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Transcriptomic and metabolomic adaptation of *Nannochloropsis gaditana* grown under different light regimes

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ABSTRACT

Omic technologies are a major source of information in understanding the cellular processes while their employment for studying microalgal biomass and productivity is rapidly expanding. Microalgae are known for their complex cellular metabolism. Environmental conditions affect intensely both their metabolic and transcriptomic profiles, resulting in production of numerous compounds with applications in pharmaceuticals, cosmetics, nutrition and biofuel. In an attempt to detect global changes occurring during environmental light alteration, an integrated omics approach was employed while the results were evaluated using different statistical approaches. An RT-qPCR based platform was utilized for the targeted transcript profiling of *Nannochloropsis gaditana* genes involved in primary and secondary metabolism, while the metabolite profiles were analyzed by GC–MS and GC-FID analytical methods. The combined transcriptomic and metabolomic results revealed extensive metabolic adaptations triggered by different chromatic qualities of light. In summary, an overall induction in both transcripts and metabolites, involved mainly in amino acid metabolism, was observed under red filtered light. Blue filtered light provoked decreased carbohydrate concentration but elevated polyunsaturated fatty acids content. Moreover, green filtered light induced the lowest responses in metabolite and gene transcript levels, indicating that its photons are poorly absorbed by *N. gaditana*. The current work suggests that spectral light changes leading to biochemical and metabolic manipulation of microalga *N. gaditana* can be accomplished by light filtering of solar irradiance, a cost-effective method which could be routinely applied in large scale photobioreactor cultivating systems.

1. Introduction

Over the past few decades, microalgae have attracted massive scientific interest due to their numerous advantages and applications. Microalgae annually contribute up to 45% of the planet's photosynthetic biomass [1]. Their ability to synthetize high value chemical compounds either by absorbing solar energy or under mixotrophic cultivation varies depending on growth conditions [2]. Optimization of parameters which regulate cell function is a critical step in microalgae exploitation. In many of these studies, the primary goal is to enhance the production of algal biomass and economically meaningful compounds while reducing production cost, concerning photosynthetic ability, light intensity, wavelength and photoperiod, which are crucial for algal growth and metabolite synthesis [3]. Therefore, light quality could stimulate photosynthetic and metabolic adaptations, presenting a powerful tool for the metabolic manipulation of industrially produced microalgal biomass.

Microalgae are capable of absorbing light, even if they are exposed to high irradiance, and can use photon energy for cell growth. However, over 150 μE m^{-2} s^{-1} photoinhibition is reported, which could be also caused by low irradiance [4,5]. A solution to the outdoor cultivation instability caused by unpredictable weather conditions is the indoor cultivation under artificial illumination, where microalgae could reveal their full potential. Consequently fluorescent lamps, LEDs or photovoltaic cells [1,6] in range of photosynthetically active region (PAR) are being used to create a platform for effective microalgal production. The

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unicellular marine strain *N. gaditana* selected in the present study, which belongs to the Eustigmatophyceae, is located globally and regularly used as aquaculture feed. *N. gaditana* is rich in lipid content, especially under stress conditions, consisting of omega-3 fatty acids [7]. The growing interest on *N. gaditana* is attributed to high concentration of eicosapentaenoic acid (EPA), which has been proven beneficial for human and animal health [8].

Growth of *N. gaditana* has been studied under different light intensities. Low and moderate intensity combined with a photoperiod of continuous light, results in better growth and higher lipid content [9,10]. Combination of moderate light and optimal temperature for microalgae growth (25 °C) favors high biomass, EPA, vaucheriaxanthin and β-carotene productivities [2], while low light intensity and low temperature cause synthesis of more EPA and slight decrease of biomass and lipid productivity [11]. *N. gaditana* can utilize a wide range of light intensities due to its photoacclimation ability [3], except for very high irradiance which causes photoinhibition [12]. High irradiance combined with nitrogen depletion hinders cell growth and triggers accumulation of neutral lipids in the form of TAG [13], while high irradiance with sufficient nitrogen supply favors biomass and TAG synthesis [14].

Research on microalgal response to light wavelength has revealed that cell growth and metabolism are both affected by light quality. Due to their light harvesting pigments, many microalgae exploit blue and red light photons more efficiently [15]. In *Nannochloropsis*, red and blue photons are absorbed by chlorophyll *a* while blue is also absorbed by the accessory pigments such as carotenoids [16]. Adaptation of light source could lead to accumulation of desirable cell products. Red light photons seem to accelerate cell cycle [6]. Photosynthetic reducing equivalents produced by *N. gaditana* were induced under red light illumination, which could be deposited as lipids and carbohydrates, enhancing microalgal lipid content [17]. Blue light was found to stimulate growth, resulting in biomass [18,19], chlorophyll *a* and accessory pigments content increase [19]. Application of more than one wavelengths has also been investigated [6] while red-blue light combination was found to enhance EPA synthesis [20]. Green light is not so effective for growth since instead of being absorbed it is reflected, though it has been previously shown to have a positive influence in lipid production [18,21].

In order to get a deeper insight into the molecular and biochemical mechanisms governing adaptation and productivity of *N. gaditana* grown under different light wavelengths, we have employed a combined omics approach including metabolomics, lipidomics and transcriptomics. Additionally, microalgal growth was also monitored in order to evaluate the effect of light quality on biomass production. Metabolomic and fatty acid profile under different light conditions verified by transcriptional analysis may reveal crucial differences in metabolic pathways which could point out valuable information for growth and high value products synthesis of *N. gaditana*. Thus, determination of the optimal light wavelength could ameliorate the commercial value of the specific strain, already being used for many applications [22–24]. It is demonstrated that light filtering is an economically feasible and practical method of applying low intensity narrow spectrum on large scale microalgal cultures, leading to cells enriched in high-value added compounds. Light filters are easily installed on photobioreactors for mass production of microalgae, avoiding high cost LED or fluorescent monochromatic illumination. Similar approaches have shown that light filtering stimulates cell growth and modulates the metabolic content of *N. gaditana* [25].

2. Materials and methods

2.1. Microalgae and culture conditions

N. gaditana strain B3 was grown in Fitoplancton Marino Company (El Puerto de Santa María, Cádiz, Spain). Stock cultures were grown in

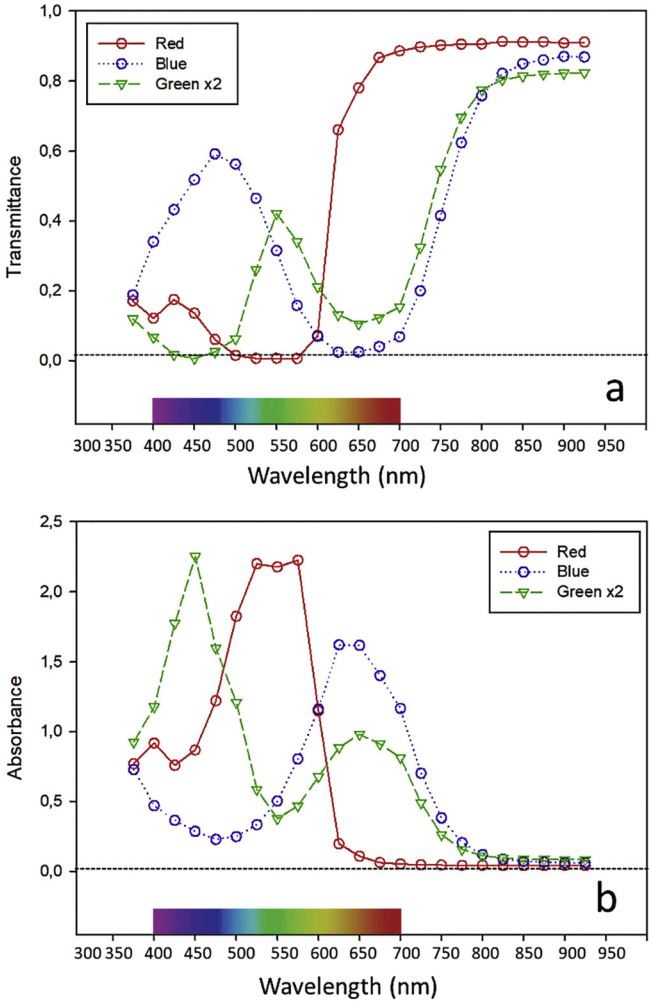

Fig. 1. Photometric measurements of a. transmitted and b. absorbed light by each light filter, within the PAR region. Data are shown as average of 3 repeats ($n = 3$).

5 L round bottom flasks and were used to inoculate the working cultures. Filtered f/2 was used as culture medium [26] using autoclaved seawater from bay of Cádiz at 27‰ salinity. Working cultures were grown in 1 L flat-bottom Erlenmeyer flasks filled with 625 mL of seawater, sealed with cotton cup and autoclaved. Culture medium was enriched with 2 mL of f/21000× nutrient solution. Flask inoculation was performed by adding 125 mL from stock cultures to obtain a final volume of 750 mL. Filtered air enriched with 5% CO_2 was constantly (24 h) supplied using glass pipettes. Low volume combined with continuous turbulence of cultures prevented auto-shading among microalgal cells. Initial cell density was measured just after inoculation in order to have the same start point for all experimental conditions.

In order to obtain different wavelengths, several colour filters were used (no filter, red, blue and green). In each case, the absorbance and transmittance wavelength range was measured between 350 nm and 900 nm (Fig. 1). The number of filters was adjusted accordingly in order to obtain uniform light intensity conditions in all filtered light treatments, which was no < 82% of the intensity of the full spectrum light. The calculation of light intensity was achieved by positioning a radiometer sensor (LI-250A underwater probe, LI-COR Biosciences, United States) and then it was adjusted to around 20 μmol m^{-2} s^{-1}. Additionally, a second radiometer instrument was used in order to verify the measurements of light intensity (MQ-501, Apogee Instruments, USA). Flasks were constantly illuminated by 2 daylight fluorescent lamps (T8 Luxline Plus, F58 W/T8/865, Sylvania). Three biological

replicates were set-up for each filtered light condition ($n = 3$). Flasks were aerated with filtered air enriched with 5% CO_2 and controlled every day for evaporation and salinity level. The cells were harvested for further processing at day 7 after inoculation, the cell density being measured with a hemocytometer.

2.2. Metabolomic analysis

Microalgal biomass was harvested from 750 mL cultures by centrifugation at 5000*g* for 5 min at room temperature. The pellet was resuspended in 50 mL of ammonium formate 0.53 M and centrifuged again. Finally the collected biomass was lyophilized and stored at −20 °C. 50 mg of lyophilized microalgal samples were grounded with liquid nitrogen. For each treatment, three independent biological repeats were analyzed, while two independent metabolite profiles were obtained for each biological repeat ($n = 6$). Pulverized biomass was then grounded with 395 μL methanol and 5 μL of 1 mg/mL ribitol. Samples were incubated at 70 °C for 15 min under continuous shaking. Then, 200 μL of chloroform were added and samples were incubated at 37 °C for 5 min under continuous shaking. Furthermore, addition of 400 μL of ddH_2O was followed by vigorous vortex and centrifugation at 13.000 rpm for 5 min at room temperature. The aqueous phase containing the polar metabolite fraction was evaporated with nitrogen gas. Final and essential step was the derivatization of samples. For derivatization, dried samples were resuspended in 50 μL of methoxyamine-HCl (15 mg mL^{-1} in pyridine) and sonicated for 10 min at room temperature. This was followed by incubation at 50 °C for 1 h and sonication for another 10 min at room temperature. Next step was the fast addition of 50 μL of *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (MSTFA +1% TMCS) and incubation at 50 °C for 1 h. An n-alkane mix was injected separately for the determination of retention indexes (RIs). GC/MS analysis was carried out on an Agilent 6890 GC coupled to a 5973i MSD. Separation was achieved on DB-5MS fused capillary column (60 m long, 0.25 mm internal diameter, film thickness 0.25 μm, J&W Scientific). Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. Split mode of injection was used and injection volume was 1 μL. The GC inlet temperature was held at 280 °C. The oven temperature program was as follows: initial temperature 80 °C (held for 2 min), followed by increase to 315 °C (held for 12 min) with rate 5 °C/min. Total run time was 60 min. The MS source was held at 250 °C and the quadruple at 150 °C, scanned from *m/z* 50–650. AMDIS (Automated Mass Spectral Deconvolution & Identification System) software, provided by the NIST (National Institute of Standards and Technology), was used to extract information of the compounds analyzed by GC–MS. Compound libraries Feihnlab and Golm metabolite databases were used for the identification of each component, according to its mass spectrum and retention time. Metabolite names accompanied with suffixes like TMS or [$+CO_2$] are compounds called analytes and are identified by Golm library. Analytes are the less polar and potentially volatile derivatized form of the actual metabolite and suitable for GC–MS profiling analysis.

2.3. Fatty acid analysis

Lyophilized microalgae were analyzed for fatty acids according to the method of Laurens et al. [27]. For each treatment, three biological repeats were analyzed ($n = 3$). Briefly, in 15 mg lyophilized microalgae, 0.2 mL chloroform/methanol (2:1 *v*/v) solution (Merck KGaA) and 0.3 mL HCl/methanol (5% v/v) (Merck KGaA) solution were added simultaneously. The mixture was heated for 1 h at 85 °C in the presence of 250 μg tridecanoic acid methyl ester (Fluka Analytical, Sigma-Aldrich Co., USA) as internal standard. The resulting fatty acids methyl esters (FAMEs) were extracted with 1 mL hexane at room temperature for at least 1 h. The FAMEs were analyzed by GC-FID (Agilent 6890 N, Centerville Road, Wilmington, USA) with an HP 88 column (60 m × 0.25 mm i.d. with 0.20 μm film thickness, Agilent). Flame ionization detector temperature was set at 260 °C, and the chromatographic analysis involved a temperature programmed run starting at 50 °C and held for 5 min. The temperature was initially increased at a rate of 5 °C/min to 150 °C followed by an increase at a rate of 1.5 °C/min to 210 °C, at which it was eventually maintained for 5 min. Hydrogen was used as the carrier gas with a linear velocity set at 30 cm/s. Each peak was identified and quantified using a 37 component FAME mix standard (Supelco, Sigma-Aldrich Co., USA).

2.4. Gene expression analysis using RT-qPCR

To detect changes in gene expression under different filtered light conditions, transcript levels were measured under continuous exposure to multichromatic light and were compared with transcript levels under filtered light. For each treatment, three biological repeats were analyzed ($n = 3$). Most genes studied coded for enzymes involved in basic metabolic processes in microalgal cells such as glycolysis, tricarboxylic acid cycle (TCA), pentose phosphate cycle, carbon fixation, fatty acid biosynthesis, photorespiratory nitrogen cycle, and sulfur and amino acid metabolism. Moreover, transcripts involved in light signaling [28], GABA biosynthesis and oxidative stress responses were tested for differences among experimental conditions.

Microalgal cells reached the desirable cell density for further processing on the seventh day. Transcript levels were measured after the exposure of cells to filtered light and compared to those exposed to full spectrum (control) in order to detect differences among light regimes. For quantitative real-time PCR (RT-qPCR) analysis, total RNA was isolated using the NucleoSpin® RNA Plant kit (Macherey-Nagel) including on-column DNase I treatment. Thereafter, cDNA was synthesized using the iScript™ cDNA Synthesis kit (Bio-Rad). In order to normalize small differences in the amount of the starting cDNA template, the expression levels of the previously identified reference genes Nga_actin (Nga07090), Nga_TUA (Naga_100021g70), Nga_UBE2N (XM_005853577.1) and Nga_UBE3C (XM_005856272.1) were used as internal controls [29,30]. All primers (Table S3) were designed with Primer Express (Applied Biosystems) software and tested for self- and hetero-dimers.

Quantitative RT-PCRs were performed on StepOnePlus™ Real-Time PCR System (Applied Biosystems) using SYBR Green with ROX PCR MasterMix (Applied Biosystems). Reaction volume was 10 μL and expression levels of the housekeeping genes were used as internal standards to normalize small differences in cDNA template amounts. PCR program included 10 min incubation at 95 °C and 40 cycles with 15 s at 95 °C and 1 min at 60 °C. Amplification efficiency (E) was assessed using the LinReg PCR program [31]. Analysis of expression data was performed using the algorithm $E^{-\Delta Ct}$ where ΔCt is Ctgene-GeoMean Ctreference genes.

2.5. Statistical analysis

All graphic presentations were developed using SigmaPlot 12.0 software (Systat Software). Statistical significance of the results was evaluated by analysis of variance (ANOVA) followed by Tukey's HSD (Honestly Significant Difference) or Fisher's Least Significant Difference (LSD) multiple comparison tests at a 95% level of significance ($p < .05$), using IBM SPSS Statistics 23. GC–MS method is used for universal metabolite detection thus strict Tukey's HSD post-hoc test was used for metabolomic analysis. For GC-FID data a less strict post-hoc test was used (Fisher's LSD), since it is a sensitive and common detection method for fatty acids. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using the software SIMCA version 15.0.2 (Umetrics). For all PCA models Hotelling's square contribution (T^2) was calculated with significance level 0.05. Heat map and hierarchical cluster analysis were calculated using the online platform MetaboAnalyst version 3.0 [32].

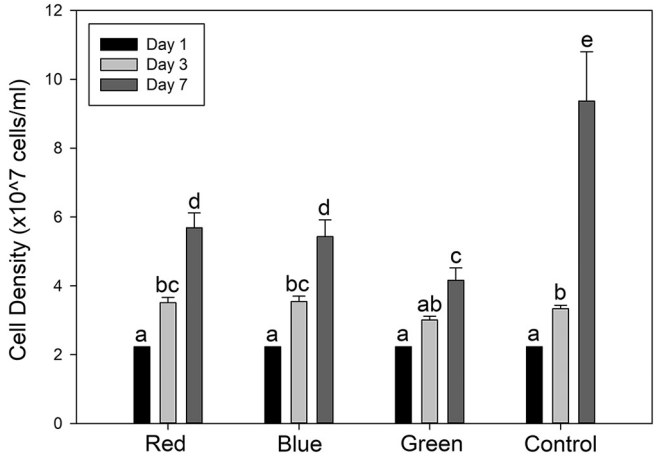

Fig. 2. Cell density of *N. gaditana* cultures grown under red, blue and green filtered light source as well as control conditions. Measurements were taken on first, third and seventh day of growth. Different letters indicate statistically significant difference ($p < .05$, Tukey's HSD). Data are shown as average +/-SD of 3 biological repeats ($n = 3$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. Cell density productivity

N. gaditana was cultured under both full spectrum (control) and filtered light illumination regime. Results from a pilot experiment, indicated that growth differentiation and highest density of algal culture are reached on the third and seventh day respectively. In terms of cell density, microalgae growth under white light proved the most efficient. According to one way ANOVA test ($p < .05$) followed by Tukey's HSD test, no significant differences were observed between cultures illuminated with red and blue filtered light. Growth rate under filtered green light was significantly lower in comparison to red and blue light. The highest cell density among different light sources was observed on the seventh day of red filtered light application with values ranging from 5.2 to 6 $\times$ 10^7 cells/mL (Fig. 2).

3.2. GC–MS based global metabolomic analysis of N. gaditana under different light wavelength

GC–MS analysis, performed in order to evaluate the global metabolomic effect of filtered light variation on *N. gaditana*, revealed the presence of > 100 compounds including sugars, amino acids, organic acids, nitrogen containing compounds, phosphates, polyols, etc. (Table S1). In order to create an overview of the X-variables with a principal component model and uncover groupings, trends and outliers in the data derived from each filtered light condition, model type PCA-X was used. The two principal components accounted for 43.7% of the total variation in these four treatments and most of the samples were categorized according to light quality. The first principal component R2X [1] accounted for 32% of the total variance in metabolite levels and separated samples of red, blue and green filtered light from those of control. However, according to first component, samples of blue filtered light exhibited similarity to those of green. The second component R2X [2], which accounted for 15.4% of the variance, resulted in similar distinction among the four conditions to the first component (Fig. 3). Control light grown cells are located mostly in the negative right quadrant of component 2 axis while red grown cells are distinctly grouped in the positive right quadrant of the same axis. Blue and green light grown cells seemed to cluster together with overlapping regions on the plot. According to Hotelling's T^2 only one value (sample 9 - blue light) was defined as suspected outlier due to its slightly increased value compared to 95% confidence limit; however it was included in the PCA model since it did not exceed the 99% confidence limit (Fig. S1).

Metabolic adaptation to light quality was evaluated by the response ratios of filtered versus control light of each compound. Concerning the metabolites tested, ANOVA ($p < .05$) detected 54 that changed significantly and more than half of these reached their highest relative response under red filtered light. Although filtered blue and red light gave almost the same cell density, red significantly affected microalgal metabolomic profile. In general, sugars and organic acids proved to be higher under control conditions while amino acids, nitrogen containing compounds, polyols and phosphates were observed to be accumulated under red filtered light. Green filtered light, apart from low growth rate also caused lower metabolite responses.

Sugar content (Fig. 4) mainly remained stable under filtered light irradiance. However, cellobiose, D-(+)-trehalose and 2-amino-2-deoxy-D-galactose levels declined significantly under all filtered light sources. Additionally sorbose and D-allose levels decreased under green filtered light. Galactose concentration also decreased under filtered light except for red. In the polyol group, significantly reduced concentrations of lactitol, mannitol and palatinol were observed under blue and green filtered light; however red filtered light induced higher accumulation of allo-inositol (almost 2-fold). Glycerol-1-phosphate concentration was remarkably high under filtered red light (> 3.5-fold). Malic and itaconic acid were only produced under control and red filtered light, 3-phenyllactic only under red filtered, and lyxonic only under white light. Moreover, succinic, citric and tartronic organic acids were significantly reduced in filtered green light treated microalgal cells.

An important metabolic adjustment of the organism subsequent to red filtered light application was the high accumulation of amino acids and nitrogen containing compounds. L-alanine, L-serine and ornithine exhibited 1.5-, 1.8- and 6.5-fold increased concentration respectively after 7 days of culture. Asparagine presented the highest accumulation (13-fold) of all amino acids under red; although it was not produced by blue and green light treated cells. Tyrosine accumulation remained unaltered under red filtered light; still it was lower under blue and green filtered irradiance. One evident exception was proline, as it was considerably increased under filtered blue light. Pipecolic acid, pyroglutamic acid and citrulline, metabolites included in the group of nitrogen containing compounds, were also positively influenced by red filtered light. Furthermore *N*-methylalanine was exclusively produced under red filtered light. Apart from 4-guanidinobutyric acid, adenosine, 2-amino-2-methyl-3-hydroxy-propanoic acid and uracil significantly reduced concentrations, green filtered light did not affect the concentration of nitrogen containing compounds. 2-amino-2-methyl-3-hydroxy-propanoic acid and uracil were also decreased under blue filtered light. The strong impact of filtered light on *N. gaditana* amino acid metabolism was also noticeable by PCA analysis, where the top contributors to metabolic variance were amino acids and nitrogen containing compounds, including proline [+CO_2], glutamine [-H_2O], guanine, *N*-methylglutamic acid and pyroglutamic acid (Fig. S2).

3.2.1. Metabolite-metabolite correlation analysis

Correlation analysis is a widely used tool for the interpretation of large-omic datasets obtained from numerous organisms [33,34]. However, it has never been used to study microalgal metabolomes. In this context, a hierarchical cluster analysis (HCA) of identified compounds was conducted to explore changes in patterns between the reviewed wavelengths, based on the Pearson *r* correlation coefficient of metabolites (Fig. 5 and supplemental excel file of the correlation matrix). Correlation has always to be interpreted according to the experiment that it is applied to [35]. In such a manner, significant correlations were considered those with either $r > 0.5$ or $r < -0.5$ according to previous metabolomic studies [36,37].

Red filtered light induced 45% negative and 55% positive correlations. Sugars were negatively correlated with many of the identified

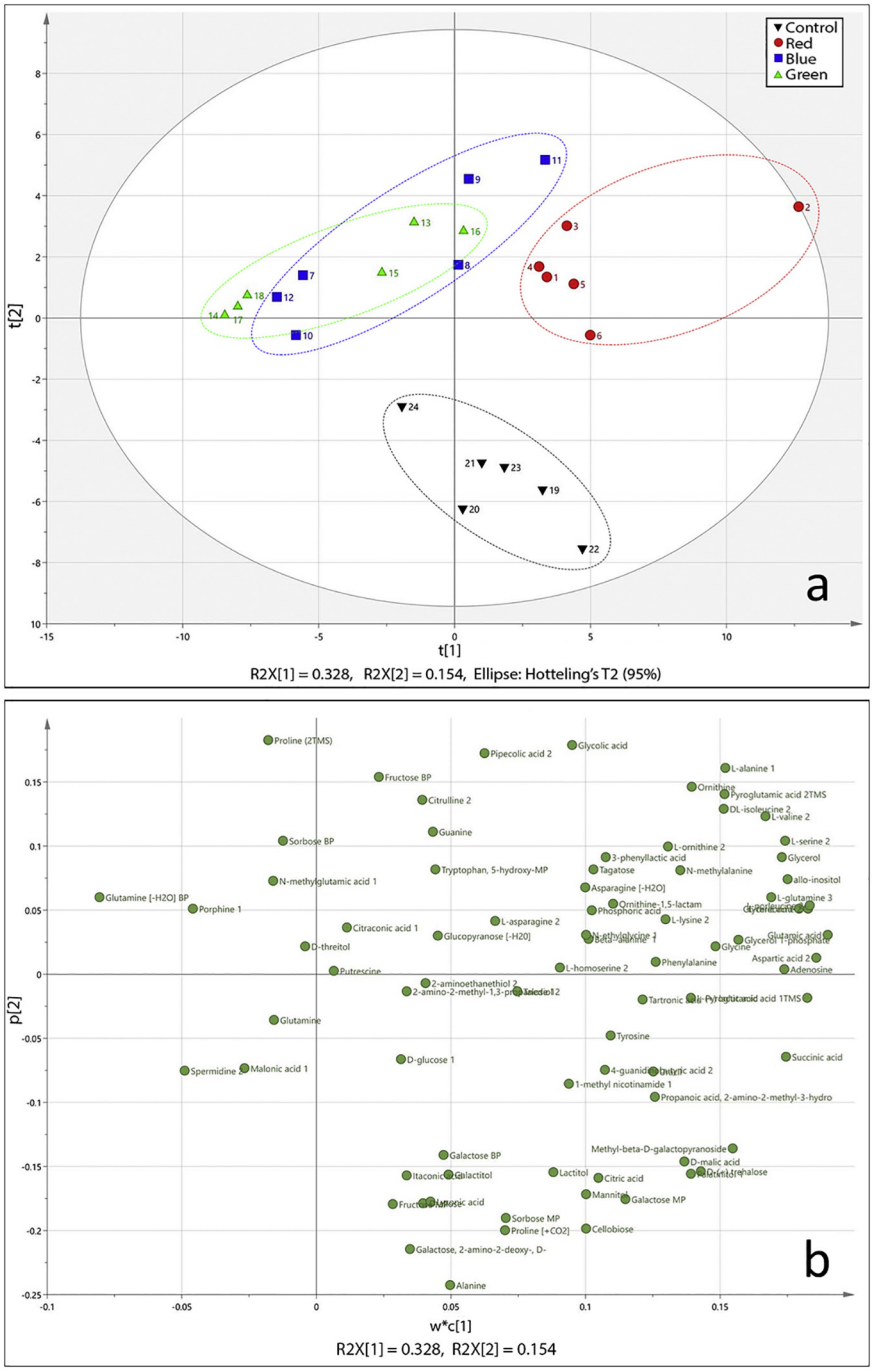

Fig. 3. PCA plots (a. Score plot, b. Loading plot) of metabolite profiles from cells treated with different filtered light conditions. The percentages listed on the axis labels describe the fraction of variance explained by the first (RX2 [1]) and second (RX2 [2]) principal component. For each treatment, three biological and 2 technical repeats for each are presented (n = 6).

metabolites. Three clear exceptions were fructose BP, talose and tagatose which were separately clustered from the rest of sugars and their accumulation was positively correlated with some of the identified compounds; however only for tagatose these positive correlations were significant. Noteworthy, negative correlations were only detected in a few cases including alanine, spermidine, galactose MP and D-(+)-trehalose, which obtained low coefficient factors under filtered red light.

Polyols were divided into two groups located in different clusters of the HCA, giving two response patterns to red filtered radiation. First group of palatinitol, glycerol and allo-inositol, whose content was increased under filtered red light, obtained significant positive correlations mostly with some amino acids and nitrogen containing compounds. Second group of D-sorbitol, lactitol, mannitol, D-threitol and galactitol was not significantly correlated with the rest of identified metabolites. The only exceptions were D-threitol and galactitol which had only a few significant positive correlations derived from the comparison with some organic acids (citric, D-malic, lyxonic), alanine, proline [CO_2], porphine 1 and 2-amino-2-deoxy-D-galactose. Exposure to filtered red light led to positive correlations of the majority of nitrogen containing compounds with amino acids, indicating that these two groups responded similarly to light quality changes. Similar to polyols, organic acids were separated by HCA into those that are significantly accumulated under red filtered light and have a strong positive correlation with some amino acids and nitrogen compounds (L-

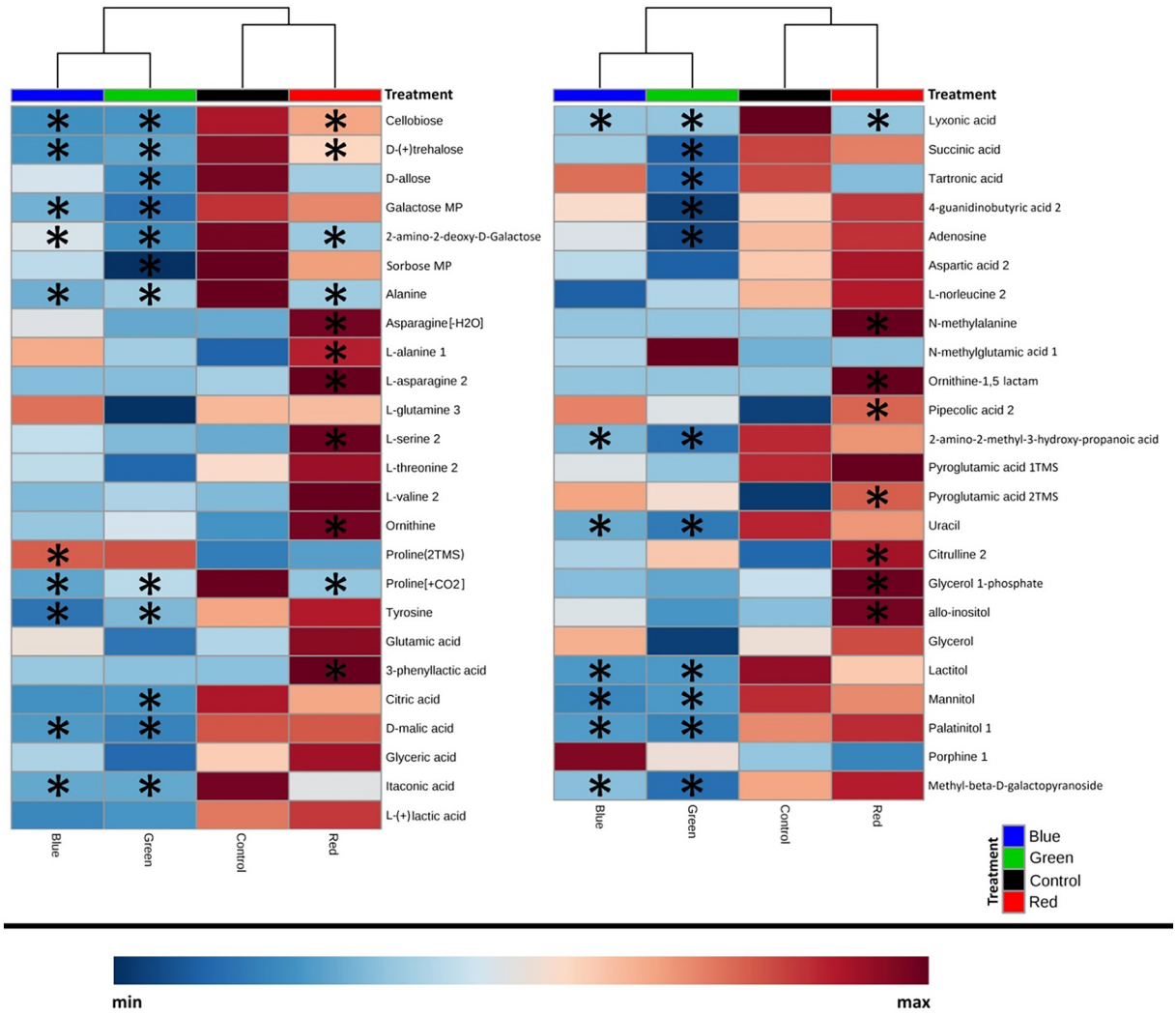

Fig. 4. Metabolic heatmap of *N. gaditana* as a response to light quality. Data are shown as means of three biological and 2 technical repeats for each (n = 6). Each metabolite was examined separately and compared to control light. Asterisks indicate significant differences of metabolites compared to control ($p < .05$, Tukey's HSD).

lactic, glyceric) and those whose concentration is not favored under the particular treatment (citric, itaconic, lyxonic, tartronic) and are mostly correlated with $r < 0$.

Although number of positive (61%) and negative (39%) correlations was comparable between blue and red filtered light, their heatmap patterns were clearly distinct (Fig. 5b). Under blue filtered radiation, a general reduction in metabolic content was observed, which in a few cases was sharp (Fig. 4). This overall decline resulted in plenty of significant positive correlations. Important negative associations were limited and most of them concerned glutamine. The small dark red pattern of heatmap derives from particularly high values of the coefficient r and mainly represents the correlations between amino acids and nitrogen compounds. However, the second dark red square of the left corner derives from strong and positive correlations mostly among polyols (D-threitol, galactitol, lactitol, mannitol, palatinitol), organic acids (L-lactic, succinic, lyxonic, citric, malic) and sugars (2-amino-2-deoxy-D-galactose, fructose MP, galactose BP, cellobiose, sorbose MP, D-allose).

Regarding cells grown under continuous exposure to green filtered light, 68% of the correlations were positive and 32% negative. The number of significant positive correlations was considerably higher compared to the aforementioned treatments. The overall negative impact of green filtered light on the metabolic rate of *N. gaditana* was clearly responsible for this specific effect. Proline, citrulline and glutamine [-H_2O] were the only metabolites being correlated with $r < -0.5$ in many cases, depicting their higher concentration under green filtered light. As already mentioned for filtered blue light, many of positive correlations derived from the comparison between amino acids and nitrogen containing compounds. However, members of the large positive correlated area on the heatmap (Fig. 5c) were also some polyols (mannitol, galactitol, palatinol), sugars (D-allose, galactose, cellobiose, sorbose, fructose) and organic acids (succinic, tartronic, citric, lyxonic, D-malic, L-lactic).

3.3. Analysis of fatty acid composition

Microalgal lipids from *N. gaditana* were extracted and converted to FAMEs whose compositions are summarized in Table 1. According to literature, C16, C18 and C20 are the most abundant fatty acids across *Nannochloropsis* strains [38]. In the current experiment identified fatty acids were in the range of C14–C24; the most abundant palmitic (C16:0), palmitoleic (C16:1) and eicosapentaenoic acid (C20:5n3) reached approximately 82% of total fatty acid methyl ester content. Other identified FAMEs were myristic (C14:0), oleic (C18:1n9), linoleic (C18:2n6) and dihomo-alpha-linolenic (C20:3n3). Except for palmitic acid concentration, which remained constant across all light regimes,

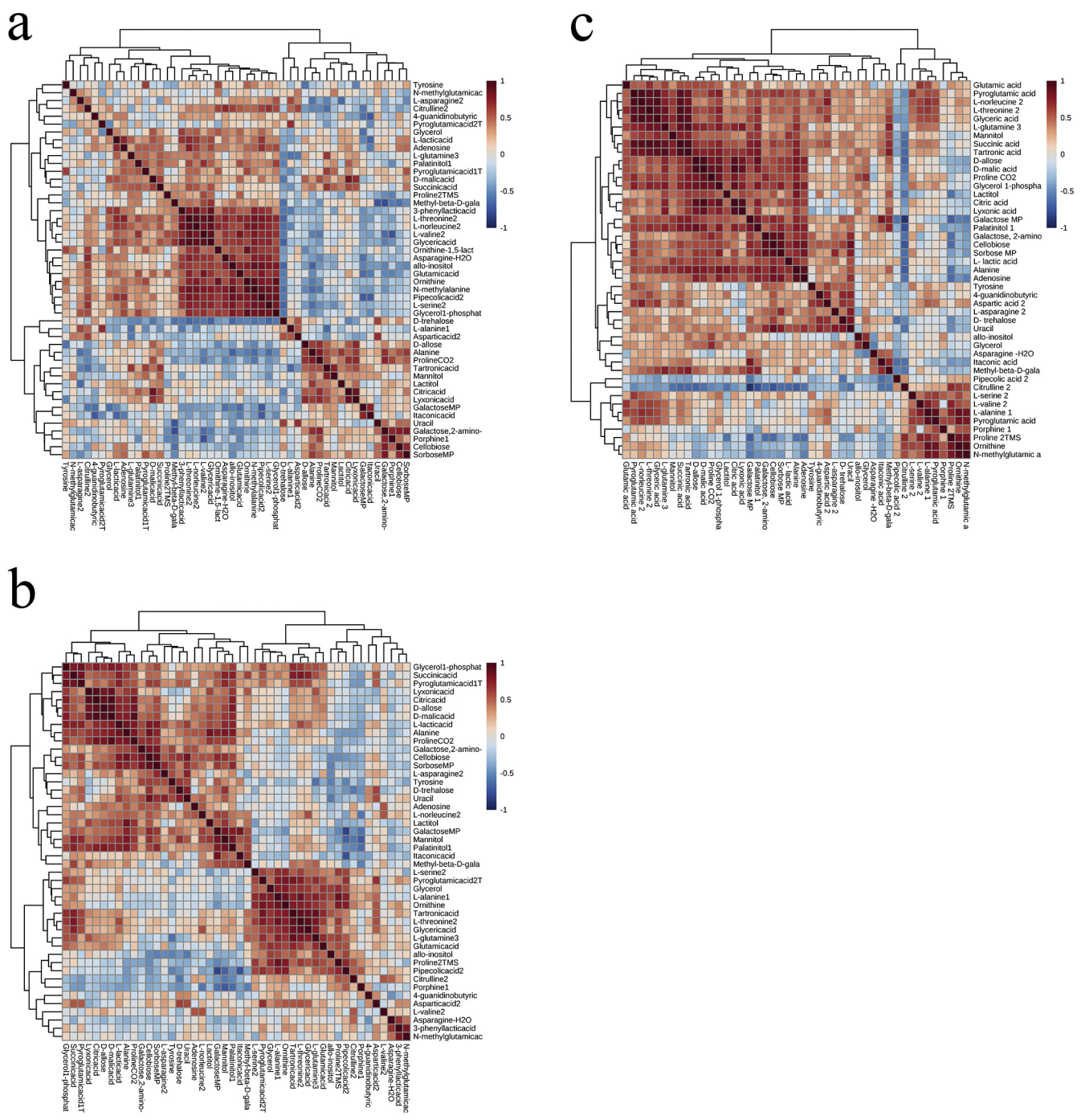

Fig. 5. Hierarchical cluster analysis of significantly changed metabolites ($p < .05$). Heatmap of metabolite-metabolite correlation is displayed in response to light quality. Correlation coefficients are shown as means of three biological and 2 technical repeats for each (n = 6), based on Pearson's linear correlation sampling coefficient (r). Positive correlation is shown with red and negative with blue. **a**. Correlations under filtered red light, **b**. Correlations under filtered blue light, **c**. Correlations under filtered green light. In all three cases correlation coefficients were calculated as response to filtered light irradiance compared to white light. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

fatty acid levels of *N. gaditana* were influenced by filtered light. Green and blue filtered light provoked a sharp decrease in myristic and palmitoleic acid concentration respectively. Oleic acid was found to accumulate under filtered green and red light compared to full spectrum. Similarly linoleic acid levels were also favored under green and red filtered light but only a slight change was detected. Dihomo-alpha-linolenic acid was significantly accumulated under all types of filtered light according to LSD post-hoc test ($p < .05$). C20:3n3 fatty acid has been previously detected in other microalgal species but in lower percentage of the total fatty acids [39]. Additionally EPA content, which is mainly responsible for putting *N. gaditana* on scientific forefront, did not exceed the conventional level of statistical significance under none of light quality regimes; though there was a definite trend ($p = .083$) under prolonged blue filtered light application.

Table 1 depicts the amount of saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acid values. Microalgal cells produced similar amount of SFA under all wavelengths. MUFAs concentration was significantly decreased under filtered blue light; however the highest amount of PUFAs was obtained under this filtered light (39.08%). In PLS-DA model, the first principal component R2X [1]

Table 1
Composition of main fatty acids (% TFA - % total fatty acid) of *N. gaditana* after a week grown under different light wavelength conditions. Data are presented as average ± SE of 3 biological repeats ($n = 3$). Different letter exponents indicate statistically significant differences ($p < .05$ - LSD).

		Control		Green		Red		Blue	
		Content (%)	SE	Content (%)	SE	Content (%)	SE	Content (%)	SE
C14:0	Myristic	7.7^{b}	0.2	6.6^{a}	0.1	7.1^{ab}	0.4	7.3^{ab}	0.5
C16:0	Palmitic	24.7	0.8	24.7	0.3	25.1	0.8	23.6	0.8
C16:1n7	Palmitoleic	34.7^{b}	0.5	33.0^{ab}	0.2	34.1^{ab}	0.7	32.4^{a}	0.8
C18:1n9	Oleic	2.1^{a}	0.1	3.5^{c}	0.1	2.5^{b}	0.1	2.2^{a}	0.1
C18:2n6	Linoleic	1.6^{ab}	0.0	1.7^{b}	0.1	1.7^{b}	0.0	1.5^{a}	0.1
C20:3n3	Dihomo-alpha-linolenic	3.6^{a}	0.1	5.1^{b}	0.1	4.8^{b}	0.3	4.7^{b}	0.3
C20:5n3	Eicosapentaenoic	24.5^{ab}	1.0	25.2^{ab}	0.1	23.8^{a}	1.7	28.2^{b}	1.8
SFA		32.4	1.0	31.2	0.3	32.2	1.2	31.0	1.3
MUFA		37.5^{b}	0.2	36.8^{b}	0.1	37.5^{b}	0.8	34.6^{a}	0.9
PUFA		32.5^{a}	2.4	37.1^{ab}	0.4	35.1^{ab}	2.3	39.1^{b}	2.3

accounted for 40.3% of variance in FAMEs levels and separated samples of green filtered light from those of control light. The second principal component R2X [2] explained 33.3% of variance and allowed the distinction among red and blue light filtered samples (Fig. 6a). The plot of the first two components, which combined explain 73.6% of variance of fatty acid compositional data, illustrates the successful clustering of all samples according to light quality. The lowest dispersion among replicates was observed for control light.

3.4. Analysis of gene expression

Targeted transcriptional profiling of *N. gaditana* cells in response to control light and filtered light source for red, blue and green was also employed to study the influence of illumination on metabolism. Quantitative real-time PCR (qRT-PCR) analysis performed using a platform containing 104 gene-specific primer pairs (Table S3) for genes involved in various functions of microalgal metabolism.

In order to detect changes in gene expression, transcript levels were measured after the exposure of cells to 7 days of filtered light and compared to those exposed to full spectrum. ANOVA revealed 52 genes that were significantly up- or down-regulated following the application of these filtered light conditions to microalgal cultures ($p < .05$) (Table S2). The score plot of the PCA analysis showed clear classification of the different illumination treatments (Fig. 7a). Variation in microalgal gene transcripts can be explained by the first two principal components which accounted for 53.6% of the variation. The first principal component R2X [1] accounted for 42.4% of the total variance in transcript levels and revealed distinction among filtered light regimes and control light conditions. The second component R2X [2], which accounted for 11.2% of the variance, resulted in similar distinction among the four conditions to the first component (Fig. 7a). Control and blue filtered light grown cells were located in between of right and left negative and right and left positive quadrants respectively of the component 2 axis. Cells grown under red filtered light were located on the upper right quadrant while those cultured under filtered green light were distinctly clustered on the upper left quadrant of the component 1 axis of the plot.

Filtered light irradiance induced differentiated expression levels for several genes (Fig. 8); however, the effect of red and green filtered light was more intense. The highest relative response for 59 genes was achieved under red filtered light, though only in some cases this differentiation was statistically significant. Despite the fact that the majority of these genes were involved in amino acid metabolism, some exceptions were noticed in almost all pathways. There were only a few significant differences in gene expression levels caused by blue while green filtered light triggered the lowest expression of almost all genes under study.

In glycolysis pathway, variance between full spectrum and filtered light irradiance was limited. Specifically, greater differences were observed mainly between filtered green light and either control or red filtered light. Two genes encoding for enolase (*ENO1*, *ENO2*) were down-regulated under green filtered light (almost 2.7-fold for *ENO1*). Pyruvate decarboxylase (*PDC*) was found to be down-regulated under all filtered light conditions while the most remarkable decrease was under filtered green light (4.6-fold). Acetyl-CoA synthetase (*ACSS1*) was more highly expressed under filtered red light (Fig. 8).

Within tricarboxylic acid cycle (TCA), transcripts for isocitrate dehydrogenase (*IDH1*), succinyl-CoA synthetase alpha subunit (*LSC2*) and succinate dehydrogenase (ubiquinone) flavoprotein subunit (*SDHA*) levels were down-regulated under green filtered illumination demonstrating the negative effect of this particular filtered light on such metabolic pathway. Moreover expression of pyruvate carboxylase (*PC*) gene was induced by filtered red light. Transcripts involved in carbon fixation metabolism remained almost invariable across treatments except for ribulose-bisphosphate carboxylase small chain (*rbcS*) and pyruvate orthophosphate dikinase (*PPDK*), which were down-regulated under filtered green and up-regulated under filtered red light respectively (Fig. 8).

Moreover, under red filtered light, transcripts for ferredoxin-$NADP^+$ reductase (*FNR*), cysteine synthase A (*cysK2*) of sulfur metabolism and thioredoxin reductase (*TXNRD_2*) of pyrimidine metabolism were found to be significantly up-regulated. Transcript levels for genes involved in oxidative stress metabolism, including two encoding for glutathione peroxidase (*GPx1*, *GPx3*) and two for superoxide dismutase (*SOD2_1*, *SOD2_3*) were significantly decreased under green filtered light (Fig. 8). Six out of 19 studied gene transcripts of fatty acid biosynthesis showed significant differences among treatments. Acyl-ACP desaturase (*ACP-DES*) was down-regulated under green filtered light compared to full spectrum. Additionally, transcriptomic levels of five genes involved in EPA biosynthesis were significantly affected by blue filtered light (Fig. 8). More specifically, endoplasmic reticulum Δ6-desaturase (*ERΔ6FAD*), endoplasmic reticulum Δ12-desaturase (*ERΔ12FAD*) and endoplasmic reticulum ω3-desaturase (*ERω3FAD*) were up-regulated, while Δ6 elongase (*Δ6-ELO*) was down-regulated under blue filtered light (Figs. 8, 9).

Prolonged exposure to red filtered light resulted in induced transcript levels of several genes involved in amino acid metabolism including one encoding for acetylglutamate kinase (*NAGK*), two for glutaminase (*GLS_1*, *GLS_2*), one for glutamate dehydrogenase ($NADP^+$) (*GDH2_2*) and one for glutamate synthase (NADPH/NADH) small chain (*gltD*). Genes which encode for ferredoxin-nitrite reductase (*NIR1*), glutamate decarboxylase (*GAD*), glycine (serine) hydroxymethyltransferase (*SHMT1* and *SHMT2*) and glycine dehydrogenase (*GLDC*) were down-regulated in cells grown under filtered green irradiance (Fig. 8). *SHMT2* concentration was too low to be displayed in Fig. 8 but it is included in Table S2 (Supplemental material). Finally, glycerol-3-phosphate dehydrogenase (*GPDH*) was significantly down-regulated under red and blue filtered light.

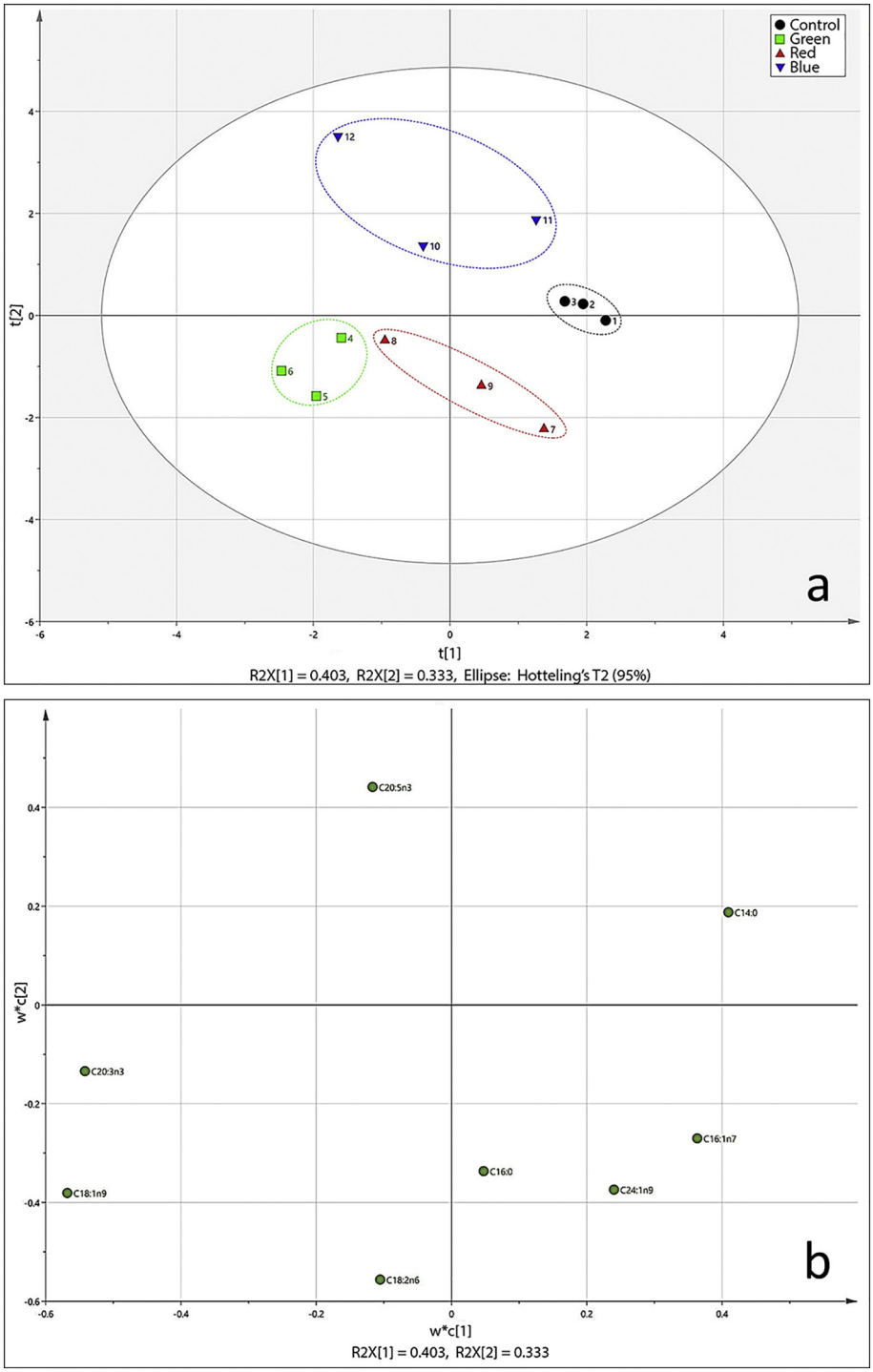

Fig. 6. a. Score plot, b. Loading plot of PLS-DA analysis of fatty acid compositional data of *N. gaditana* grown under different filtered light conditions. The percentages listed in the axis labels describe the fraction of variance explained by the first (RX2 [1]) and second (R2X [2]) principal component respectively. For each treatment, three biological repeats are presented ($n = 3$).

4. Discussion

It has been well documented that light represents one of the fundamental parameters affecting cellular physiology, metabolism and productivity in microalgae. In addition, light conditions have been shown to influence metabolic enzyme activities in *N. gaditana* [7]. However each species responds differently to light quality due to a high diversity of the photosynthetic apparatus [40]. At the molecular level, irradiance alterations are known to provoke reprogramming of gene expression in photosynthetic organisms [41]. Up to our date, research interest is mainly focused towards establishing the optimum light properties that could lead to optimization of production conditions for application-based microalgae biomass through metabolic induction strategies. Along this line, previous studies have indicated that changes in light quality could alter cellular growth, photosynthesis and fatty acid content of microalgae [42,43]. However, little is yet known about the global molecular and metabolic adaptations in microalgae, triggered by different light quality regimes. To that end, targeted

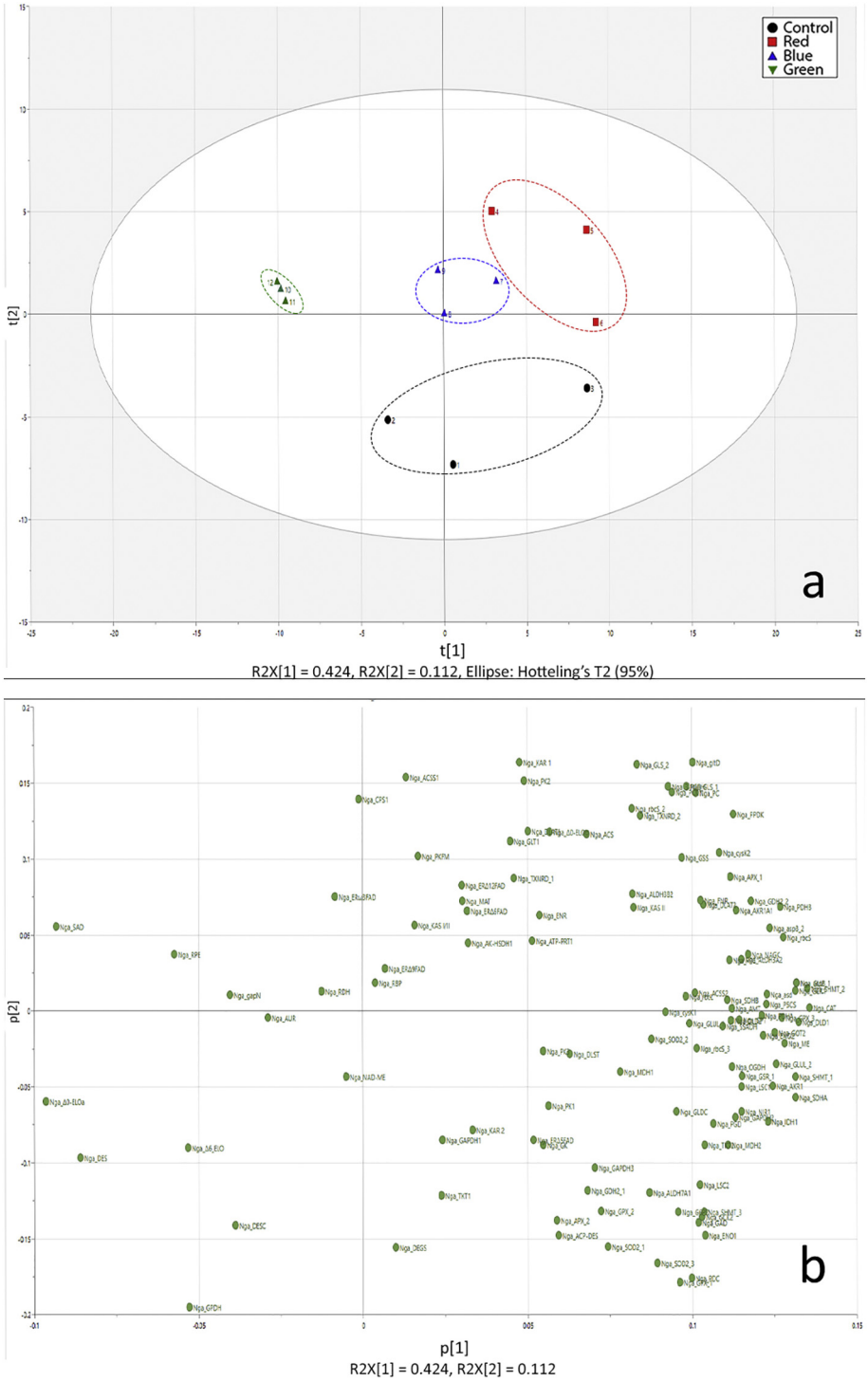

Fig. 7. PLS-DA plots (a. Score plot, b. Loading plot) of selected transcript levels from cells treated with different filtered light conditions. The percentages listed in the axis labels describe the fraction of variance explained by the first (R2X [1]) and second (R2X [2]) principal component respectively. For each treatment, three biological repeats are presented (n = 3).

transcriptomic and global metabolomic study were combined in order to obtain a better understanding of *N. gaditana* metabolic reprogramming as a response to light quality. Specifically, lipidomic and metabolomic profiling as well as a RT-qPCR transcriptomic platform have been employed for probing microalgal metabolism under different filtered light wavelengths. Light filtering could possibly be proven an effective metabolic manipulation strategy to microalgae for accumulating different cell products of economic interest even at a mass cultivation algae photobioreactor.

4.1. Light wavelength strongly affects various metabolic processes in N. gaditana

Under our experimental conditions, *N. gaditana* demonstrated decreased cell density compared to full spectrum. Nevertheless, in all three filtered light conditions, irradiance was sufficient to keep the cultures growing for many days and also to provide enough biomass yield for the experiments. Several methods have been employed in order to evaluate the effect of certain treatments on photosynthesis and

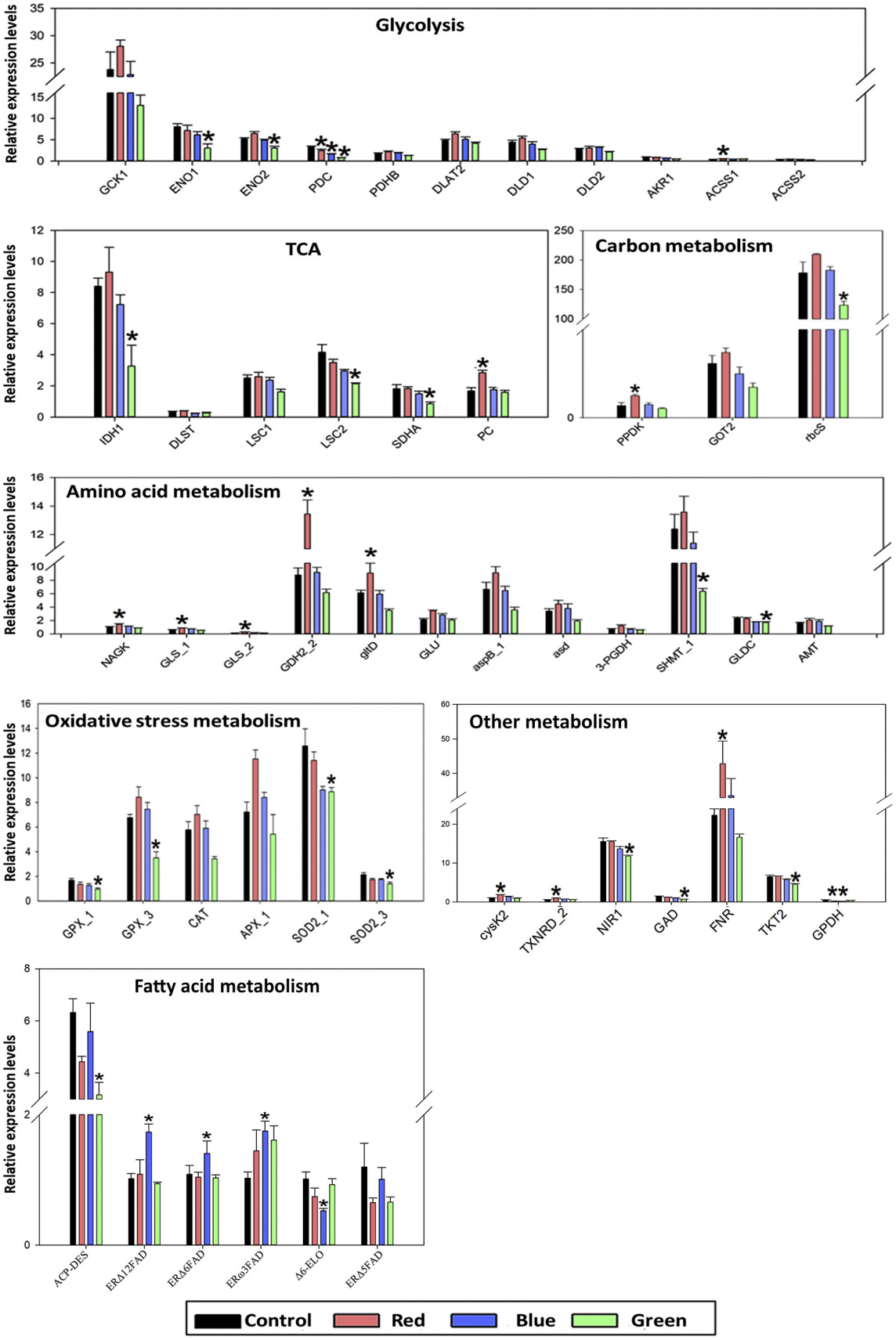

Fig. 8. Transcript levels of studied genes are displayed, normalized in respect to housekeeping genes and categorized into pathways. Statistically significant differences with regard to control are marked with an asterisk ($p < .05$, Tukey's HSD). Data are shown as average ± SE for 3 biological repeats (n = 3).

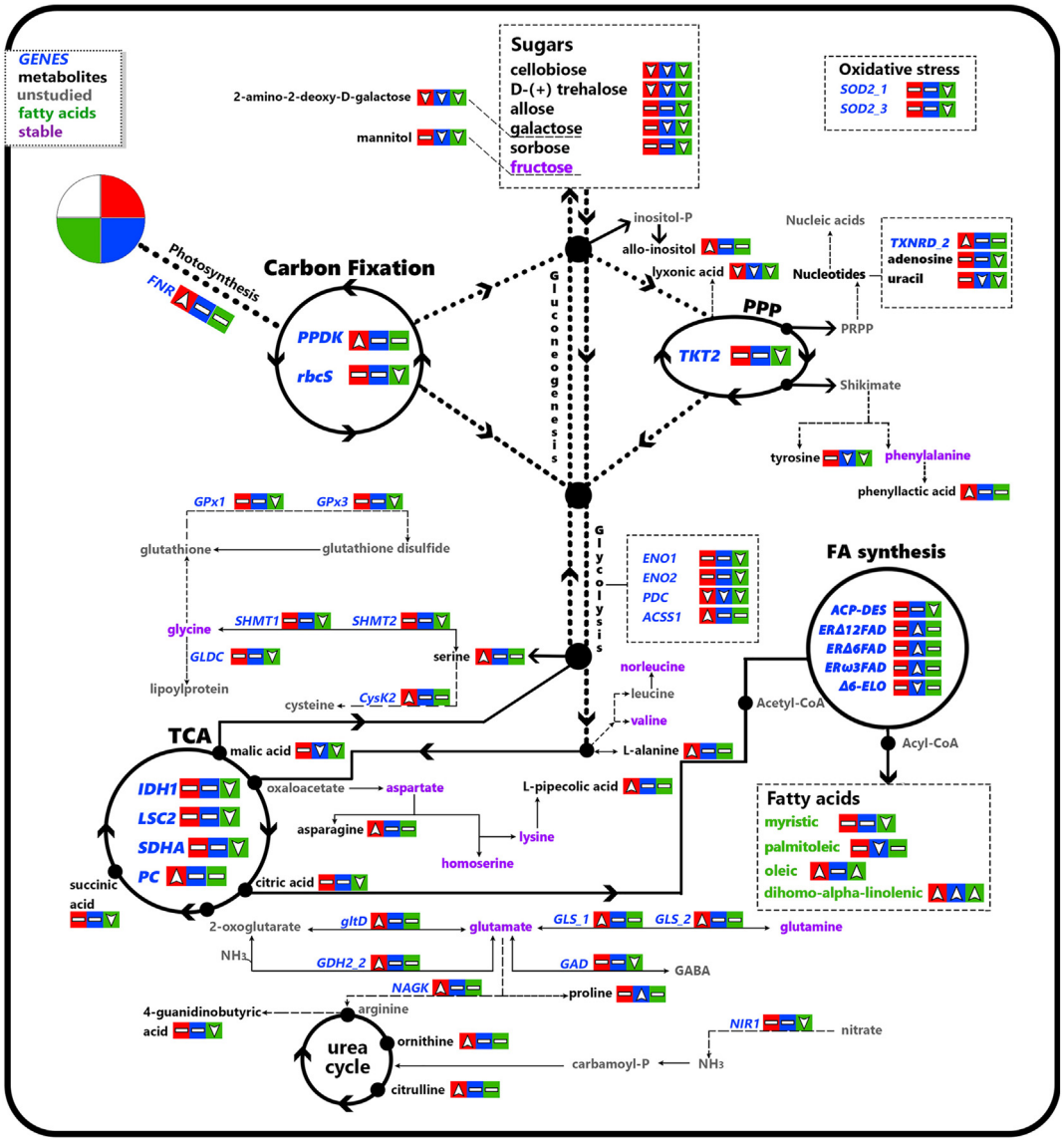

Fig. 9. Global effect of filtered light on *N. gaditana* metabolism. Blue letters represent genes and refer to the transcriptional study. Black and green correspond to metabolites detected by the GC–MS and GC-FID method respectively. Gray letters correspond to metabolites that are not yet studied. Finally, purple letters are used for metabolites with an unaltered response profile across all light quality regimes compared to white irradiance. The three colored squares next to each gene, metabolite or fatty acid correspond to the three light regimes tested (red, blue and green filtered irradiance) and include arrows in case of increasing or decreasing their accumulation while the unaffected levels are represented with a dash. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

growth of microalgal cultures. In similar studies concerning growth and lipid productivity of *N. gaditana* under filtered light, biomass dry weight measurement method was performed [25,104]. In the current study, narrow wavelength was generated by light filtering instead of monochromatic light source while the result was obtained by spectrophotometry. Similar growth was observed under red and blue filtered light, which could be attributed to partially overlapping spectra among these two light qualities (Fig. 1). In order to clarify this point, PAM analysis could be part of a follow-up more specialized manuscript. As reported by Vieler, Wu et al. *Nannochloropsis* was shown to perceive blue light; however there was no evidence whether it could also perceive red and green irradiance [45]. This hypothesis was based on lack of identified genes coding for phytochrome or rhodopsin proteins. In the current study, statistical analysis revealed many significant changes in specific microalgal transcripts and metabolites levels in response to light spectrum. In addition, GC-FID based fatty acid analysis revealed notable variations in fatty acid content among different light wavelengths.

Integrated metabolomic and transcriptomic analyses revealed increased levels in a range of metabolites and gene transcripts under red filtered light. Interestingly, a number of reports have demonstrated that light in the red spectrum exert a physiological effect on microalgae physiology. However, these results were mainly obtained by the use of monochromatic red light instead of red filtered light. Using this approach, differential regulation of gene expression has been demonstrated for several microalgae species including *Chlamydomonas reinhardtii* [46], *Cyanidioschyzon merolae* [41] and *Volvox carteri* [47]. In this work, with only a few exceptions (cellobiose, D-(+)-trehalose, 2-amino-2-deoxy-D-galactose), *N. gaditana* cells showed mainly unaltered response for sugars content (Table S1), accompanied by stable transcript levels of genes involved in glycolysis under filtered red illumination (Table S2).

Acetyl-CoA synthetase enzymatic activity is associated with many metabolic pathways including TCA cycle or fatty acid biosynthesis.

Moreover, it has been proposed that overexpression of acetyl-CoA synthetase gene introduced into the marine microalga *Schizochytrium* sp. TIO1101, might improve the utilization of carbon resource [48]. Thus, induction of acetyl-CoA synthetase under red filtered light represents a central metabolic adaptation and it could improve the properties of *N. gaditana* by altering the carbon flux towards metabolic pathways of interest. Interestingly, most of sugars measured, exhibited a negative correlation coefficient *r* with many of the other identified metabolites, thus showing a different response compared to the overall trend of cellular metabolism. Similarly, polyols with the exception of allo-inositol, were not significantly affected by narrow red light. Sugars and polyols are considered as carbohydrate protectors under abiotic stresses [49–51]. Thus, their unaltered concentration could indicate that red filtered light is not perceived as stressful agent by microalgal cells.

Monochromatic red light (600–700 nm) has already been reported as suitable wavelength for microalgal photosynthesis and biomass productivity [43,52], which results from the fact that chlorophylls, the most abundant pigments in plenty of species, absorb more efficiently red photons compared to remaining light wavelengths [52,53]. In such a way, red light has been employed in order to enhance biomass production of various microalgal species including *Chlorella vulgaris*, *Gloeothece membranacea* and *C. pyrenoidosa*. However, the longer the wavelength the lesser light energy is generated, making it harder for photons to penetrate high density or deep cultures. Notably, chlorophyll *a* is a major constituent of algal pigment composition in *Nannochloropsis* genus (Eustigmatophyceae) [54]. Our results showed that ferredoxin-$NADP^+$ reductase (*FNR*) and pyruvate, orthophosphate dikinase (*PPDK*) expression levels were significantly up-regulated, while ribulose-bisphosphate carboxylase small chain (*rbcS*) expression was also slightly increased under red filtered light compared to full spectrum (Fig. 8). *FNR*, *PPDK* and *rbcS* are known as key components of photosynthetic pathway and they are strongly influenced by light [55–58]. These results indicate that filtered light in the range of red spectrum could positively affect sugar metabolism and content in *N. gaditana* by enhancing photosynthesis.

Organic acid content remained unaffected during growth under red filtered light in comparison to the full spectrum light. Except for pyruvate carboxylase (*PC*), no important increase was detected in TCA cycle genes and metabolites under narrow red light (Figs. 4, 7, 9). Interestingly, statistical analysis revealed significantly increased content for various amino acids and nitrogen containing compounds in microalgae grown under that condition (Table S1). Moreover, up-regulation of amino acid biosynthesis pathway has been reported for the red microalga *C. merolae* as well; however it was caused by simultaneous application of monochromatic red and blue irradiance [41]. Several amino acids are produced from TCA cycle intermediates, while *PC* enzyme is responsible for carboxylation of pyruvate, forming oxaloacetate and participates in numerous metabolic pathways that depend on the availability of oxaloacetate such as gluconeogenesis, amino acid biosynthesis, glycogen synthesis, lipogenesis, etc. [59]. In conjunction, these results indicate that in *N. gaditana PC* could represent the main enzymatic activity responsible for the replenishment of TCA intermediates during active amino acid biosynthesis.

Transcript levels for several genes involved in amino acid biosynthesis were affected by the application of filtered red light. Even though L-glutamine concentration remained unaltered, transcript levels of two genes encoding for glutaminase (*GLS_1*, *GLS_2*), an enzyme which plays the primary role in ammonium ion incorporation into glutamine and glutamate, were significantly up-regulated under red filtered light. Glutamic acid also exhibited a minor increase in cells grown under narrow red irradiance. Concomitant with the above changes, NADH-glutamate synthase (*gltD*), and glutamate dehydrogenase - $NADP^+$ (*GDH2_2*) transcript levels were also increased under this specific light regime. In plants NADH-glutamate synthase genes are regulated by light and there is evidence that combined interaction of light and carbon signals influences transcript levels of several genes involved in nitrogen assimilation [60]. Moreover, NADP-specific glutamate dehydrogenase of *C. sorokiniana* has been shown to require light for both its induction and its continuous accumulation in fully induced cells [61]. In *Chlamydomonas* and *Chlorella* cells alanine and also malic, aspartic and glutamic acid have accumulated ^{14}C after prolonged growth under red light [62]. In *N. gaditana* accumulation of L-alanine and L-asparagine was substantially increased under prolonged red filtered light (Table S1). High accumulation of ornithine and citrulline, both intermediates of urea cycle, could be related to the up-regulation of acetylglutamate kinase (*NAGK*) and also to the general induction of nitrogen metabolism under red filtered light (Fig. 9). Increased accumulation of cysteine synthase A (*cysK2*) is probably related to the enhanced content of serine under narrow red irradiance, since *cysK2* uses serine as substrate for the L-cysteine synthesis. Subsequently, some nitrogen containing compounds also reached higher concentration under red filtered spectrum (Fig. 9).

Amino acids, in addition to their role as precursors and components for proteins, are involved in several cellular functions and therefore affect a number of physiological processes [63–65]. The absorption of nitrates into amino acids and nitrogenous compounds requires reduced equivalents and carbon backbones. Increased transcription of genes encoding enzymes involved in amino acid metabolism under filtered red light could be indicative of the tight interaction between carbon and nitrogen metabolism in *N. gaditana*. Thus, stimulation of photosynthesis through the application of red filtered light may possibly provoke increased flux of photosynthetic carbon directed to glycolysis, TCA, pentose phosphates and citric acid pathways and consequently to amino acid synthesis. It has been shown that amino acid content of algae depends on environmental conditions [105,67]. However the effect of light quality on amino acid and nitrogen metabolism of microalgae has been poorly examined. A study on the effect of UV-B radiation has demonstrated accumulation of mycosporine-like amino acids, though no clear correlation between accumulation and UV-B resistance of the photosynthetic apparatus was determined [106]. Thus, further investigation is needed to elucidate the microalgal cellular mechanism employed in order to adapt to different light conditions. The unaffected expression levels of gene transcripts involved in oxidative stress metabolism combined with the induction of photosynthetic *FNR* and *PPDK* genes under red filtered spectrum, suggest that this specific light regime is adequate for the photosynthetic needs of the culture. Therefore, reprogramming photosynthetic carbon flow in *N. gaditana* under filtered red light could be employed as an effective tool in order to enhance cellular amino acid production.

In addition, our results showed that application of filtered red light induced higher production of oleic and dihomo-alpha-linolenic fatty acids compared to full spectrum. A recent study has shown that a red LED light source of 30 μmol m^{-2} s^{-1} irradiance, placed 30 mm from the bioreactor has positively influenced growth and fatty acid production of *N. gaditana* leading to 2-fold higher FAME content than white light [17]. Moreover, filtered LED light obtained by a 4:1 mixture of red and yellow pigments, improved biomass productivity and FAME yield of *N. gaditana* cells compared to cells grown under white light [25].

Previous results have shown that blue spectrum could have a significant effect on microalgae growth. It has also been proposed that blue light causes enhanced chlorophyll production by changing the chloroplast ultrastructure [19,69]. However, its influence varies markedly among different microalgae species, as it has been shown to either promote or have no impact on cell density in a species depending manner [21,43,70,71]. In addition, below photosaturation irradiance *N. oculata* absorbs blue light more efficiently than red and can effectively direct it to photosystem II [15]. However, *Nannochloropsis* sp. growth has been shown to be negatively affected under blue light [19]. To our knowledge, there are no available studies addressing the effect of blue light derived from the filtering of full spectrum. To this end, our results showed that application of blue filtered light on *N. gaditana* cells

for 3 days had no significant impact on cell density, but after 7 days a significant decrease in population growth was observed. As reported previously for *Nannochloropsis*, in contrast to red light, blue light is not only absorbed by chlorophyll *a* but also by accessory pigments. Additionally, the strongly absorbed blue part of the spectrum was found to favor pigment and protein synthesis with little apparent growth and a high package effect in other microalgae species [72]. As a result, a possible biological interpretation of this cellular behavior could be the package effect. According to this phenomenon, accumulation of proteins coding for pigments induces reduction of absorption efficiency, leading to lower photosynthetic rate [73,74]. Blue wavelengths are more abundant when going into deep water. We know that under natural light conditions, high-energy blue light usually gets wasted and dissipated as heat if excess light energy is provided on the surface of high-density cultures. So, even though blue light is one of the best absorbed wavelengths it can cause photoprotection mechanisms. However, our experimental conditions ensure the uniform distribution of light by providing turbulence as means of CO_2 enriched air bubbling in small volume microalgal cultures. Additionally exposure to blue light is reported to result in delayed cell division [75,76]. Thus, even though filtered blue light might be efficiently absorbed, prolonged exposure to this light quality might trigger a decrease in cell density while *N. gaditana* cell diameter continue to increase.

According to our results, filtered blue light barely affected gene expression in *N. gaditana* in comparison to white control light (Fig. 9). Furthermore, transcript levels of blue light sensing genes studied remained stable under all different light conditions (Table S2). In contrast, metabolomic profiling revealed significantly declined metabolite responses of specific sugars, polyols and organic acids. The above metabolic adaptation could be attributed to higher utilization of stored cellular carbon in order to equilibrate the increased metabolic rate under blue light. Decreased carbohydrate concentration under blue light was also observed for other microalgal species [77]. Interestingly, proline exhibited increased content under this light regime (Fig. 9). In many plant species proline accumulation has been observed as a response to various environmental abiotic stress, were it effectively acts as a compatible solute [78]. In addition, GC-FID analysis showed a significant decrease of MUFAs and palmitoleic acid concentration, whereas, dihomo-alpha-linolenic acid was shown to accumulate under these conditions. Previous studies using monochromatic blue light (450–495 nm) have clearly demonstrated its positive impact on growth and crude oil production rate of *Nannochloropsis* sp. [21,79]. Moreover, a binary combination of LED-Blue and LED-Red gave the highest EPA productivity of *N. oceanica* CY2 [20]. In the current study, total PUFAs concentration indicated a moderate increase by filtered blue light which could be attributed to the relatively low intensity filtered light. Moreover, EPA content was shown to slightly increase under our experimental conditions. According to literature, stress conditions drive microalgae to use C16:0 and C18:0 to synthesize triacylglycerols as storage lipid form rather than to synthesize long chain PUFAs such as 20:5 [80]. Thus, the increase in 20:5 under filtered blue light seems to be indicative of a decrease in the proportion of 20:5 - poor triacylglycerol, which might reflect carbon reallocation from triacylglycerol storage form. Metabolic levels of glycerol remained stable under all three filtered light conditions (Table S1, Fig. 4), which could be related to the unaltered expression levels of glycerol kinase (*GK*) (Table S2). However, glycerol 1-phosphate was significantly increased under red filtered light (Table S1, Fig. 4), suggesting the involvement of glycerol phosphate dehydrogenase. Transcriptomic results of the current study demonstrated that glycerol phosphate dehydrogenase (*GPDH*) was significantly down-regulated under red and blue filtered light (Table S2, Fig. 8). Thus, increase of glycerol 1-phosphate could be provoked by another gene involved in its synthesis like glycerol-3-phosphate oxidase or sn-glycerol-1-phosphate dehydrogenase.

Transcripts of three genes coding for endoplasmic reticulum Δ6-desaturase (*ERΔ6FAD*), endoplasmic reticulum Δ12-desaturase (*ERΔ12FAD*) and endoplasmic reticulum ω3-desaturase (*ERω3FAD*), which are involved in EPA biosynthesis [81], were up-regulated during prolonged blue filtered light application. Lipid content of various microalgal species including *Tetraselmis* sp., *Nannochloropsis* sp., *Isochrysis galbana* and *Arthrospira platensis* cultivated under blue light was higher compared to white light [79,82,83]. Similarly, diatoms respond to abiotic stresses by inducing lipid synthesis and yield [84,85]. Increased expression levels of the aforementioned desaturases could be attributed to light stress since only a part of full spectrum was provided to the *N. gaditana* cells. It has been suggested that the ability of membrane lipids to desaturate fatty acids is important for the photosynthetic organisms to tolerate stress [86]. Moreover, Δ6 elongase (*Δ6-ELO*) was down-regulated under blue filtered light.

Under our conditions, the significant lower growth rates along with the global down-regulation of gene expression and metabolite responses, indicated that filtered green light is poorly absorbed and exploited by *N. gaditana*. The global decline of metabolism is also portrayed by the high percentage of positive correlations among identified metabolites under filtered green spectrum (Fig. 5c). Under these conditions, we observed decreased responses in many sugars, including cellobiose (17.5-fold), D-allose (7.5-fold) and galactose (10-fold). Similar results have been obtained using monochromatic green light, which had a negative effect to the growth of *Nannochloropsis* and other microalgal species. Furthermore, cultivation of *Nannochloropsis* sp. under green photons has been shown to gradually deteriorate to an unsustainable culture [19].

In particular, we observed that carbohydrate metabolism was affected at the transcription level under filtered green light, as key transcripts involved in glycolysis including *ENO1*, *ENO2*, and *PDC* were significantly down-regulated (Fig. 8). Expression of *rbcS* gene was also down-regulated, indicating reduced C-fixation through photosynthesis. Additionally, *FNR* and *PPDK* expression levels were slightly lower compared to white light (Fig. 8). Thus the significantly lower sugar content of the microalgal cells could be attributed to both the lower photosynthetic efficiency and the reduced fluxes through the main carbohydrate metabolism. Similarly to microalgae, the utilization of monochromatic green light (LEDs) has also provoked photosynthetic limitation in higher plants [87,88]. Interestingly, under our experimental conditions we observed a significant decrease in cellobiose content under both green and blue light (Supplemental Table S1). Cellobiose is known to be successfully utilized as a carbon source in heterotrophic cultures of microalgae such as *Neochloris oleoabundans* and *Scenedesmus obliquus* [89,90]. Moreover growth of *C. pyrenoidosa* and *S. obliquus* was enhanced by the combination of photosynthesis and cellobiose availability as a substrate [89]. In addition, *N. gaditana* genome contains specific genes which are involved in cellulose metabolism through which cellobiose could be produced [91]. Consequently, hydrolyzation of cellobiose into individual monosaccharides could be employed as an alternative carbon source during photosynthetic limitation caused by filtered light. Interestingly, sugar decline was followed by a significant decline or even absence of some organic acids (Fig. 9). This decrease was accompanied with the significant down regulation of several genes of the TCA cycle, including isocitrate dehydrogenase (*IDH1*), succinyl-CoA synthetase (*LSC2*) and succinate dehydrogenase (*SDHA*) (Fig. 8). According to various studies green light leads to the down-regulation of several transcripts of TCA cycle [92,93]. Moreover the succinate dehydrogenase gene of *Arabidopsis thaliana*, involved in TCA pathway, has been shown to be influenced by different light qualities [94]. The observed decrease of citric, D-malic and succinic acid contents combined with the down-regulation of *IDH1*, *LSC2* and *SDHA* gene transcript, indicates that green filtered light has a negative effect on *N. gaditana* TCA cycle flux (Fig. 9).

In addition, several transcripts involved in various aspects of *N. gaditana* metabolism and cell viability were found to be down-regulated under green light. Transcripts of glycine (serine) hydroxymethyltransferase (*SHMT1* and *SHMT2*) and glycine dehydrogenase

(*GLDC*) genes were down-regulated under filtered green light, even though serine and glycine levels remained stable (Fig. 9). Also, ferredoxin-nitrite reductase (*NIR1*) transcripts were found to be significantly down-regulated under green light. Down-regulation of ferredoxin-nitrite reductase activity in *C. reinhardtii* is suggested to correlate to the lack of carbon skeletons and protein degradation caused by illumination conditions [95]. In spite of the use of filtered light instead of monochromatic irradiance, our results are consistent with the aforementioned study, as gene expression levels of ferredoxin-nitrite reductase (*NIR1*) and carbohydrate content have both been reduced under green filtered light (Fig. 9). Interestingly, although all studied gene transcripts involved in oxidative stress responses remained unaltered under red and blue filtered source, several of them including *GPx1*, *GPx3*, *SOD2_1*, and *SOD2_3* exhibited significantly lower expression levels under filtered green compared to white light. Antioxidant enzymes act as a primary mechanism to control concentrations of ROS in all organisms exposed to oxygen [96]. Despite the fact that light presence is crucial for algal photosynthetic apparatus, light and photosynthesis are major contributors of ROS [97]. Janknegt et al. demonstrated that SOD activity in some microalgae species is decreased in response to high light irradiance. Moreover, the variation in SOD activity as a response to high irradiance was correlated to the cell size [98]. Thus, the observed down-regulation of major genes of the antioxidant apparatus could be indicative of the reduced photosynthetic and metabolic activity under filtered green light conditions. However, under high intensity, green photons can be as productive as absorbed photons of other light wavelengths, which is attributed to their ability to penetrate deeper into dense cultures compared to other light qualities [99]. Thus, a range of light intensities must be examined in order to elucidate the effect of green filtered light on *N. gaditana* metabolism. Finally, previously published fatty acid profiles of three *Nannochloropsis* species under various monochromatic light sources showed that growth under green light induces the highest lipid content [18]. Similarly, under our experimental conditions, contrary to the general trend of cell metabolism, filtered green light showed a positive influence on oleic and dihomo-alpha-linolenic acids content, but it also caused a decrease of myristic acid levels.

Green filtered light induced a metabolic profile with many similarities to filtered blue light. However, some significant changes were also observed including mainly sugars (D-allose, Galactose MP, L-glutamine 3, 2-amino-2-deoxy-, D-Galactose, Mannitol and Methyl-beta-D-galactopyranoside). A possible interpretation of this result could be that spectra of the respective filtered light conditions were partially overlapping (Fig. 1). According to literature, light quality affects sugar content in several photosynthetic species [100,101]. Additionally, sugars modulate internal regulators and environmental signs which control growth and development [102]. Variation of sugar profile could be an indication of diverse photosynthetic activity among blue and green filtered spectrum.

During environmental changes affecting living cells, metabolic content in general responds faster than gene transcription, indicating that a disruption in the early stages of application of the treatment agent occurs [103]. However, in the subsequent stages, the metabolic dependence on the surrounding conditions is revealed. In this case microalgae biomass was harvested on the seventh day of the light treatment. Therefore, due to prolonged exposure of the cultures to filtered light irradiance, it is considered that results of the transcriptomic and metabolomic analysis are comparable and linked. A very promising approach for future research could include two phase cultivation systems of microalgae [18]. Future work should assess the combination of two wavelengths or the enrichment of white irradiance with a filtered light sources as methods for enhancing microalgal growth and manipulating metabolism [42].

5. Conclusions

In the current study, complementary -omic technologies and correlation analysis were employed to study the impact of filtered light on *N. gaditana* growth and metabolism. The integrated -omic approach revealed extensive metabolic adaptations triggered by light quality. Photosynthetic limitation caused decreased cell density in all treatments. Red filtered light strongly affects *N. gaditana* metabolism even at the level of gene expression enhancing the concentration of amino acids and nitrogen containing compounds, indicating that this strain perceives and exploits red light efficiently. Thus full spectrum enriched in red photons could enhance *N. gaditana* metabolism and be part of a new competitive growth system for commercial applications. In contrast, green photons were under-utilized by *N. gaditana*, leading to a low cell density culture with a low metabolic potential. In between, filtered blue spectrum while shown to be efficient for *N. gaditana* growth, caused a decline in the carbohydrate content. It also induced the expression of 3 fatty acid desaturases as well as the synthesis of polyunsaturated fatty acids which are valuable due to their beneficial role in human and animal health. Future work will aim to optimize the illumination properties in the direction of biomass and metabolic productivity.

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Authors' contribution

M. Patelou participated in the experimental design and performed most of the experiments and wrote the manuscript. C. Infante participated in the experimental design and microalgal growth, cell harvest and sample preparation. F. Dardelle participated in biomass growth, cell harvest and sample preparation. D. Randewig designed the RT-qPCR primer platform. E.D. Kouri performed the derivatization and GC–MS analysis of the metabolite samples. M. K. Udvardi participated in the metabolomic analysis. E. Tsiplakou participated in the analysis and evaluation of fatty acid profiles. L. Mantecón participated in the experimental design and microalgal growth. E. Flemetakis directed the project, the experimental design, analysis and overall evaluation of results, and participated in manuscript writing. All the authors read and approved the final manuscript.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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