EFFECTS OF LIGHT WAVELENGTH AND SALINITY ON GROWTH AND LIPID PRODUCTION IN THE MICROALGA *NEOCHLORIS OLEOABUNDANS*

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INTRODUCTION Over the recent decades microalgae have received much attention as a potential feedstock rich in fatty acids for sustainable biodiesel production [1], [2]. The use of microalgal biomass as feed in aquaculture has also been of great interest; replacing fish meal based feed with microalgal biomass would greatly improve the sustainability of the industry [3]. Compared to conventional crop plants, microalgae exhibit faster growth rates, higher biosynthetic efficiency, higher rates of carbon dioxide fixation as well as higher biomass and lipid accumulation rates [5]. Further, microalgae have simple requirements for their growth environment and they can be cultivated using e.g. waste water of different origins [6] or seawater [7], thus promoting circular bioeconomy and avoiding use of freshwater resources. Microalgae are readily capable of adapting to environmental cues via metabolic changes [8]. It is known that the lipid content and fatty acid composition as well as the expression of pigments, proteins and secondary metabolites in microalgae alter significantly in response to abiotic stress [9],[10]. The subject of our experiments *Neochloris oleoabundans* UTEX 1185 (family Chlorophyceae; from here on *Neochloris*) is a green unicellular, halotolerant freshwater alga. The aim of the current work was to study how a) the chromatic quality of light and b) salinity of the growth medium affect the cell growth, lipid production and fatty acid profile in *Neochloris* cells. RESULTS IN BREIF: It was observed that in blue LED light production of neutral lipids was strongly induced, cells grew bigger and concomitantly reached higher biomass while red light led to higher cell density. Further, both red and blue ligth led to different fatty acid profile in *Neochloris;* blue light could suit for microdiesel production while red light would be beneficial for food/feed and nutraceutical industries. Saline growth medium induced stress and concomitant lipid production, while red light led to higher level of carotenoids and more efficient photoprotection. Based on the obtained results, it is evident that the growth conditions have a great impact on the viability of any application utilising microalgal biomass.

Neochloris grown in freshwater medium under white/red/blue LED light

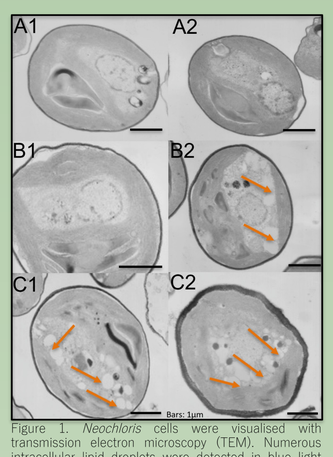

Figure 1. *Neochloris* cells were visualised with transmission electron microscopy (TEM). Numerous intracellular lipid droplets were detected in blue light cells (C1,2). Few droplets were detected in some red light cells (B1,2) while in white light the accumulation of lipids was negligible (A1,2). Lipid droplets are indicated with orange arrows.

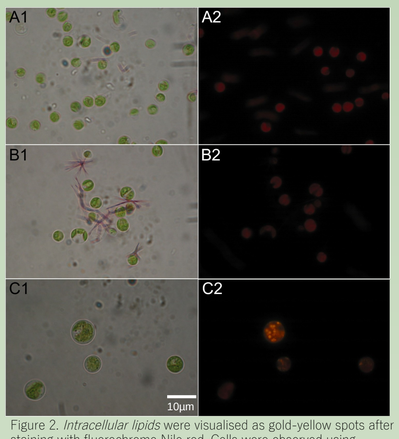

Figure 2. *Intracellular lipids* were visualised as gold-yellow spots after staining with fluorochrome Nile red. Cells were observed using excitation with a 485nm filter. Numerous small lipid droplets were seen in blue light grown cells (C2), while in red (B2) and white light (A2) no lipid droplets could be observed.

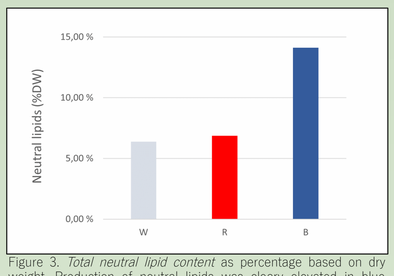

Figure 3. *Total neutral lipid content* as percentage based on dry weight. Production of neutral lipids was cleary elevated in blue light.. W=white light, R= red light, B= blue light.

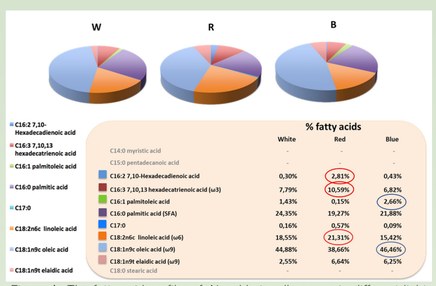

Figure 4. *The fatty acid profiles* of *Neochloris* cells grown in different light conditions were analysed with gas cromatography. The analysis revealed that the chromatic quality of growth light changes the fatty acid profile; blue light led to increase in monounsaturated fatty acids while in red light the level of polyunsaturated fatty acids was elevated.

Accumulation of lipids was significantly pronounced in *Neochloris* cells grown under blue light. Numerous small lipid droplets were observed within majority of the cells with TEM (Fig.1 C1,2) and by Nile red staining (Fig.2 C2). Red light induced the lipid production to some extent and lipid droplets could be seen in a few cells (Fig.1 B2; Fig.2 B2), however, to a much lesser degree than in blue light. In white light lipid accumulation was negligible (Fig.1 A2; Fig.2 A1,2). In accordance, the total neutral lipid content was highest in blue and lowest in white light (Fig. 3). Interestingly, chromatic quality of the growth light had a clear impact also on the fatty acid profile, as was revealed by a gas chromatography analysis (Fig. 4.). The fraction of monounsaturated fatty acids (MUFAs) was increased in blue light, consisting of 55% of total fatty acids vs. 49% and 39% in red light. In particular oleic acid ($\omega 9$) and palmitoleic acid ($\omega 7$) were pronounced. In red light the level of polyunsaturated fatty acids (PUFAs) was increased, being 35% of total fatty acids compared to control (26%) and blue light conditions (23%). In particular levels of 7,10,13-hexadecatrienoic acid ($\omega 3$) and linoleic acid ($\omega 6$) were elevated in red light.

Cell growth was observed as optical density (Fig. 5A), cell density (Fig. 5B) and dry biomass (Fig. 5C). By the end of the experiment highest dry biomass was obtained from blue light cells, while highest cell density was reached in red light. Further, cell size appeared to be larger in blue light (Fig.1 C1,2; Fig.2 C2) in comparison to cells grown in white and red light conditions.

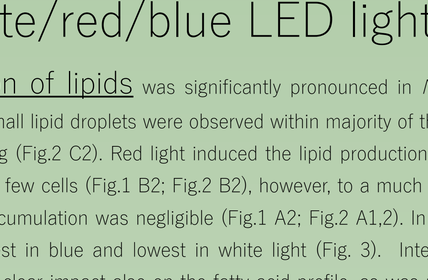

Figure 8. UTEX LED light platforms (red 626nm; blue 470nm) were used as light source for the experiment. Growth conditions: continuous light, 25°C, no CO_2 addition.

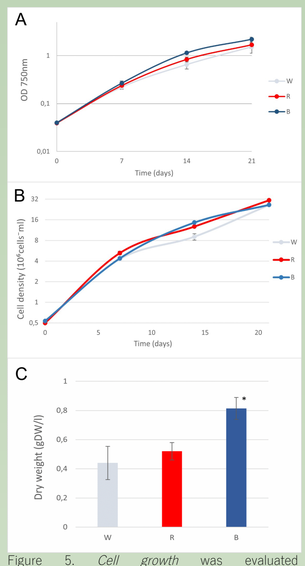

Figure 5. *Cell growth* was evaluated spectrophotometrically (A), by counting cell density (B) and by measuring the dry biomass (C). Highest cell density was achieved in red light while largest dry biomass was obtained in blue light. The asterisk indicates statistical significance ($P<0.05$) W=white light, R= red light, B= blue light.

Photosynthetic performance

of *Neochloris* cells was estimated by measuring the PSII maximum quantum yield (Fv/Fm, Fig.6) throughout the experiment. It was observed that in blue light the Fv/Fm -value was declining towards the end of the experiment, while in white and red light the PSII maximum quantum yield would reach normal level (in microalgae about 0.6) This indicates that in blue light the photosynthetic efficiency is lower due to PSII imbalance caused by stress derived from blue light irradiance. This observation was further corroborated by analysis of the photosynthetic pigments, chlorophylls *a* and *b* and carotenoids (Fig.7); in blue light light the content of carotenoids (D) was highest while the chorophyll/carotenoid -ratio (C) remained lowest throughout the experiment, indicating increased accumulation of photoprotective pigments in the cells. Chl*a* and Chl*b* levels (A, B) were highest in blue light, but this could be due to larger cell size and subsequent rise in the pigment content.

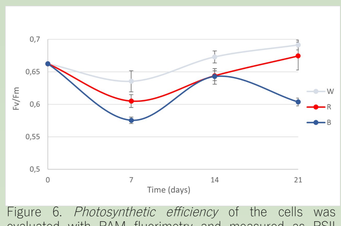

Figure 6. *Photosynthetic efficiency* of the cells was evaluated with PAM fluorimetry and measured as PSII maximum quantum yield (Fv/Fm). In blue light the Fv/Fm value decreased signficantly towards the end of the experiment compared to red and white light, implying that the cells were exposed to stress due to blue light irradiance. W=white light, R= red light, B= blue light.

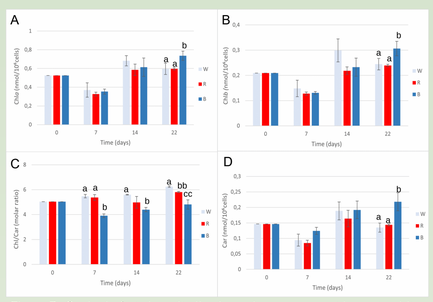

Figure 7. *Content of photosynthetic pigments* was analysed throughout the experiment. It was seen that the accumulation of carotenoids (D) was highest in blue light. Subsequently the chlorophyll/carotenoid -ratio (C) remained lowest, notwithstanding the higher level of chlorophylls *a* and *b* (A, B) in comparison to white and red light cells. The letters a vs. b vs. c indicate statistical significance ($P < 0.05$) . W=white light, R= red light, B= blue light.

Neochloris in saline medium (25‰) under white/red LED light

Figure 13. *Experimental setup* to grow *Neochloris* in red LED light. Growth chamber adjusted with LED light strips was used for the experiment. Growth conditions: 16:8 light:dark photoperiod, 25°C, no CO_2 addition.

Cell growth was evaluated regularily as optical density (Fig.9A) and cell density (Fig.9B) and at the end of the experiment dry biomass (Fig.9C) and cell diameter (Fig.9D) were measured. The growth of the cells followed more or less similar pattern in both light conditions. In red light both dry weight and cell diameter were slightly higher compared to white light by the end of the experiment, however, the difference in values was not statistically significance ($P>0.05$). All in all, the growth was hampered in saline medium in respect to freshwater medium.

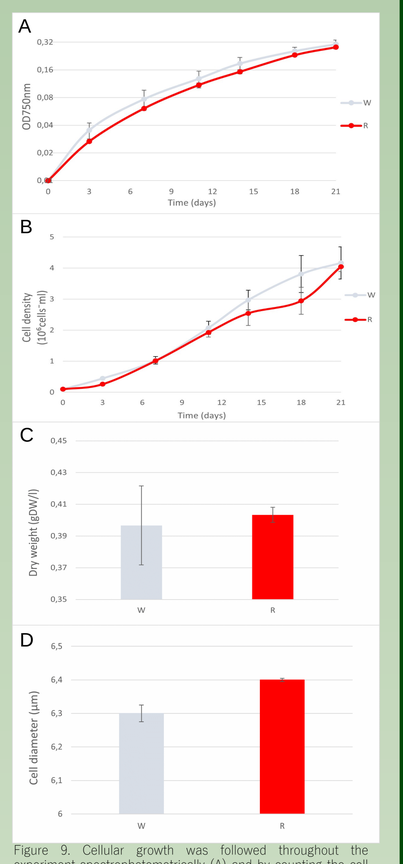

Figure 9. Cellular growth was followed throughout the experiment spectrophotometrically (A) and by counting the cell density (B) while dry biomass (C) and cell diameter (D) were measured at the end of the experiment. The cells seemed to grow in a similar manner in both light conditions. In red light the cells obtained slightly higher cell dimeter and dry biomass by the end of the experiment. W= white light, R= red light

Photosynthetic performance of *Neochloris* cells grown in white and red LED light was evaluated with PAM fluorimetry and measured as PSII maximum quantum yield (Fv/Fm value; Fig.10). It was observed that in both light conditions the Fv/Fm -ratio would decline during the first days of the experiment remaining lower in red, however, still around typical value of 0.6. The Fm/Fv -ratio would continue to fall but seemed to stabilise around ~0.53 in red light. This might be explained by higher carotenoid content and thus increased photoprotection in the red light cells (Fig.11C). This effect remains to be verified. Chlorophyll *a* and *b* levels remained similar in both light conditions throughout the experiment (Fig. 11A,B).

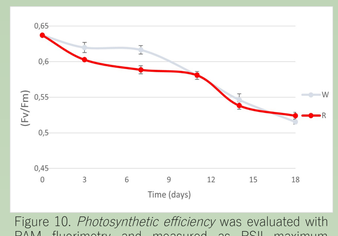

Figure 10. *Photosynthetic efficiency* was evaluated with PAM fluorimetry and measured as PSII maximum quantum yield (Fv/Fm). The Fv/Fm -value was seen to decline in both light conditions and fall below the typical microalgal value of 0.6 from day 11 onwards. by the end of the experiment the Fv/Fm –ratio seemed to stabilise at 0.53 in red light white in white light the value continued to fall.

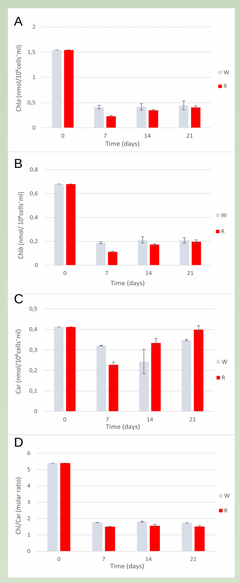

Figure 11. *Content of photosynthetic pigments* was analysed throughout the experiment. Increased level of carotenoids (C) and lower chlorophyll/carotenoid -ratio (D) was observed in red light while chlorophyll *a* and *b* levels woud remain similar in both light conditions.

Accumulation of lipids

was studied by staining with fluorochrome Nile red. It was seen that small lipid droplets would accumulate within the cell cytoplasm irrespective of the chromatic quality of the light, however, more precise analysis should be done in order to verify this observation. It is expected that the production of lipids is due to the salinity of the growth medium, but e.g. the effect of red light wavelength in respect the fatty acid profile remains to be studied.

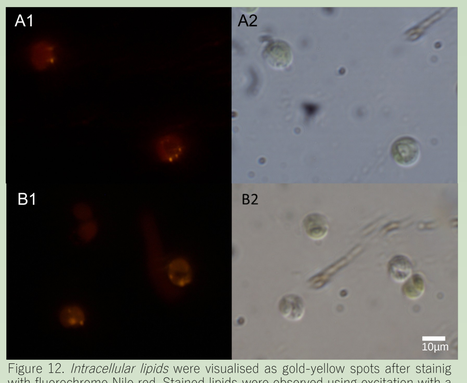

Figure 12. *Intracellular lipids* were visualised as gold-yellow spots after stainig with fluorochrome Nile red. Stained lipids were observed using excitation with a 485nm filter. Several small lipid droplets could be seen in both white (A1) and red (B1) light cells.

MATERIALS & METHODS

Growth conditions: BG11-medium was used as freshwater medium and BM (brackish medium; epsag.uni-goettingen.de) as saline medium (25‰ salinity). LEDs were used as light source for the experiments (blue 470nm, red 626nm). Cell growth was studied spectrophotometrically at 750nm (Pharmacia Ultrospec 2000 UV-Vis) and by counting the cell number with a Thoma haemocytometer. For dry biomass an aliquot of cell culture was filtered through pre-dried and weighed glass-fibre filters (1.2 μm pore size; Whatman GF/C). Cell diameter was measured with Image J -sofware. Transmission electron microscopy: Sections of samples embedded in Araldite-Epon 812 resin were observed with Hitachi H800 electron microscope. Lipid content was analysed using modified Folch's method with chloroform:methanol (2:1) extraction. Fatty acid profiles were determined by methyl ester derivation and GC analysis with a gas chromatograph (HP Agilent 4890D) equipped with a flame-ionisation detector at a temperature of 260°C, a split/splitless injector (175°C) and a capillary column SP–2560 (100m, 0.25 mm and 0.2 μm). PSII maximum quantum yield (Fv/Fm) was measured using a PAM fluorometer (ADC-OS1-FL; ADC Bioscientific Ltd) after 15min of dark adaptation. Pigments were extracted with MeOH at 80°C and quantified spectrophotometrically (Chla 666nm, Chlb 653nm, Car 470nm) using equations proposed by Wellburn [11].

CONCLUSIONS

In the current work we found that the use of different light wavelengths causes changes in cell metabolism of *Neochloris oleoabundans*. In particular, blue light in combination with freshwater medium leads to: 1) higher biomass, 2) larger cell volume, 3) pronounced accumulation of lipids and 4) higher level of carotenoids. Further it was oserved that blue growth light promotes production of monounsaturated fatty acids while red light induces accumulation of polyunsaturated fatty acids in *Neochloris*; thus, blue light could suit for microdiesel production while red light would support production of high-quality raw material for food/feed and nutraceutical/pharmaceutical industries. Saline medium (salinity 25‰) was seen to induce stress in *Neochloris* cells. It was observed that lipids were produced in response to environmental stress irrespective of growth light wavelenght. The PSII quantum yield decreased and level of carotenoids increased towards the end of the experiment, indicating (oxidative) stress. Accumulation of carotenoids was slightly pronounced in red light, which could indicate that red light wavelength promotes the photoprotective mechanisms based on carotenoids in microalgae. Studying microalgae in saline growth medium is important in order to recognise the effects natural sea- or brackish water have on microalgal cultures, so as to avoid use of freshwater resources in future application. Further, the results obtained show that finding optimal combinations of a microalgal strain and growth conditions it is possible to elevate the sustainability and viability of industrial applications harnessing the diversity of microalgae.

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