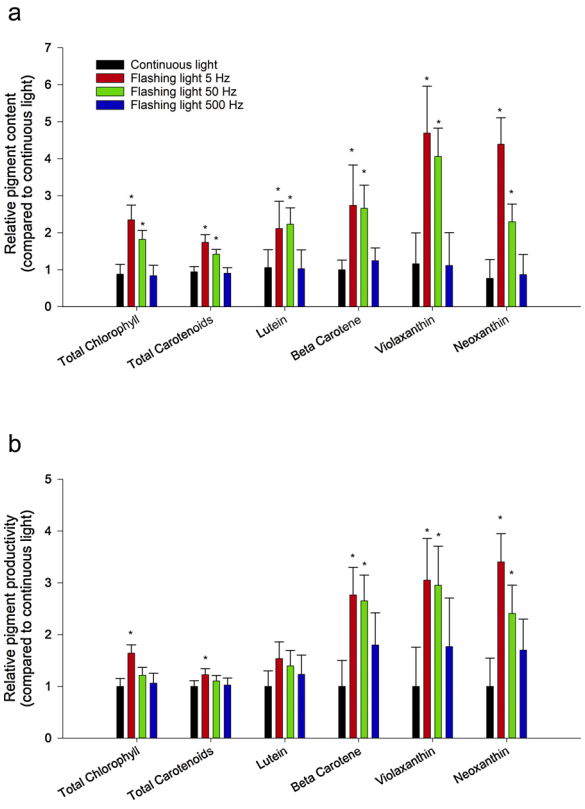

Fig. 5. Relative pigment contents (a) and productivities (b), combined for the three algae exposed to flashing light ($f = 5$, 50, 500 Hz ; $DC = 0.05$). Data for each microalgal species were normalised to results obtained under continuous light (= 1) and given as adjusted means ±95 % confidence interval obtained from Tukey's post hoc test (ANCOVA) from data of the one- and two-stage cultivations ($n = 3$). Asterisks indicate significant differences compared to continuous light. Neoxanthin was only detected in *T. chui* and *K. antarctica*. A detailed pigment profile and statistics about the effects of biomass concentration on pigment contents are available in the supplementary material (Table S1).

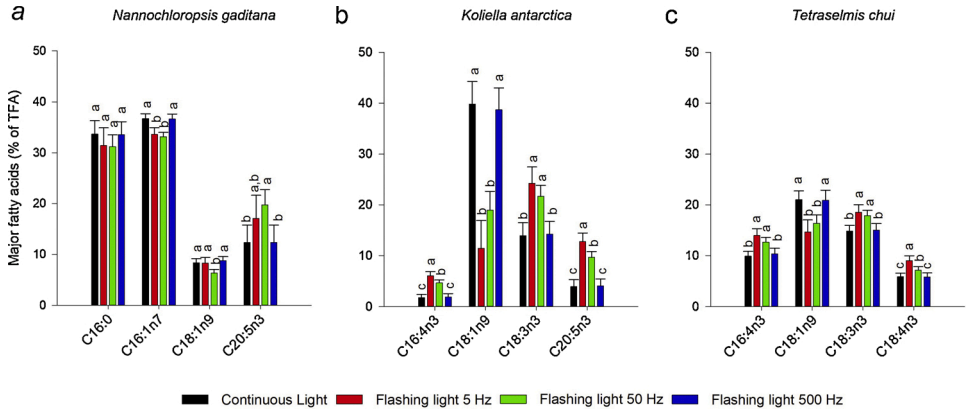

Fig. 4. Major fatty acids of *Nannochloropsis gaditana* (a), *Koliella antarctica* (b) and *Tetraselmis chui* (c) exposed to flashing light and continuous light. Data given are adjusted means ±95 % confidence interval obtained from Tukey's post hoc test (ANCOVA) from data of the one- and two-stage cultivation ($n = 3$). Treatments that do not share the same letter are significantly different to each other. A detailed fatty acid profile and statistics about the effects of biomass concentration on fatty acid profiles are available in the supplementary material (Table S1). Abbreviations: C16:0, palmitic acid; C16:1*n*7, palmitoleic acid; C18:1*n*9, oleic acid; C20:5*n*3, eicosapentaenoic acid; C16:4*n*3, hexadecatetraenoic acid; C18:3*n*3, α-linolenic acid; C18:4*n*3, stearidonic acid.

phases) (Fig. 1, Table 1). Taken together, these results indicate that the prevailing growth stage had the strongest effect on the fatty acid profiles of a given microalga (Table S1) and that low-frequency FL induced PUFA in total fatty acids, an effect similar to responses to low-light in microalgae (He et al., 2015; Schüler et al., 2017).

3.2.3. Pigments

The contents of most pigments were significantly higher in all microalgae exposed to FL 5 and FL 50 compared to those under CL and FL 500 ($p < 0.05$; Fig. 5a). The carotenoids β-carotene, violaxanthin in all microalgae and neoxanthin in *T. chui* and *K. antarctica* exposed to FL were on average 3–4 times higher compared to cultures under CL. Total chlorophyll and total carotenoids as well as lutein increased moderately by 1.7–2.3 times in FL 5 compared to CL treatments (Fig. 5a). Interestingly, the productivity of neoxanthin, violaxanthin and β-carotene were on average two to three times higher in all microalgae under low-frequency flashing light (FL 5 and FL 50) compared to cultures under CL or FL 500 (Fig. 5b). However, productivities of total carotenoids, chlorophyll and lutein were only slightly enhanced (1.1–1.6 times higher) under FL 5 and FL 50 compared to cells exposed to CL.

Intracellular contents of total chlorophyll and carotenoids in the tested strains ranged from 1.8 to 13.8 mg g DW^{-1} and 0.7–7.4 mg g DW^{-1}, respectively. The major carotenoid in *N. gaditana* was violaxanthin (0.24–2.07 mg g DW^{-1}), in *K. antarctica* lutein (0.27–2.05 mg g DW^{-1}) and in *T. chui* lutein (0.29–2.2 mg g DW^{-1}) and β-carotene (0.40–1.25 mg g DW^{-1}, Table S1), which is in accordance with what has been described for these species (Ahmed et al., 2014; Fogliano et al., 2010; Simionato et al., 2013b). Under FL 5, *N. gaditana* was most productive in terms of chlorophyll, total carotenoids and violaxanthin (2.97, 1.34 and 0.45 mg L^{-1} d^{-1}, respectively), whereas *T. chui* was efficient in producing chlorophyll, neoxanthin, lutein and β-carotene (2.97, 0.04, 0.47 and 0.35 mg L^{-1} d^{-1}, respectively; Table S1). On the other hand, *K. antarctica* did not reach any of these productivities. Similarly, previous studies have reported that low-frequency FL (e.g., $f < 30$ Hz) can induce pigments and improve their production, for example, in *Chlamydomonas reinhardtii* ($f = 0.00138-1$ Hz, $DC = 0.5$) (Takache et al., 2015) and in *Haematococcus pluvialis* (f=25-200 Hz, DC=0.17-0.77; f=3.49 Hz, $DC = 0.47$) (Katsuda et al., 2008; Kim et al., 2006).

The violaxanthin-antheraxanthin-zeaxanthin (VAZ) cycle is an important regulator for the adaptation to different light intensities. Shifts towards the biosynthesis of zeaxanthin occurs in plants and algae under high-light stress to avoid photodamage of the photosynthetic apparatus (Jahns et al., 2009). Conversely, violaxanthin accumulates under low-light stress and allows efficient light harvesting (Jahns et al., 2009). Therefore, the observed increase in violaxanthin levels with decreasing FL frequencies in this study may be indicating an adaptation of cells to low-light conditions. In addition, microalgae adapted to low-light also increase their number of thylakoids to harvest light more efficiently (Berner et al., 1989). This requires more PUFA containing membranes and light harvesting complexes (LHC), which in turn requires more proteins to bind pigments, as seen in chlorophytes such as *Koliella* and *Tetraselmis* and eustigmatophytes (i.e., *Nannochloropsis*) (Basso et al., 2014; Jahns et al., 2009). Therefore, the concomitant increase in protein (Fig. 2), PUFA (Figs. 3 and 4) and pigment (Fig. 5) contents could be attributed to an increase in photosynthetic units in cells treated with low-frequency FL, which indicates an acclimation of cells to low-light conditions (He et al., 2015; Schüler et al., 2017). Indeed, a low-light adaptation was suggested earlier for microalgae exposed to low-frequency FL (Grobbelaar et al., 1996; Yarnold et al., 2016). However, low-frequency FL also induced higher levels of β-carotene and lutein, pigments that are usually connected to both photoprotection (Mulders et al., 2014) under high-light and accessory light harvesting. This let suggest that photoinhibition may play an additional role for cells exposed to low-frequency FL. Such high-light response may be connected to the high-light flash intensities of, e.g., 6000 μmol s^{-1} m^{-2} during a period of $t_l = 1$ or 10 ms (e.g., $f = 5$, 50 Hz, $DC = 0.05$, $I_a = 300$ μmol s^{-1} m^{-2}). However, for a better understanding of possible high-light responses of microalgal cells exposed to low-frequency FL, we suggest additional studies that would focus on high-light linked pigments such as zeaxanthin (e.g., in *Nannochloropsis*) or diatoxanthin (e.g., in diatoms), including the associated metabolic pathways on regulatory levels, using transcriptomic approaches.

Furthermore, biomass concentration correlated negatively with all the pigments (r= -0.4 to -0.9, $p <$ 0.01-0.7; significance strain-dependent; Table S1). Generally, cells in aging cultures are subjected to nutrient depletion or light limitation, leading to downregulation of their photosynthetic activity and decrease in photosynthetic pigments, e.g. chlorophyll, violaxanthin (Oukarroum, 2016). Our statistical analysis revealed that FL significantly counteracts this effect because higher pigment contents were observed under low-frequency FL even after considering growth stage (or culture maturation) effects. Therefore, we conclude that the long flash duration (e.g., 1−10 ms) and high instantaneous light intensity ($I_l = 6000$ μmol s^{-1} m^{-2}) of FL 5 and FL 50 treatments may still stimulate protein, PUFA or pigment biosynthesis even at advanced growth stages where otherwise proteins and pigments decrease or the fatty acids tend to saturate.

4. Conclusions

Low-frequency FL of 5 Hz and 50 Hz partially inhibited growth of *Nannochloropsis*, *Tetraselmis* and *Koliella* but induced protein, PUFA, chlorophyll, violaxanthin, lutein or β-carotene compared to CL or FL 500 Hz treatments. The productivity of these compounds was up to three times higher under low-frequency FL than under CL when using a two-stage cultivation. Nonetheless, statistics revealed that FL treatments were often less significant than the prevailing growth stage to induce biomolecules, revealing the importance to statistically consider biomass concentration-related effects. Microalgae producing industries could employ low-frequency FL shortly before harvesting to maximize high-value metabolite contents in biomass produced. Among the tested strains, *Nannochloropsis* was most promising for industrial production of pigments, proteins and fatty acids, *Koliella* for carbohydrates and proteins and *Tetraselmis* for carbohydrates.

Author contributions statement

S.L. (serena.lima@unipa.it) and P.S. (peterschulze@greencolab.com) contributed equally to this study. They designed the experiments, performed the laboratory work, analysed protein and carbohydrate contents and drafted the manuscript. P.S. carried out the statistical data as well as the fatty acid analyses and revised the article for intellectual contents. S.L. and L.S. (lmschueler@ualg.pt) analysed the pigment profiles (HPLC) and revised the article for intellectual contents. T.S. (tfsantos@ualg.pt) analysed total lipids and elemental composition (CHN) of biomass. H.P. (hugopereira@greencolab.com), R.R. (ralf.rautenberger@nibio.no) and D.M-S. (daniela.morales-sanchez@uit.no) contributed to the artwork and revised the intellectual content of the manuscript. J.V. (jvarela@ualg.pt) and F.S. (francesca.scargiali@unipa.it) contributed to the critical review of the manuscript and gave administrative support for the project. R.W. (rene.wijffels@wur.nl) and K.V. (kiron.viswanath@nord.no) conceived the main project, contributed to the design, reviewed and edited the article and provided the administrative support for the research project. All authors agreed with the authorship of this work and gave the final approval of the version to be submitted.

Declaration of Competing Interest

The authors declare no competing financial interests.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jbiotec.2020.11.019.

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