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Research Article

EFFECT OF DIFFERENT LIGHT SPECTRA ON GROWTH AND FATTY ACID COMPOSITION IN THE EUSTIGMATOPHYCEAN MICROALGA *TRACHYDISCUS MINUTUS*

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Summary: We examined the effects of illumination with lights of three different spectral characteristics (LED diodes, FLUORA and LUMILUX light tubes) on growth, sequestration of nitrates and phosphates, composition of fatty acids, and long-chain polyunsaturated fatty acid accumulation in the eicosapentaenoic-rich eustigmatophycean microalga, *Trachydiscus minutus*. When using LED diodes, cultures grew fastest, followed by FLUORA and LUMILUX, and this was confirmed by nitrate and phosphate sequestration rates. Whereas there were only slight differences in the overall degree of saturation of fatty acids, individual fatty acid compositions were substantially affected. LUMILUX specifically inhibited eicosapentaenoic acid accumulation compared to LED and FLUORA tubes, by 50 and 40%, respectively. Linoleic and alpha-linolenic acids were three and four times higher, respectively in algae cultivated under LUMILUX than under the other light sources. Consequently, the ω 3:ω 6 polyunsaturated fatty acid ratio was highest in cultures illuminated by LED (3.235) followed by FLUORA (2.790) and LUMILUX (1.099). We concluded that LED diodes were the best source of light for growth, and eicosapentaenoic acid and arachidonic acid productivities, probably due to their continuous emission across the visible light spectrum.

Keywords: Arachidonic acid; eicosapentaenoic acid; light; long chain polyunsaturated fatty acids; *Trachydiscus minutus*.

Abbreviations: ALA – α-linolenic acid; ARA – Arachidonic acid; DHA – Docosahexeaenoic acid; EPA – Eicosapentaenoic acid; FA – fatty acid; LC-PUFA – Long chain PUFA; MLI – Mean light intensity; PUFA – Polyunsaturated fatty acid.

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INTRODUCTION

Polyunsaturated fatty acids (PUFAs) have recently gained interest in the food industry as an important component of so-called functional foods. However, as a consequence of increasing environmental and economic risks associated with PUFA production from sea-fish (Worm et al. 2006; Mahaffey et al. 2008), attempts at biotechnological production using microorganisms have risen. The 2014 global market for microalgal-based omega-3 oils was USD $375.5 million, corresponding to approximately 7,280 metric tons of microalgal biomass. Globally, applications in infant formulae represent the most important end use for microalgal omega-3 oils, followed by dietary supplements, food and beverage, and animal feed (Shanahan 2014). Although long-chain (LC)-PUFAs are primarily present in lipids from a variety (mostly marine) of cryptophyte, dinophyte, rhodophyte, haptophyte or ochrophyte microalgae (Renaud et al. 1999; Lang et al. 2011) and even cyanobacteria (Hu et al. 2011), only a few species have been used for commercial production. Apart from obligatory heterotrophic thraustochytrids (*Schizochytrium* sp.), the marine dinophyte *Crypthecodinium cohnii* has been used for heterotrophic production of docosahexaenoic acid (DHA)-rich oils (Winwood 2013). The production of eicosapentaenoic acid (EPA)-rich (>65%) algal oil was commercialized (product name A2 EPA Pure™) in 2013, using a proprietary strain of the eustigmatophyte *Nannochloropsis* sp., cultivated autotrophically in large seawater open ponds (Aurora algae 2015). A small number of other potential microalgal producers of LC-PUFAs have been investigated in detail, in laboratory or large-scale pilot plants, especially the diatom *Nitzschia laevis* (Wen and Chen 2000, 2001), the haptophyte *Isochrysis galbana* (Fidalgo et al. 1998; Liu et al. 2013) or, most recently, the eustigmatophyte *Trachydiscus minutus* (Řezanka et al. 2010; Cepák et al. 2014).

As well as searching for new production strains, and developing innovative cultivation systems, optimization of cultivation conditions and the identification of environmental factors are of great importance. Recently, a deficiency in nutrients (Řezanka et al. 2010; Řezanka et al. 2011), and effects of various nitrogen forms, temperatures, salinity and light intensities (Cepák et al. 2014) were studied with respect to EPA and other PUFA contents, and productivity in *T. minutus*. Results showed that both low light and temperature substantially increased total cellular PUFA content, including EPA that constituted more than 50% of total fatty acids (FAs). However, under these conditions productivities were markedly reduced due to growth restrictions (Cepák et al. 2014).

As light seems to be one of the major factors influencing FA composition, we focused on the spectral quality of light as a possible factor in the further optimization of LC-PUFA content in *T. minutus* without limitations in growth (and therefore productivity).

Previously, information was not available on the effects of light spectral quality on growth and LC-PUFA accumulation in any relevant microalgal producer, including *T. minutus*. Here, we compared the effects of light sources

with three different spectra (LED diodes and two types of light tubes) on growth and FA composition in this microalga.

MATERIAL AND METHODS

Organism and preparation of inoculum

Trachydiscus minutus CCALA 838 was obtained from the Culture Collection of Autotrophic Organisms (CCALA, http://ccala.butbn.cas.cz/index.php). It was maintained on agar slants (2% in Zehnder medium, Staub 1961) at a light intensity of 23 µmol m^{-2} s^{-1} of photosynthetic active radiation (PAR) and at a temperature of 12ºC. Light intensity was measured using a digital lux-meter PU 550 (Metra, Blansko, Czech Republic) equipped with a PAR sensor, calibrated according to LI-185 quantum sensor (LI-COR, Lincoln, USA). To prepare an inoculum for cultivation experiments, cells were transferred from an agar slant to a glass cylinder (diameter 4 cm) containing 100 ml of 1/2 SŠ medium (Zachleder & Šetlík 1982). Algae were grown for ten days at a temperature of 28°C, and under increasing irradiance up to 460 µmol m^{-2} s^{-1} PAR provided by white fluorescent FLUORA tubes L 36W/77 (OSRAM).

Cultivation experiments

Stock cultures were inoculated into planar-parallel glass vessels (the light path within the suspension was 4 cm) filled with 6.5 l of 1/2 SŠ medium, of pH 7.4. The initial biomass was 0.127 g l^{-1} of DW. A temperature of 28 ± 0.5ºC was maintained using a thermostatic water bath and cultures were aerated by air enriched with 2% CO_2 (v/v). Cultivations were carried out under continuous light for 14 days. To fully adapt the cultures to high-light conditions, the incident light intensity was set at 230 µmol m^{-2} s^{-1} PAR at the onset of the experiment; after 3 days, the light intensity was increased to 300 µmol m^{-2} s^{-1} PAR, and after 6 days, to 460 µmol m^{-2} s^{-1} PAR until the end of experiment. We used three different light sources, (1) a panel with four LED diodes (LED chip XM - L2 Cool white, Dioptra Turnov, Czech Republic) (2) a panel of FLUORA T8 light tubes (Tubular fluorescent lamp, L 36W/77, Osram, Germany) and (3) a panel of LUMILUX T8 light tubes (Tubular fluorescent lamp, L 36 W/830, Osram, Germany). The spectra of light sources were measured using an optical probe and USB 2000 spectrometer (Ocean Optics, USA). The spectral specifications of the light sources are given in Fig. 1. The mean light intensity (MLI) was calculated as follows: $MLI = (I_i - I_t)/\ln(I_i / I_t)$, where I_i is the incident light intensity and I_t the transparent light intensity.

Growth estimations

Dry weight was determined as follows: culture aliquots (1-2 ml) were centrifuged in pre-weighed microtubes at 2 000 g for 10 min. and the sediment was dried at 105ºC for 12 h. Samples were weighed on an analytical weights Mettler Toledo balance (XS 205 DualRange, Germany). Cell number was quantified using a Bürker counting chamber (Assistent, Sondheim, Germany). At least 500 cells were counted for each sample. Chlorophyll *a* was determined as follows: 10 ml of algal sample were spun down for 10 min (2000 g). The supernatant was decanted and to the sediment was added

0.5 ml of glass beads (diameter 0.14-0.39 mm) and 0.5 ml of phosphate buffer (pH 7.6-7.8). Cells were disrupted on a Vortex vibrator blender (4 × 1 min.) and immediately mixed with 4 ml of 100% acetone, briefly shaken and centrifuged. The supernatant was decanted into 10 ml graduated test tubes. Then 5 ml of 80% acetone were added to the sediment, shaken, centrifuged and pooled together with the previous extract and topped up to 10 ml with 80% acetone. The absorbance of the acetone extract was measured using a UV-1650PC spectrophotometer (Shimadzu, Japan) and the chlorophyll *a* content was calculated using the following equation: chl. *a* (mg l^{-1}) = 12.7 $D_{663\ nm}$ – 2.69 $D_{645\ nm}$, where D_{663} and $D_{645\ nm}$ represent absorbance values at 663 and 645 nm, respectively (Arnon 1949).

Analyses of nutrients

After centrifugation of a culture sample (10 min, 2000 g), nutrient uptake from the growth medium was analyzed in the supernatant. Phosphorus (as dissolved reactive phosphorus – PO_4-P) and nitrogen (as nitrate – NO_3-N) were determined using a Flow Injection Analyser (FIA, TECATOR, Sweden; Ruzicka and Hansen 1988). PO_4-P was analyzed by reaction with ammonium molybdate and reduction by stannous chloride to phosphomolybdenum blue (Proctor and Hood 1954). NO_3-N was analyzed by reaction with sulphonamide (Paasonen-Kivekas et al. 1999). Determination of the sum of soluble nitrate and nitrite was carried out over the range 50-100 mg L^{-1}.

Fatty acids analysis

For FA analysis, biomass was lyophilized using a Heto PowerDry PL3000 freeze dryer (Thermo Scientific, Waltham, MA, United States) for 3 days at –57ºC and 0.022 kPa. Lyophilized samples were extracted with 2–5ml of dichloromethane:methanol (1:1, v/v) by sonication, centrifugation and evaporation in a reaction vial. After saponification (0.05 M NaOH in methanol at 80°C for 30 min) and methylation (14% BF_3 in methanol was used as a catalyst), analysis of FA methyl esters (FAME) was performed using an HP 6890 Series gas chromatograph with an SP-2560 (100×0.25 mm; 0.20 μm), polar column, followed by flame-ionization detection (Hewlett-Packard, USA). The detailed procedure was described by Cepák et al. (2014).

Statistical analysis

Cultivation experiments and all growth and biochemical analyses were carried out in duplicate. Statistically significant differences between treatments were computed by ANOVA and F test using Sigma Plot 11.0 (Systat Software, USA).

RESULTS AND DISCUSSION

Recently, several studies on fatty acid composition in *Trachydiscus minutus* have shown that this microalga is rich in LC-PUFAs, especially EPA and arachidonic acid (ARA) (Iliev et al. 2010; Řezanka et al. 2010; Cepák et al. 2014). In this work, we have studied the effects of light sources (LED, FLUORA, LUMILUX) with three different spectral qualities on growth and the accumulation and composition of fatty acids in *T. minutus*. The spectral profiles of the light sources were clearly different. The LED

were characterised by one slim distinct peak with a maximum intensity at 450 nm, and another, broad lower peak between 500 and 630 nm, with a maximum at approximately 545 nm. These spectral characteristics were similar to those reported by Ho et al. (2014). An emission signal was detectable throughout the entire VIS spectrum (400-700 nm). For FLUORA light tubes, distinct peaks at different wavelengths (435 and 548 nm) were present, whereas the spectrum of LUMILUX light tubes consisted of three distinct peaks (at 433, 550, and 615) and several minor peaks, with intervals displaying no emission signal (Fig. 1).

Apart from effects of monochromatic LED light (continuous or intermittent) on growth and FA composition in *Isochrysis galbana* (Yoshioka et al. 2012), there is no literature available dealing with light quality effects on FAs in microalgae. Recently, growth and lutein production were studied in *Scenedesmus obliquus,* cultivated under different light sources. The best growth performance occurred using TL5 fluorescent lamps, while no significant difference between light sources was found for the maximum lutein content (Ho et al. 2014). As far as we are aware, spectral properties of light on other microalgal growth parameters, biochemistry or physiology have not been studied in Eustigmatophyceae to date.

Cultures of *T. minutus* were simultaneously grown under different lights, as described above, and mean light intensities (MLI) were measured during the experiments (Fig. 2). Within the first three days, MLI dropped sharply from an initial value of 185 µmol m^{-2} s^{-1} to 50 µmol m^{-2} s^{-1}, reflecting growth of cultures. An increase in incident light intensity on days 3 and 6 resulted in a transient increase in MLI, which then steadied around 50-70 µmol m^{-2} s^{-1}. The kinetics of *T. minutus* growth were different depending on the light source (Fig. 3). While cultures under LED grew fastest and reached a final biomass of up to 6.5 g l^{-1} DW after 14 days, growth under FLUORA was slow for 9 days, and only achieved 5.2 g l^{-1} DW after 14 days. Growth under LUMILUX was more heterogeneous, being slow for at least 4 days, but after 14 days reaching almost the same final biomass as cultivation under FLUORA (Fig. 3a). In all variants, growth was slower than in previous experiments with the same strain (Cepák et al. 2014). This was probably due to gradual light adaptation during the

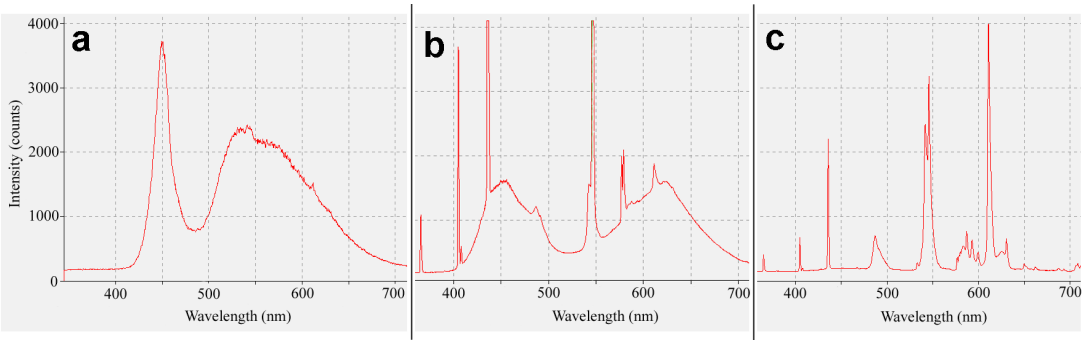

Figure 1. Spectra of light sources used in the growth experiments with *Trachydiscus minutus*. **a.** LED diodes, **b.** FLUORA fluorescent tubes and **c.** LUMILUX fluorescent tubes .

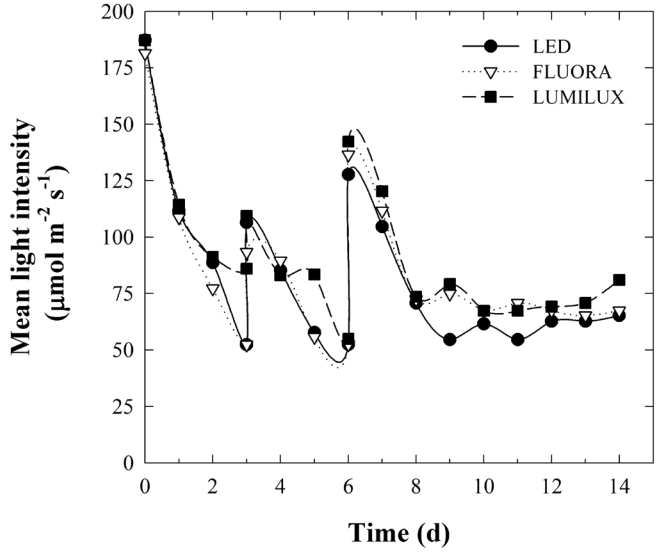

Figure 2. Mean light intensities during experiments with *Trachydiscus minutus* cultivated under different light sources. The initial incident light intensity was 230 µmol m^{-2} s^{-1} PAR and was increased to 300 µmol m^{-2} s^{-1} PAR after 3 days and to 460 µmol m^{-2} s^{-1} PAR after 6 days.

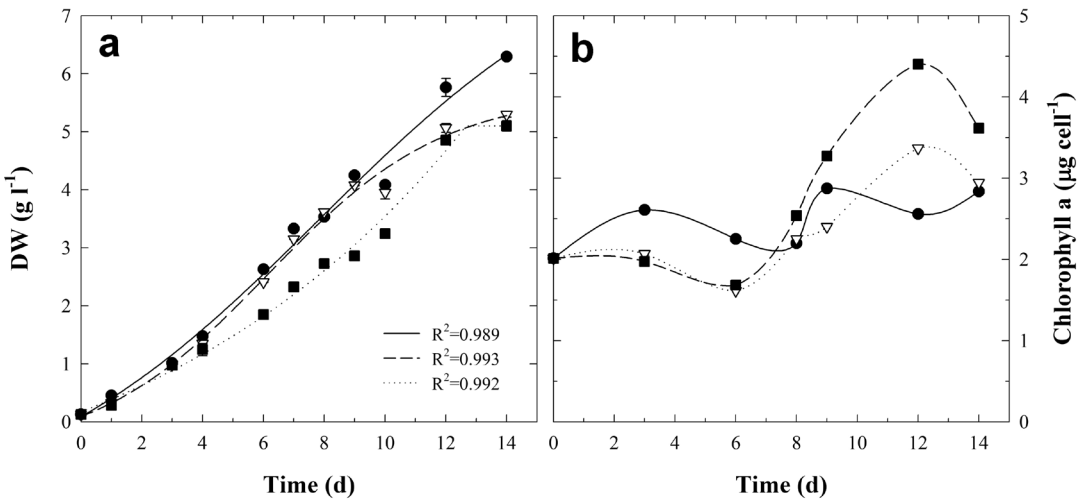

Figure 3. Time course of growth (**a**) and cellular chlorophyll content (**b**) in *Trachydiscus minutus* cultivated under different light sources: LED (filled circles), FLUORA (open triangles), LUMILUX (filled squares).

experiment (see Material and Methods and Fig. 2) resulting in a lower total light supply over the whole cultivation period. In contrast to our results, a study using *Scenedesmus obliquus* reported that a TLP fluorescent lamp surpassed LED with respect to maximum specific growth rates, biomass production, and biomass productivity (Ho et al 2014). Similar growth kinetics as for DW biomass were found when chlorophyll *a* was measured in cultures grown under different light sources (not shown), however differences in cellular chlorophyll content were the reverse, being highest in cells cultivated on LUMILUX (Fig. 3b). This was due to the fact that cellular chlorophyll content is positively correlated with cell size

(compare Fig. 3b and Fig. 4). Generally, cell size increased sharply, by 21-37% (all light variants), within the first 3 days, and then decreased to close to the initial values (FLUORA and LED), or remained constant (LUMILUX), until the end of the experiment (Fig. 4). Sequestration rates of both nitrates and phosphates from the growth medium corresponded to growth rates under the different light sources. Whereas no difference was found between LED and FLUORA lights, regardless of time and ion measured ($P>0.05$), the sequestration rates of both ions were significantly lower ($P<0.01$) under LUMILUX until the 12th day of the experiment. Between days 12 and 14 however, sequestration rates were not different ($P>0.01$) between all light sources (Fig. 5). Sequestration rates of

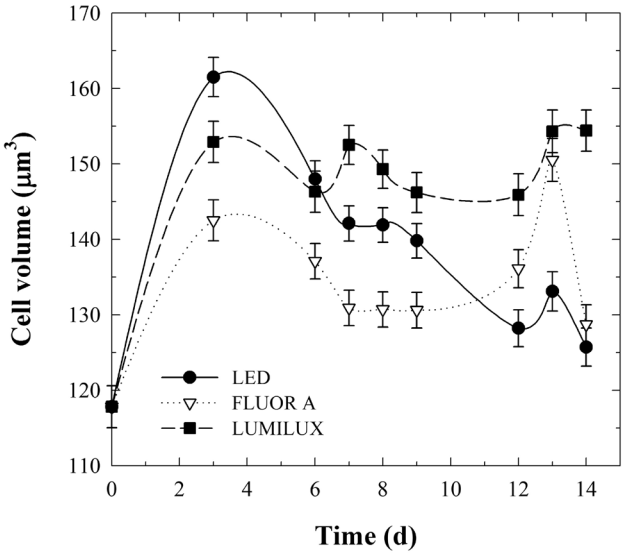

Figure 4. Changes in *Trachydiscus minutus* cell volume during cultivation under different light sources: LED (filled circles), FLUORA (open triangles), LUMILUX (filled squares).

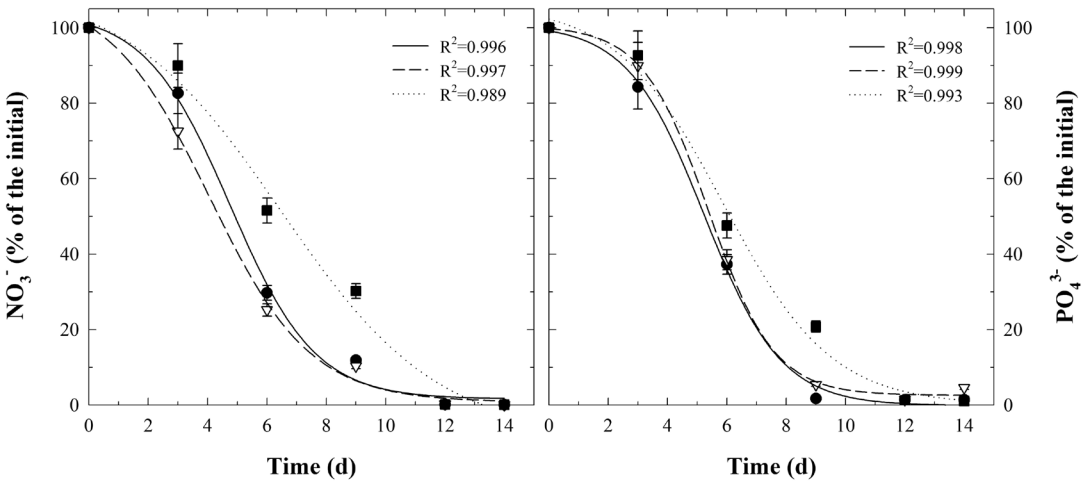

Figure 5. The rate of sequestration of nitrates (left) and phosphates (right) from the culture medium by *Trachydiscus minutus,* cultivated under different light sources: LED (filled circles), FLUORA (open triangles), LUMILUX (filled squares).

both nitrates and phosphates were shown previously in *Chlorella vulgaris,* and their kinetics' were similar to *T. minutus*. However, both nutrients were completely depleted earlier (2-3 days) by *C. vulgaris* (Přibyl et al. 2012) than by *T. minutus*. Differences in depletion rates could be explained by higher growth rate of *C. vulgaris*.

Fatty acid composition was estimated from lyophilized biomass harvested after 14 days of cultivation and is presented in Table 1. The complete FA profile was identified in the inoculum culture (23 individual FA), while in the stationary cultures, several FAs had disappeared (5-8 individual FAs) from the profile, depending on the light source used. It was clear that light spectra had a pronounced effect on the metabolism of some PUFAs.

Table 1. Effect of light sources on the complete FA profile in *Trachydiscus minutus* measured at the onset of the experiment (inoculum) and in the stationary growth phase (after 14 days). Values are % of total FAs. SFA – saturated fatty acids, MUFA – monounsaturated fatty acids, PUFA – polyunsaturated fatty acids.

Fatty acids	Inoculum	LED	FLUORA	LUMILUX
C08:0	0.29	—	0.25	—
C10:0	0.21	—	0.40	—
C12:0	1.11	0.74	1.75	0.77
C14:0	17.82	11.28	16.75	11.59
C14:1	0.20	—	—	—
C15:0	0.20	—	—	—
C16:0	9.89	6.75	8.74	11.32
C16:1	3.69	5.26	3.86	2.32
C17:0	0.20	—	—	—
C18:0	1.23	0.76	0.90	0.87
C18:1n9c +c18:n11c	3.87	3.07	2.05	5.42
C18:2n6c	7.62	7.03	6.89	22.29
C18:2n6t	0.21	—	—	1.78
C18:3n3	2.31	2.71	2.24	8.52
C18:3n6	0.31	0.22	0.37	1.60
C20:0	0.37	—	0.25	—
C20:2	0.25	0.41	0.41	1.10
C20:3n6	0.20	0.48	0.53	—
C20:4n6	6.68	8.77	8.42	5.44
C20:5n3	40.05	50.64	42.97	25.67
C22:0	2.72	1.43	3.22	1.31
C22:2	0.20	—	—	—
C24:0	0.37	0.45	—	—
SFA	34.41	21.41	32.26	25.86
MUFA	7.76	8.33	5.91	7.74
PUFA	57.83	70.26	61.83	66.40
TOTAL	**100**	**100**	**100**	**100**
ω-3/ω-6	2.818	3.235	2.790	1.099

The LED and FLUORA lights induced the accumulation of EPA (C20:5n3), which was the major FA (50.64%, 42.97%, respectively), in contrast to LUMILUX (25.67%), where EPA synthesis was inhibited during cultivation. The linoleic acid (C18:2n6c) content however, was more than 3 times higher in cultures grown under LUMILUX light (22.29%) than under LED or FLUORA lights (7.03%, 6.89%, respectively) and the α-linolenic acid (ALA, C18:3n3) content was almost 4 times higher under LUMILUX light (8.52%) than under LED and FLUORA lights (2.71%, 2.24%, respectively) (Table 1). The suppression of EPA synthesis under LUMILUX can be only partly explained by the putative inhibition of Δ6 desaturase, and thus the accumulation of amino laevulinic acid (ALA), in the conventional Δ6-pathway (Pereira et al. 2003), as shown by a quantitative analysis of ALA and EPA (Table 1). More likely, inhibition of the complete Δ6-pathway took place under LUMILUX treatment. Total PUFA contents were, however, not dramatically influenced by the light source used; a similar situation was also found for total MUFAs. Only the relative amount of total saturated FAs in cultures grown under LED diodes was slightly decreased compared to FLUORA and LUMILUX lights (Fig. 6, Table 1). Hence the overall degree of saturation of FAs was more or less independent of the light source used, unlike light intensity, as is known for the Eustigmatophyceae (Pal et al. 2013; Cepák et al. 2014). However we found distinct differences in ω-FA ratios of PUFAs in response to the light source used. When LED, FLUORA and LUMILUX lights were used, the ω3:ω6 ratios were 3.235, 2.790, and 1.099, respectively (Table 1).

We also analyzed the effects of different light sources on the productivities of commercially important LC-PUFAs, EPA and ARA. Both EPA and ARA productivities significantly increased, in the order LUMILUX < FLUORA < LED. The maximal specific

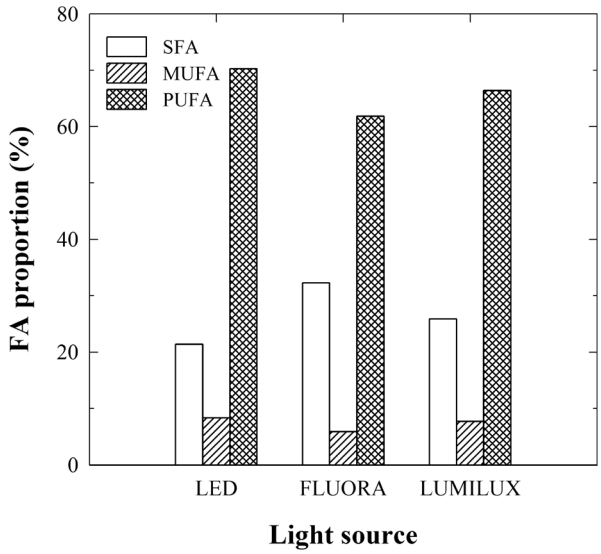

Figure 6. Fatty acid categories after 14 days of *Trachydiscus minutus* cultivation under different light sources.

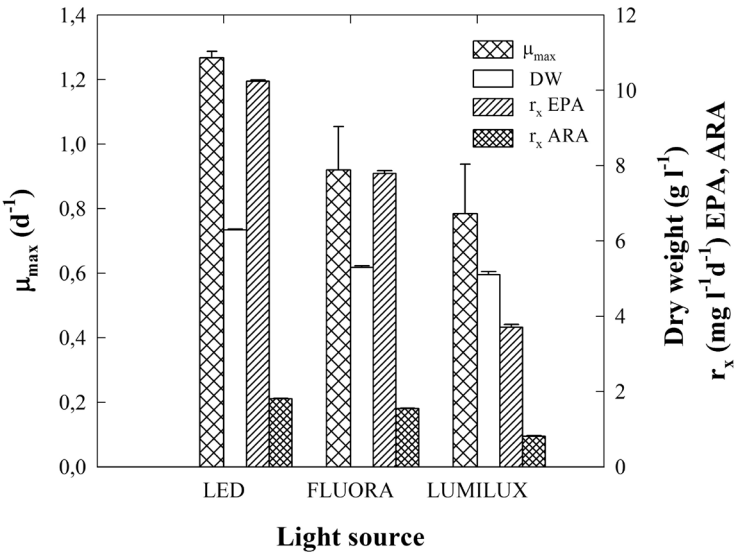

Figure 7. Maximal specific growth rate ($\mu_{max.}$), dry weight (g l^{-1}), and overall EPA and ARA productivities (r_x) at the end of experiments with *Trachydiscus minutus* cultivated under different light sources.

growth rate (μ_{max}) was significantly higher ($P<0.05$) in cultures under LED (over 1.3 d^{-1}) compared to the other light sources, whose values were not significantly different ($P>0.01$) from each other. The same was found for the final biomass DW (Fig. 7). These findings were consistent with the apparent growth stimulation by LEDs (Fig. 3a). Enhanced productivities of both EPA and ARA under LED diodes, compared with FLUORA and LUMILUX, were probably at least partially due to growth rate. Lower EPA and ARA productivities under LUMILUX light compared to FLUORA light were however light source-specific since growth parameters under LUMILUX and FLUORA lights were not different (Fig. 7).

The LED diodes proved to be the best light source in terms of growth and LC-PUFA productivities. An obvious reason for this effect could be that the LED emission spectrum covered the entire visible spectrum (VIS, 400-700 nm), and thus was the ideal of all light sources. The least ideal was LUMILUX lights, where growth was slowest and LC-PUFA synthesis was specifically inhibited. The underlying mechanisms however remain unresolved.

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