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Combined effect of nutrient and flashing light frequency for a biochemical composition shift in *Nannochloropsis gaditana* grown in a "quasi-isoactinic" reactor.

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ABSTRACT

Artificial lighting is a growing attractive for the cultivation of microalgae. In particular, Light Emitting Diodes (LEDs) can be employed to tailor the lighting to the microalgal culture in a controlled mode in order to create flashing light. In order to establish the effect of the flashing frequency on growth and biochemical composition of a model microalga, a "quasi-isoactinic" reactor, in which the light distribution is almost homogeneous, was set up. In this work, it was employed for the cultivation of the heterokont *Nannochloropsis gaditana* in two growth media with limiting and not limiting nutrients. The combined effect of nutrient concentration and flashing frequency on the growth, lipid content, fatty acid content and pigment content was for the first time assessed. Results indicate that both nutrient concentration and flashing frequency influence the above-mentioned parameters. In particular, under flashing light conditions, an increase of lipid content and a decrease of polyunsaturated fatty acids (PUFAs) and chlorophyll are observed when nutrients are deficient, while opposite effects are shown when nutrients are abundant.

Keywords: *flashing light; Nannochloropsis gaditana; flat-plate photobioreactor; lipid composition; chlorophyll; carotenoids.*

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1. INTRODUCTION

Microalgae belong to a polyphyletic group of photosynthetic microorganisms with deeply different characteristics. Nowadays, they are employed in several fields, such as nutraceutics and aquaculture, and are involved in other emerging applications in the fields of bioenergy, biomaterials, bioremediation and production of heterologous proteins[1]. Beside their great potential, the state of art for industrial cultivation of microalgae is still not optimized, mainly because of limitations in culture scale-up. Light availability is the main limiting factor in autotrophic microalgae cultivation. In outdoor cultivations light is supplied by the Sun; in alternative it can be provided by artificial sources. The use of artificial light shows several advantages: for example it allows a better system control and can lead to an increased biomass productivity. The drawbacks, instead, are mainly connected to the electric energy cost and replacement of lamps[2]. Furthermore, artificial light may trigger biomolecules production, as recently reviewed[3]. In microalgal cultivation, moreover, the inefficient light energy usage due to photoprotective mechanisms employed under high-light intensities may cause an inefficient light energy usage[4]. In this work, we propose to employ flashing lights as a promising approach for optimizing the energy usage by matching the photosynthetic reaction kinetics in microalgae. Flashing light, in fact, could have the same effect on triggering biomolecules production compared to high-intensity continuous light[5,6], with the advantages of saving energy and reduce production costs. A flashing light regime is characterized by a *frequency* (f) and a *Duty Cycle* (DC). The frequency is the repetition rate of the light–dark transition, and the duty cycle is the relative proportion of the light flash period. The flashing light usage for microalgal cultivation was already investigated[7–9] and encountered a renewed interest in recent years[10,11]. In our experiments light was provided by LEDs, whose usage for microalgae cultivation was reviewed[12]. In order to study the actual effect of light on a culture, it is needed employing very short light-paths (<1 cm) and diluted culture, as shown in previous studies[13,14].

In this work, a "quasi-isoactinic" photobioreactor, in which the radiant field is almost homogeneous, was employed for the cultivation of the oleaginous algae *Nannochloropsis gaditana*. The growth response to different flashing frequencies was tested for the first time in this reactor, under nutrient depletion and under nutrient abundance (nitrate and phosphate ten times more concentrated). Flashing light effects were statistically analyzed taking into account effects of nutrient concentration. Results indicate that flashing light and nutrient concentration have a combined effect on the biochemical composition of *Nannochloropsis gaditana.* In particular, under flashing light conditions, when nutrients are limiting, starvation seems to have

the prevailing effect, showing an increase of lipid content and a decrease of PUFAs and chlorophyll. On the contrary, when nutrients are abundant, the flashing light effect becomes prevailing and opposite effects are observed in comparison with the previous conditions. Although the effect of both flashing light and nutrient concentration were previously individually studied (e.g. [15–18]), in this work their contribution were considered in association in order to assess their combined effect.

2. MATERIALS AND METHOD

2.1. Algal growth

Liquid cultures of *Nannochloropsis gaditana* (CCAP 849/5 Scottish Association for Marine Science, Oban, Scotland), Eustigmatophyceae, were maintained in Erlenmeyer flasks with f/2 medium[19]. For the experiments, two different versions of the same medium were employed in this work: the first one is called "basic medium" and consists of artificial sea water (6.3 mM KCl, 2.0 mM $NaHCO_3$, 7.1 mM KBr, 0.36 mM H_3BO_3, 0.024 M Na_2SO_4, 9 mM $CaCl_2$ $2H_2O$, 0.046 M $MgCl_2$ $6H_2O$, 0.35 M NaCl) supplemented with a modified f/2 medium differing from the original for an increased $NaNO_3$ concentration, for the absence of Na_2SiO_3 and for slightly modified concentrations of micronutrients with the following final composition: 3.5 mM $NaNO_3$, 0.036 mM NaH_2PO_4 H_20, 0,12 µM $FeCl_3$ $6H_2O$, 0,12 µM Na_2EDTA, 0.04 µM $CuSO_4$ $5H_2O$, 0.076 µM $ZnSO_4$ $7H_2O$, 0.042 µM $CoCl_2$ $6H_2O$, 0,91 µM $MnCl_2$ $4H_2O$, 0.025 µM Na_2MoO_4 $2H_2O$); the second one is called "enriched medium" and has the same composition as the first one but with ten times increased concentration of $NaNO_3$ and NaH_2PO_4 (35 mM and 0.36 mM, respectively).

A microalgal pre-culture was set up by inoculating 10 ml of a back-up culture in 100 ml of the same liquid medium used for the main experiment. When the cells were in late exponential phase (around 10 days of cultivation), they were used to inoculate the "Quasi-isoactinic" reactor[13] in order to reach an initial concentration of approximately 0.1 AU (λ=750 nm).

The "Quasi-isoactinic" reactor[13], consists in a flat plate photobioreactor with the width of 1.5 cm lit by two side by aluminium panels with LEDs stripes (KWB 5050 RGB IP44).

The cultures inside the reactor were mixed by supplying microfiltered air (0.22 µm) passing through a sparger with micro-holes. When the pH was below 8.0, pure CO_2 was supplied through the same sparger until it reached the value of 7.0. Each experiment was carried out for

13 days. The concentration of the microalgal suspension was daily monitored by manually counting the cells in a Burker chamber. The suspension was diluted in order to have between 100 and 200 cells per square. The number was then multiplied by the dilution factor and by a multiplier (10^4) in order to obtain the concentration in cell/ml. Cultures were performed once for each condition, while measurements were done in triplicate (n=3) and the average value was retained and reported together with the standard deviation.

2.2. Light conditions

Flashing light is a way of supplying light that consists in a sequence of light and dark periods. A flashing light is characterized by a *Duty Cycle* (DC), namely the lightened portion of a light cycle, and a *frequency* (f), the number of cycles in the unity of time (s), measured in hertz. Three different flashing light conditions were employed in this work: 25, 250 and 2500 Hz with the same DC of 0.25. An average light intensity of 70 μmol m^{-2} s^{-1} was applied in all the tested conditions including the continuous light control. The maximum light intensity in the three flashing light condition is the same and equal to 280 μmol m^{-2} s^{-1}. The light distribution was measured on the surface of the reactor in 9 equally-spaced points by means of a Delta Ohm-HD 9021 equipped with Photosynthetic Active Radiation (PAR) probe (Delta Ohm LP 9021 PAR) (Data not shown).

2.3. Determination of specific growth rate μ

In order to estimate the specific growth rate (μ), the cell density was plotted on a semi-log diagram versus the cultivation time in days. Growth rates values were calculated by the determination of the slopes of the regression lines obtained with the points of exponential growth in the different conditions. This value gives an estimation of the amount of increase as cell/ml per day.

2.4. Sample preparation

After 13 days from the cultivation start, the cell suspension was centrifuged and the obtained biomass was frozen in liquid nitrogen and freeze-dried for 48 h in a bench lyophilizator (FreeZone 2.5L, LABCONCO, US). The biomass was then stored at -20°C for further analysis.

2.5. FTIR analysis

Biomass samples were analyzed by Fourier Transform Infrared Spectroscopy (FTIR) in order to investigate the approximate biochemical composition. The method was adapted from Stehfest et al.[20]. About 2 mg of freeze-dried biomass were weighted and transferred in a mortar together with 100 mg of potassium bromide (KBr) to prepare glassy sample discs. Then, the mixture was vigorously crushed and a pellet was made using a hydraulic press (CrushIR, PIKE Technologies, US). The pellet was then scanned in a Cary 630 Spectrometer (Agilent Technologies, US). This technique correlates one or more peaks to the corresponding biochemical macromolecule, thanks to the vibrational frequency of the related functional group. By integrating the area under the curve, a semi-quantitative analysis of the macromolecule is obtained. The ratios between different areas were analyzed in order to make comparable different samples. In Table 1 the employed wavelengths to integrate different peaks are shown.

Table 1: Reference wavelengths for peaks integration connected to macromolecules by the vibrational frequency of the related functional group.

Wavelenght (cm^{-1})	Assignment	Macromolecule
2799-300	CH of saturated CH	Lipids
1584-1725	Amide I C=O of amides from proteins	Protein
1490-1584	Amide II N-H of amides from proteins	Protein
950-1200	C–O–C of saccharides	Carbohydrates

2.6. Extraction of lipids and analysis of Fatty Acids

The extraction of lipids was realized by crushing 20 mg of dry microalgae biomass in a mortar with 5 ml of chloroform/methanol (2:1, v/v) and 1 ml of NaCl 1%. The mixture was vigorously mixed and centrifuged until the formation of two phases. The lower phase (chloroform phase) was transferred in a pre-weighted tube and the solvent was evaporated under a nitrogen stream. After complete evaporation of the solvent, the total lipids were determined gravimetrically. Then, they were transesterificated by adding 1 ml of sodium metoxide (1 g NaOH in 100 ml MeOH) and 1 ml of hexane for 1 h at 60°C. The upper phase (hexane phase) was then analyzed by gas chromatography by means of a GC 7890B System (Sigma-Aldrich, US) supplied with a

FID detector and a capillary column Omegawax 250 (Sigma-Aldrich, US). Initial temperature was 50 °C, increased to 220°C as working temperature. Total analytic time was 79.5 minutes and argon was used as eluent gas. The quantification of lipid was done by comparing samples chromatograms with the standard. *Supelco 37-Component FAME Mix* (Sigma-Aldrich, US) was used as standard.

2.7. Spectrometric Pigment Analysis

For the chlorophyll and total carotenoid extraction, after biomass was disrupted in methanol by crushing in a mortar approximately 20 mg of biomass. The methanol extract was separated from the algae pallet via centrifugation and spectrophotometrically analysed (Cary 630 Uv/Vis spectrophotometer, Agilent) against a methanol blank. All analyses were done under dimmed light. Chlorophyll *a* (C_a) and total carotenoids (C_{carot}) were determined according to Lichtenthaler and Wellburn[21] and Henriques et al.[22] by applying the OD measurements at 666 and 470 nm (A_{666}, A_{470}) from the methanol extracts to Equations **I** and **II**:

$$C_a = 15.65\ A_{666} \quad \textbf{I}$$

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

2.8. Data analysis

Two-way ANOVA analysis was performed to detect differences in the realized analysis among *light treatments* and *strains*. The output F-values together with p-values were used to describe the impact of treatment on the variables. Bonferroni´s correlation (p value) was used to quantify the variability between control and treatments. Data were considered significant for p-values smaller than 0.1. Results are shown as means and standard deviations are reported as error bars.

3. RESULTS AND DISCUSSION

3.1. Growth performance

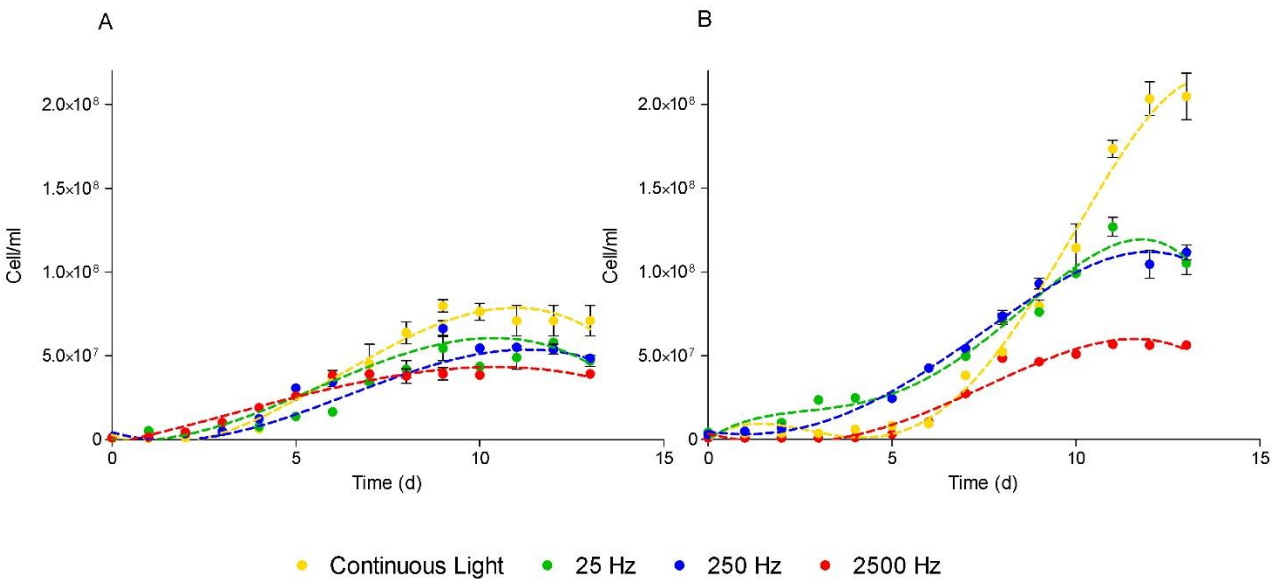

Figure 1: Growing curves of *Nannochloropsis gaditana* grown in *basic medium* (A) and *enriched medium* (B). Three flashing light conditions (25, 250 and 2500 Hz) are shown in green, blue and red and the continuous light control in yellow. Values are shown as means (n=3) and error bars report the standard deviation.

Figure 1 shows the growth curves of *N. gaditana* cultured in two different media, with limiting (Figure 1 A) and not limiting concentration of nitrate and phosphate (Figure 1 B), under three flashing light conditions of 25, 250 and 2500 Hz and a control of continuous light. Algal cells grew more in the enriched medium than in the basic one; in fact, the maximum concentration in the enriched medium was of about 200 milions cells/ml in the continuous light control, *i.e.* almost three times more than the corrispondent control in the basic medium. Similar cell concentrations were obtained in other studies found in literature about the same algae[23,24]. Also all the other light conditions showed a more prominent growth in the enriched medium than in the basic one, and this is easily explained by the higher concentration of nutrients. In both the growth media, cells exposed to the continuous light control grew better than in all other conditions. By contrast, the cells grown under higher flashing frequences (*i.e.* 2500 HZ) showed worse growing performance. In fact, the highest concentration occurred under the 25 Hz frequency was of about 50 millions of cells/mL in the basic medium and of about 120 millions of cells/mL in the enriched medium. Under the 250 Hz, the reached concentrations were of about 50 millions of cells/mL in the basic medium and of about 100 millions of cells/mL in the enriched one. Under the 2500 Hz, the reached concentrations were of about 40 millions of cells/mL in the basic medium and of about 60 millions of cells/mL in the enriched one. It is

worth noting that the low and medium frequency flashing light conditions (25 and 250 Hz) had a similar growth performance in both the media. The growth performance was also measured by the calculation of the *specific growth rate μ;* as reported in Figure 2. In both media, the *specific growth rate μ*, in day^{-1}, was lower in the flashing light conditions with low and medium frequency compared to the continuous light control, even if in the basic medium there is no statistical difference in the 250 FL condition. However, this value progressively increased with the flashing frequency, reaching, under 2500 Hz, a statistically equal value compared to continuous light control in the basic medium, and a higher value than the control in the enriched medium. The specific growth rate is a measurement of reproduction speed during the exponential phase; thus it means that cells under 25 and 250 Hz flashing lights were slower than the control and in the same time they reached a lower final cell concentration. In the third flashing light condition, 2500 Hz, cells had the same (or higher, in the enriched medium) specific growth rate during the exponential phase but they reached the lowest final concentration compared to all the other conditions.

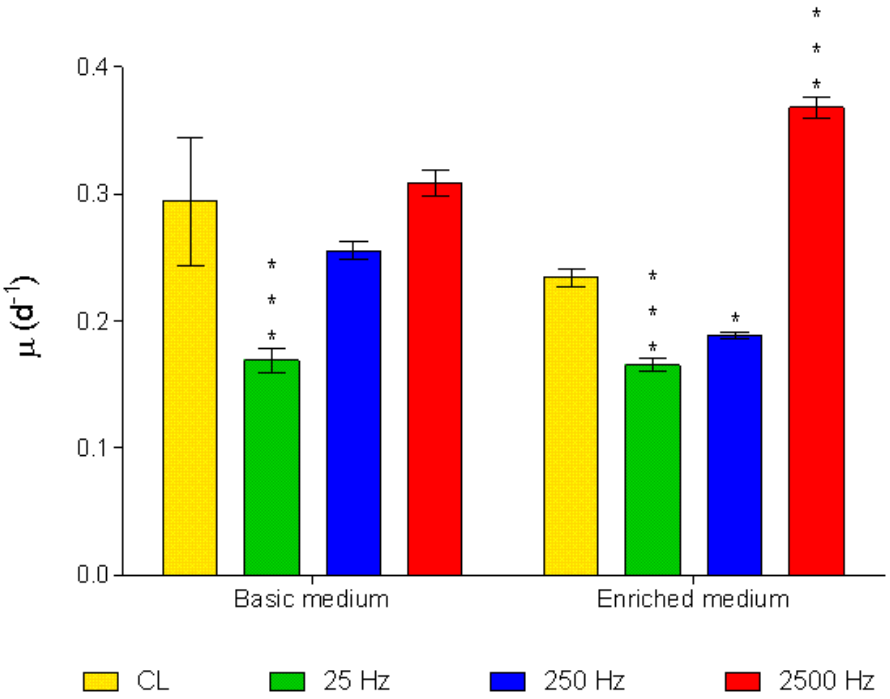

Figure 2: *Specific growth rate μ* of *N. gaditana* grown in basic and enriched medium under three flashing light conditions and a control of continuous light. Values are reported as means (n=3) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different from the continuous light control. One asterisk indicates a P value <0.1 and three asterisks < 0.001.

The flashing light effect has been widely studied in literature, as reviewed by some authors[25,26]. Several authors found a correlation between the flashing frequency and the growth performance. For example, in a very similar way than in the present work, Vejrazka and co-

workers observed in *Chlamydomonas reinardtii* that specific growth rate decreased from continuous light to flashing light and increased by increasing the flashing frequency (DC= 0.1, f=5-100 Hz)[27]. Interestingly, at the highest frequency of the work, 100 Hz, the *specific growth rate* μ returned to be similar than the one of continuous light. Other authors found no increased growth and no increased μ in cultures exposed to flashing light compared to continuous light, in a range of duty cycles and frequencies and in several microalgae species[9,10,28]. On the contrary, there are a few works in which authors observed an increase of the growth performance under flashing light compared with continuous light irradiation. For example, Lunka and Bayless[11], during the cultivation of *Scenedesmus dimorphus*, observed a higher biomass increase under flashing light compared to under the continuous light control (DC=0.2, f=10 KHz). In another case, Yoshioka et al.[15] observed in *Isochrysis galbana* an increased *specific growth rate* μ until the sixth day of cultivation and an increased final cell concentration in flashing light compared to continuous light (DC=0.5, f=10KHz). There is, therefore, a significant uncertainty on the effects of the flashing lights on microalgal cultures. The different results in this study can be due to several factors such as differencies on used flashing conditions (i.e DC and f) and algal strains. On the other way, Simionato et al.[29] hypotized that the lenght of the light pulse is one of the main parameters affecting biomass productivity and that the optimum is around 10 ms; in our work the duration of light pulses decreases when frequency increases: in 25 Hz condition it lasts 10 ms, in 250 Hz condition 1 ms and in 2500 Hz condition 0.1 ms. Our results are therefore in accordance with Simionato's theory, that could be adopted as an explanation for the different response to flashing light of microalgal cultures. It is anyway to point out that the lack of biological replicates in the present work represents a limitation in the interpretation of the results; further work should be done in this direction.

3.2. Biochemical characterization

3.2.1. FTIR analysis

Fourier Transform Infrared Spectroscopy was employed in order to obtain a gross analysis of the composition of the biomass harvested at the end of the experiments. This methodology, broadly employed in the characterization of microalgae biomass[20,30,31], connects the presence of vibrationally active functional groups with correspondent macromolecules. The related peaks are integrated and the results are reported as ratios between areas in order to make different samples comparable. By comparing three different ratios it is possible to approximately

understand the composition of the biomass. Results are reported in Figure 3 and original spectra are in supplemental material (Figure A1-A8). The reported areas are Lipid/amide I (L/A) and Carbohydrate/amide I (C/A).

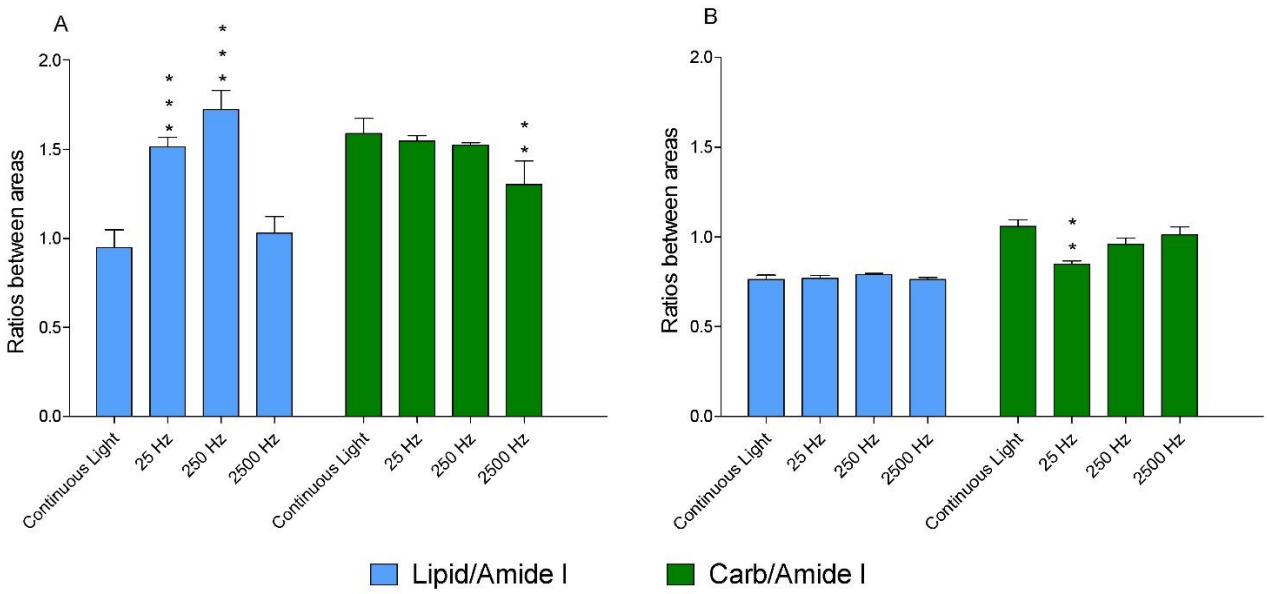

Figure 3: FTIR analysis on the microalgal biomass grown in *basic medium* (A) and *enriched medium* (B) and under three different flashing light conditions and a continuous light control. Ratios between areas under peaks related to macromolecules are reported. Values are reported as means (n=3) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different from the continuous light control. Two asterisks indicate a P value <00.1 and three asterisks < 0.001.

Under a statistical point of view, L/A ratio is influenced more by the *medium* (F=308.4, p<0.001) than by the *light treatment* (*F*=39.99, *p*<0.01). This ratio is also affected by the *interaction* between the other two variables (*F*=40.23, *p*<0.01). C/A ratio is affected only by the *medium* (*F*=317.7, *p*<0.01). This analysis allows to hypotize that lipids content is affected both by the richness of nutrients and by the frequency of flashing light, while carbohydrates content is mainly affected by the nutrients. In fact, is well known that a lack of nutrients, in particular nitrate, facilitates an accumulation of lipids (e.g. in *Nannochloropsis oceanica*[32]) or carbohydrates (e.g. in *Tetraselmis sp.*[33]). Furthermore, several studies showed a correlation between lipids accumulation and flashing lights, for example in *Isochrysis galbana*[15] and in *Chlorella vulgaris*, *Acutodesmus obliquus* and *Micractinium reisseri*[34].

In the case of *N. gaditana* grown in basic medium (Figure 3 A) the L/A ratio increased from the continuous light to the flashing light conditions of 25 and 250 Hz but decreased under the 2500 Hz condition. The C/A ratio, on the other hand, was constant under all light treatments except for the 2500 Hz one, in which it slightly decreased. Probably, the lipid content increased from the control to the 25 and 250 Hz light conditions, and decreased in the 2500 Hz treatment.

For what concerns the biomass cultivated in enriched medium (Figure 3 B), the effect is different compared to the previous one. In fact, L/A ratio is stable under all lighting conditions, while the C/A ratio decreases in 25 Hz condition. It is possible to hypothesize that a decrease of lipid content occurred together with an increase of the protein one, thus the ratio did not vary between the lighting conditions. The potential changes in the composition of the biomass grown in richness of nutrients were not detectable by using this method.

Although this method presents some limitations, these results show that flashing lights have an effect on biomass composition that is influenced by the richness of nutrients in the growth medium.

3.2.2. Total lipids quantification

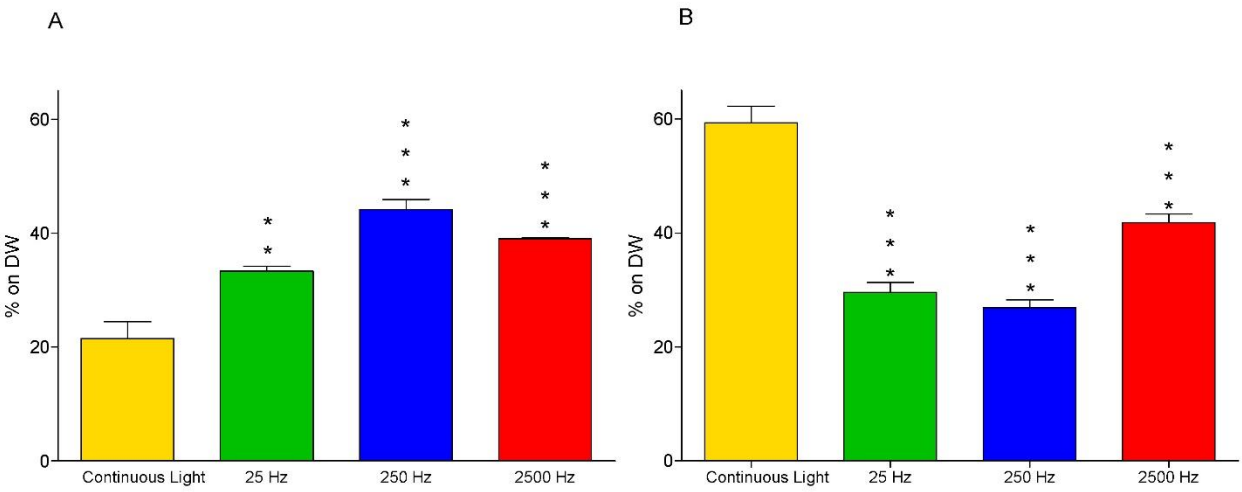

Figure 4: Total lipids on Dry Weight (DW) of microalgal biomass grown in *basic medium* (A) and *enriched medium* (B) and under three different flashing light conditions and a continuous light control. Values are reported as means (n=3) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically from the continuous light control. Two asterisks indicate a P value <0.01 and three asterisks < 0.001.

The dry biomass at the end of the cultivation was analyzed in order to assess the percent of total lipids on Dry Weight (DW). Results are shown in Figure 4.

The detected contents of total lipids in the dry biomass of *N. gaditana* ranged from 21.5±5.1% to 59.3±5.1% confirming previous results from literature data on the same algae[35,36].

According to ANOVA analysis, both *light treatment* and *medium* affected the lipid quantity in a comparable way (F=9.433, p<0.01; F=11.87, p<0.01). The analysis revealed also that the *interaction* between these two parameters accounted for approximately 79% of the total variance (F=72.44, p<0.01). This means that there is less than the 0.01% chance of randomly

observing this interaction in an experiment of this size. In other words, the interaction is extremely significant.

As shown in Figure 4 A and confirmed by FTIR analysis of Figure 3 A, when cells were cultured in the basic medium, the lipids quantity gradually increased from the continuous light control to the light treatments of 25 and 250 Hz. The flashing light condition of 2500 Hz has a higher lipid content than the control, but slightly lower than in 250 Hz light condition. On the other way, the observed effect in cells cultivated in the enriched medium was the opposite: the lipids content decreased from the continuous light control to the flashing light treatments. In particular, it gradually decreased from 25 Hz to 250 Hz and slightly increased in the 2500 Hz flashing light treatment. This result reinforces the one of the statistical analysis, that indicates a strong interaction between the nutrient concentration and the flashing light frequency.

Several studies addressed the correlation between lipid content and flashing light. Some of them did not find any relevant difference in the lipid content under continuous light and under flashing light[15,37,38]. Some others found interesting differences. For example, Simionato et al., analyzed the response of *N. salina* under a range of frequencies and duty cycles (DC=0.1, 0.33; f=1-30 Hz). It was assessed that the lipid content is lower or higher than the one of the CL condition depending on frequencies and duty cycles[29]. In a very similar way that in the present work work, another study observed in *Dunaliella salina* grown in nitrogen excess a decrease in lipids/cell when increase ng frequency (DC=0.33, 0.4, 0.5; f=0.017-5)[18]. These results taken together demonstrate that there are several factors involved in the microalgae composition in response to flashing light treatments, and nutrient richness is one of the parameters to take into consideration. A possible interpretation of the already exposed results is that when grown in starvation of nutrients, the effect of flashing is combined to the effect of the starvation that brings to accumulation of lipids. When grown in richness of nutrients, the light energy provided by flashing lights probably flows along other biochemical routes rather than to the production of lipids.

3.2.3. Fatty acid composition

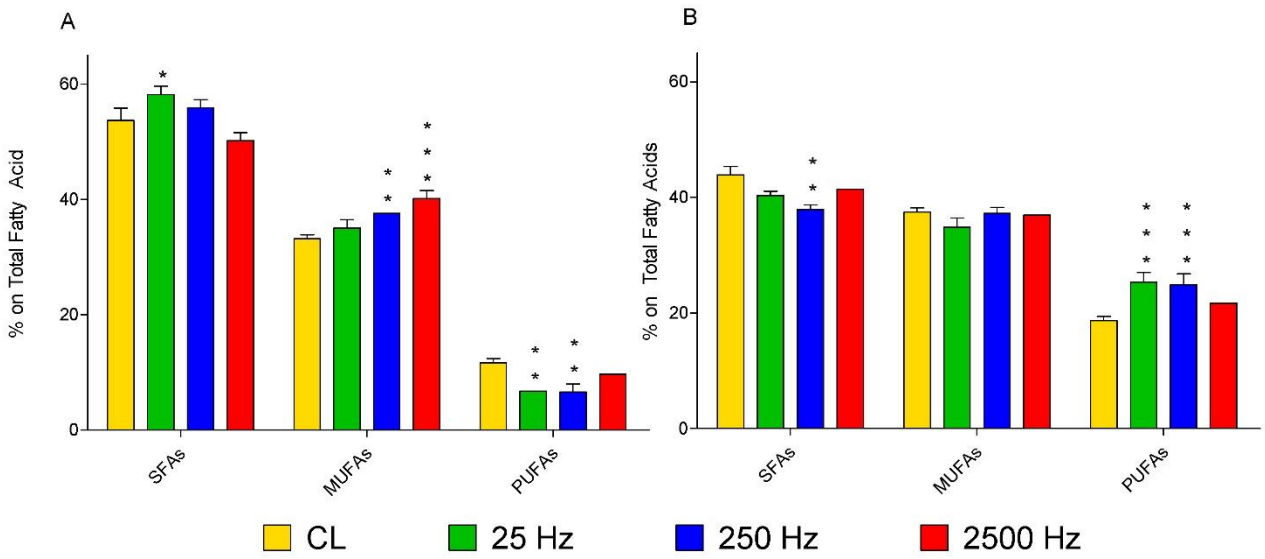

Figure 5: Fatty acid composition of microalgal biomass grown in *basic medium* (A) and *enriched medium* (B) and under three different flashing light conditions and a continuous light control. The composition is reported as percent of Saturated Fatty Acids (SFAs), Monounsaturated Fatty Acids (MUFAs) and Polyunsaturated Fatty Acids (PUFAs) on total fatty acids. Values are reported as means (n=3) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different from the continuous light control. One asterisk indicates a P value <0.1, two asterisks <0.01 and three asterisks < 0.001.

The fatty acid content of dry microalgal biomass grown in the basic and in the enriched version of the medium under three flashing light conditions and a control were assessed and results are reported in Figure 5 A and B. The analyzed fatty acid content of *N. gaditana* is coherent with other literature studies[24,39,40].

Under a statistical point of view, the Saturated Fatty Acid percent on total fatty acids is influenced markedly by the *medium* (F=375.7, p<0.01) and much less by the *light treatment* (F=5.356, p<0.01). Oppositely, Monounsaturated Fatty Acid (MUFAs) content is influenced by the *light treatment* (F=10.29, p<0.01) and not by the *medium* (F=0.08047, p<0.01). Polyunsaturated Fatty Acid (PUFAs) content depends on the *medium* (F=641.1, p<0.01) and not by the *light treatment* (F=0.4319, p=0.7359).

By looking at the results in Figure 5 A and B, the SFAs content did not show big changes between the control and the flashing light conditions, with the exception of the light conditions of 25 Hz in the basic medium and of 250 Hz of the enriched medium. MUFAs content varied in the 250 and 2500 Hz light condition of biomass grown in the basic medium and did not change in algae grown in the enriched medium. In microalgae grown in the basic version, PUFAs content, interestingly, was the same between the continuous light control and the 2500 Hz light condition, while it decreased in the 25 and 250 Hz flashing light conditions. Oppositely,

when cultivated in the enriched medium, they showed an increase in the 25 and 250 Hz flashing light conditions compared to the continuous light control that was again the same compared to the 2500 Hz light condition.

Previously, some authors studied the effect of flashing light on fatty acid composition of microalgae[37,38]. Between them, Yoshioka et al. observed a similar shift in SFAs, MUFAs and PUFAs compared to the present work when *I. galbana* was grown under intermittent light (DC=0.5, f=10 KHz)[15].

The increase of PUFAs content can be connected to a low-light response[41], as they are included in the thylakoid membranes that multiply with the aim of harvesting much light as possible[42]. This may indicate that low-frequency flashing light conditions may bring to a low-light response, indicating that cells do not acclimate to the average light intensity, as commonly believed[43], but that the time they spend in darkness has a major effect on the acclimation. A similar assumption is made by Yarnold et al.[44] which studied the acclimation of *Chlamydomonas reinhardtii* in fluctuating light regimes and observed that when cells were grown in cycles including a large dark fraction, a low-light acclimation response was observed[44]. The same effect is observed in the present work under low frequencies when cells are cultured in the enriched medium, with no nutrient limitation (Figure 5 B). The observed effect in the basic medium (Figure 5 A), on the other way, is opposite. This means that, even though the illumination conditions, the effect of the nutrient starvation is higher, as confirmed by ANOVA analysis. In fact, other studies demonstrates that in nitrogen depletion PUFAs content decreases[45,46].

3.2.4. Pigment composition

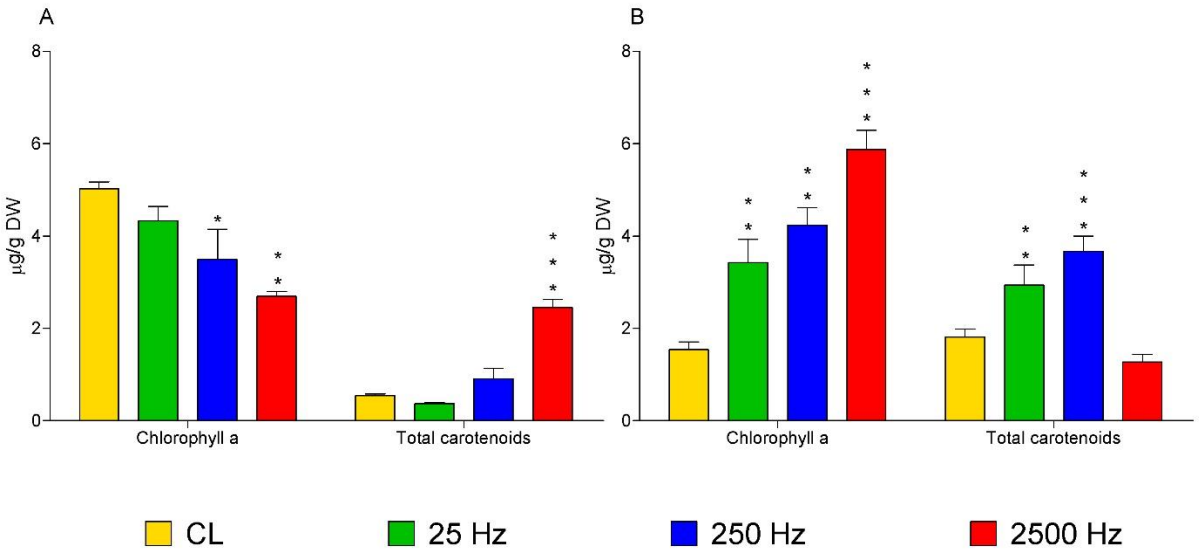

Figure 6: Pigment composition of microalgal biomass grown in *basic medium* (A) and *enriched medium* (B) and under three different flashing light conditions and a continuous light control. Chlorophyll a and Total carotenoids are reported as µg/g Dry Weight (DW). Values are reported as means (n=3) and error bars report the standard deviations. Asterisks indicate if the treatment is statistically different from the continuous light control. One asterisk indicates a P value <0.1, two asterisks <0.01 and three asterisks < 0.001.

Microalgal biomass grown in the basic and the enriched version of the medium under three flashing light conditions and a control was spectroscopically analyzed in order to assess the content in Chlorophyll a and Total carotenoids on Dry Weight (DW). Results are reported in Figure 6 A and B.

The chlorophyll content ranges from 5.88±0.7 to 1.54±0.29 µg/g DW, while total carotenoids from 3.67±0.57 to 0.37±0.05 µg/g DW. These values are in line with the ones found in literature for the same genus[36].

Under a statistical point of view, the chlorophyll content is not influenced by the *medium* or by the *light treatment* (F=2.379, p=0.1080; F=0.1907, p=0.6682). It is instead affected by the *interaction* between these two parameters, that accounts for 77.88% of the total variation (F=27.37, p<0.01). On the other way, total carotenoid content is affected more by the *medium* (F=67.96, p<0.01) than by the *light treatment* (F=7.916, p=<0.01), while the *interaction* accounts for the 46.10% of the total variation (F=30.71, p<0.01).

As observed in Figure 6 A, the biomass grown in the basic medium showed a quantitative decrease of the content in chlorophyll from the continuous light to the flashing light conditions, while total carotenoid content increased in the same way. Oppositely, in Figure 6 B, the biomass grown in the enriched version of the medium showed a strikingly different quantitative increase

of chlorophyll content from the continuous light to the flashing light conditions and an increase of total carotenoid content in 25 and 250 Hz flashing light conditions.

It is interesting how the chlorophyll content in cells grown in continuous light with nutrient deplete and replete conditions are markedly different (5.88±0.7 and 1.54±0.29 μg/g DW, respectively). It is worth noting that, although a decrease of chlorophyll content has been reported for phytoplankton grown under nitrogen and phosphorous deficiency[47], other authors observed the opposite effect with monocultures. In particular, the chlorophyll content was found to increase under nitrogen deficiency, in agreement with present work results, for *Chlorella vulgaris*[48] and *Spirulina platensis*[49]. It is not straightforward to offer an explanation for the observed effect; it can however be remarked that it recalls similar increases under stressful condition of other biomass components such as lipids.

As already observed, cells grown in the basic medium showed an increase of lipid content and a decrease of polyunsaturated fatty acids (PUFAs) and chlorophyll under flashing light compared to continuous light. This may be explained again as a combination of the effect of flashing with the effect of the starvation, that brings to accumulation of lipids and a degradation of chlorophyll. In fact, as observed by Simionato et al., *N. gaditana* grown in nitrogen depletion accumulates less chlorophyll and more carotenoids than in non-limiting conditions[24]. In the same way, also Forján et al[50] observed an accumulation of carotenoids in nitrate and phosphate limitation in *Nannochloropsis*, as well as Solovchenko et al.[51] observed a decrease in chlorophyll and an increase in carotenoids in *Parietochloris incisa* in nitrogen starvation. Thus, it is possible to hypothesize that by increasing the frequency of flashing light the effect of nutrients starvation is enhanced. On the other way, when cells are cultivated in nutrient replete condition, flashing light seemed to lead to the carotenoids accumulation instead of lipids, especially in the 25 and 250 Hz conditions. This observation confirms that there is a combined effect of flashing light frequency and nutrient concentration, furthermore confirmed by the statistical analysis of the chlorophyll content that appears to be affected by the interaction between these parameters and not by one of them.

The effect of flashing light on pigments accumulation has been studied before. For example, similarly to this work, Sforza et al. observed an increase in the quantity of chlorophyll/cell in *N. gaditana* grown with 1.5 g/L $NaNO_3$ under flashing light compared to the continuous light[10]. Other studies were addressed to the production of carotenoids, in particular

astaxanthin, from *Haematococcus pluvialis*, indicating an increase of the production when the algae were cultivated under flashing lights[5,6,52]. Another point is that the accumulation of pigments and in particular of chlorophyll, together with the already cited PUFAs increase, can be interpreted as a low-light acclimation response[53,54]. Considering that in all the analysed flashing light conditions the average light intensity is the same, the display of a low-light acclimation response may lead again to the hypothesis that rather than the average light intensity, the time cells spend in darkness affects the acclimation. To support this hypothesis, in another work by Abu-gosh et al.[55] observed a smaller high-light response in *Dunaliella salina* exposed to FL conditions compared to the continuous light control at the same average light intensity[55]. In particular, they observed that when frequency was increased from 10 to 50 Hz, the carotenoid/chlorophyll ratio decreased, indicating a lower light acclimation. Furthermore, when the duty cycle was decreased from 0.5 to 0.25 (with frequency and average light intensity stable), corresponding to higher dark period, the carotenoid/chlorophyll ratio increased showing a higher light acclimation response. It is interesting to point out that in the cited work the average light intensity employed was of 500 μmol s^{-1} m^{-2} against the 70 μmol s^{-1} m^{-2} employed in the present work. The impossibility to increase the average light intensity connected to the employed experimental apparatus is a big limitation of this study, and further work should be done in this direction to light up more effects of acclimation and photoinibition. These data, together with the ones exposed in the present study, may confirm the hypothesis that rather than to the average light, light acclimation is affected by the time the cells spend in darkness. Anyway, the acclimation response appears to vary when changing the flashing frequency, that consequently may have a role connected to the length of the dark or light periods.

In conclusion, the accumulation of pigments from *N. gaditana* is strongly influenced by the medium in which the algae were grown and also by the flashing light treatments. In fact, ANOVA analysis indicates a strong interaction between these two parameters in affecting chlorophyll and carotenoids content.

4. CONCLUSIONS

This work assessed the effect of flashing light on the growth and biochemical composition of *N. gaditana* grown in two versions of f/2 medium, the first one limited in nitrate and phosphate content and the other one enriched in them. *N. gaditana* had a worse growth performance under

flashing light conditions than under continuous light in both the basic and the enriched version of the medium. The effect of flashing light and nutrient concentration on biomass composition was also investigated. Results show that low and medium frequency flashing light increases total lipids content when cells are cultured in the basic medium. The opposite is observed for cells grown in the enriched medium. The fatty acid content was also analysed in order to assess lipid composition differences. Results point out that flashing light increases SFAs and MUFAs content for microalgae grown in the basic medium as well as PUFAs content when grown in the enriched medium. Pigment composition is also affected by flashing light. Under nutrient limitation flashing light decreases chlorophyll content and increases the carotenoids content, while, in the enriched medium flashing light increases both chlorophyll and carotenoids content. The above observations point out a combined effect of flashing frequency and nutrient concentration, not reported so far in the open literature to the best of authors' knowledge. In this case, the flashing light increases the effect of nutrient starvation, *i.e.* increasing lipid and carotenoids content. When the cells were cultivated in nutrient replete conditions, flashing light had the prevailing effect, showing a low-light acclimation response, *i.e.* increasing PUFAs and pigments. In conclusion, this work shows how, by combining nutrient starvation and flashing light effect, it is possible to stimulate the production of one or another high-value compounds.

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