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Synergistic effect of UV radiation and nutrient limitation on *Chlorella fusca* (Chlorophyta) cultures grown in outdoor cylindrical photobioreactors

I. Malpartida[1], C. G. Jerez[1], M. M. Morales[2], P. Nascimento[3], I. Freire[3], J. Ezequiel[4], R. M. Rico[1], E. Peralta[1], J. R. Malapascua[1], Y. Florez[1], J. Masojidek[5], R. Abdala[1], F. L. Figueroa[1], E. Navarro[6,*]

[1]Departamento de Ecología, Facultad de Ciencias, Universidad de Málaga, 29071 Málaga, Spain

[2]Department of Chemical Engineering, University of Almería, Almería, Spain

[3]Department Microbiology and Parasitology, Faculty of Biology-CIBUS, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

[4]Departamento de Biologia and CESAM – Centro de Estudos do Ambiente e do Mar, Universidade de Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal

[5]Academy of Sciences, Institute of Microbiology, Opatovickýmlýn, 37981 Trěboň, Czech Republic

[6]Pyrenean Institute of Ecology (CSIC), Av. Montañana 1005, Zaragoza 50059, Spain

ABSTRACT: This study assessed the interactive effects of UVR and nutrient depletion on *Chlorella fusca* cultures on the production and accumulation of particular biomolecules. To accomplish this, algae were grown for 5 d in outdoor thin-layer cascade cultivators under 3 nutrient treatments (full nutrients, –N and –S) and then transferred to outdoor cylindrical photobioreactors for another 5 d. Cultures were then exposed to full solar radiation (PAB) and decreased UVR. During the last 5 d, bio-optical properties, photosynthetic activity, pigments, biochemical composition and oxidative stress were assessed. Initially, nutrient depletion caused changes in productivity and cell number in a manner that affected biochemical composition. After 3 d, the percentage of lipids in the cultures under N deprivation reached values appropriate for being used as feed or food additives or for energy applications (35% of lipid content), regardless of the light conditions. A longer exposure (5 d) resulted in interactive effects of light and nutrient conditions. Specifically, PAB increased lipid content in all cases (1.3- to 2.3-fold), but particularly under S deprivation. Longer exposure to PAB also increased oxidative stress in UVR and nutrient-limited treatments (–N and –S). These results showed that the benefits expected from nutrient depletion (increase in biomolecule content e.g. lipids, carbohydrates and pigments) were modulated by the negative effects of algal UVR acclimation costs.

KEY WORDS: Bio-optic · *Chlorella fusca* · *In vivo* chlorophyll fluorescence · Photosynthetic pigments · UV radiation · Lipids · Lipid peroxidation · Proteins · Biochemical composition

INTRODUCTION

During the last 50 yr, microalgae have been cultivated in both out- and indoor systems to produce biomass used as food or feed or for the extraction of high-value molecules. Today, about 20 different genera of algae are used to produce compounds of interest, including carotenoids, fatty acids, polysaccharides and antioxidant substances, or to obtain biofuels (Tredici 2010, Stengel et al. 2011, Wilhelm & Jakob 2011, Sharma et al. 2012). Accordingly, the economic sectors impacted by such biotechnology

*Corresponding author: enrique.navarro@ipe.csic.es

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range from the food, cosmetic, energy, agri- and horticultural sectors to human health (De Pauw & Persoone 1988, Stengel et al. 2011, Adarme-Vega et al. 2012). Microalgae are cultured using different systems, commonly called photobioreactors, which allow for the control of the environmental variables affecting algal growth. Changes from optimal conditions (i.e. in light quantity and quality and nutrient limitation) may result in algal stress, requiring biochemical and metabolic adjustments that may result in the synthesis and accumulation of some of these molecules of interest. A few reports are available on the effects of UVR, nutrient availability, or other physiological processes (oxidative stress, membrane damage, carbon [C] allocation and photosynthesis), considering species from the genus *Chlorella* (Malanga & Puntarulo 1995), *Nannochloropsis* (Sobrino et al. 2005), *Scenedesmus* (Kasai & Arts 1998, Germ et al. 2002), *Platymonas* (Yu et al. 2004) and the cyanobacteria *Nostoc* and *Arthrospira* (Helbling et al. 2006). To date, however, no study has focused on outdoor microalgal culture systems in the context of modifying both UVR intensity and nutrient availability.

In outdoor cultures, microalgae can be exposed to elevated irradiance (>2000 µmol photons m^{-2} s^{-1}) of photosynthetic active radiation (PAR, λ= 400 to 700 nm) and UVR (λ = 280 to 400 nm). Solar UVR is an environmental variable with a range of deleterious effects on microalgae. In particular, UVR, through different mechanisms, causes DNA damage (Buma et al. 1996, Helbling et al. 2006) and decreases C incorporation rates by reducing photosystem II (PSII) efficiency, the RUBISCO pool (McKenzie et al. 2011) and the carboxylation process (Beardall & Raven 2004). However, positive effects involve the increase of C uptake under relatively low UVR levels (Nilawati et al. 1997, Barbieri et al. 2002) or DNA damage repair mediated by UVA radiation (Karentz et al. 1991). Indeed, many planktonic organisms are rather resistant to UVR, with only negligible cellular effects (Cabrera et al. 1997). Indirect effects might be viewed as positive, such as the breakdown of dissolved organic matter by UVR, which may result in an increase in nutrient supply. The vulnerability of plants to UVR is the result of a balance between photodamage, photoprotection and the photorepair mechanisms of DNA mediated by PAR and UVR (Mitchell & Karentz 1993, Murata et al. 2007), to the accumulation of lipidic and water-soluble antioxidants and the activation of antioxidant enzymes (Cockell & Knowland 1999) and to the accumulation of UV screen photoprotectors (Korbee et al. 2010). Since the irradiance of UVB radiation reaching Earth's surface is expected to change in the next decades (Hegglin & Shepherd 2009, Watanabe et al. 2011), concerns have focused on assessing and forecasting the potential impacts of such changes on the productivity of cultivated plants (Schultz 2000, Golaszewski & Upadhyaya 2003). In addition, because of the ecological and economic importance of algae and macrophyta, their responses to UVR have been extensively assessed in natural environments (Häder & Figueroa 1997, Wulff et al. 2000, Helbling et al. 2003, Navarro et al. 2007, Pessoa 2012) and under artificial conditions (Sobrino et al. 2004, Korbee et al. 2010). Other studies demonstrated that algal acclimation to UVR entails metabolic costs in the form of reduced growth that may facilitate the effects of other stressors, such as heavy metals (Navarro et al. 2008). Furthermore, UVR may promote the accumulation of secondary metabolites in algae (i.e. high-value compounds), while reducing biomass productivity (Figueroa et al. 2008). In contrast, culture under artificial light or in greenhouses with UV cut-off filters reduces the accumulation of high-value compounds, but conversely, productivity can increase (Figueroa et al. 2006). Thus, although mass algal cultivation is concentrated at latitudes with high global solar exposure throughout the year (Tredici 2010, Acién Fernández et al. 2012), insufficient information is available about the effects of UVR on the productivity of outdoor microalgae cultures and even less is known about the synergistic effects of UVR and nutrient limitation.

Nutrient deprivation (–S, –P, –N, etc.) results in a decrease of growth rate and photosynthetic rates by both direct (reduction of the synthesis of certain biomolecules) and indirect effects (reduction of protection or repair mechanisms). S is needed in protein synthesis (Grossman & Takahashi 2001) but also in a wide range of secondary cell compounds, including glucosinolates and sulpholipids (Leustek & Saito 1999). S deprivation may result in the cessation of algal cell division (Hase et al. 1959) and in the degradation of endogenous protein and starch (Melis et al. 2000, Zhang et al. 2002, Kosourov et al. 2003). The depletion of phosphate can increase photoinhibition and reduce the capacity for photoprotection against UV radiation (Carrillo et al. 2008). N is needed for the synthesis of proteins, and N deprivation increases the sensitivity of photosynthesis to UVR in several organisms (Litchman et al. 2002, Bouchard et al. 2008) due to less efficient repair of UVB damage that depends on N compounds. Fluorescence-based measurements of phytoplankton photosynthesis have been used to assess N limitation, which causes a decrease in the PSII photochemical quantum yield that

reduces the efficiency of light-harvesting, energy transduction and CO_2 fixation (Kolber et al. 1988, Berges et al. 1996, Geider et al. 1998, Young & Beardall 2003). Aquatic organisms have several mechanisms to counteract and repair UVR effects, such as the accumulation of UV-absorbing substances with antioxidant properties, i.e. mycosporine-like amino acids (MAAs) (Shick & Dunlap 2002), phenols and carotenoids (Goiris et al. 2012), or the effective dissipation of excess energy by the action of the xanthophyll cycle (Demmig-Adams & Adams 1996). Therefore, a lack of N would decrease the rate of repair, slowly and progressively decreasing photosynthetic efficiency (Litchman et al. 2002).

Three different methods may lead to increased yield of algal biomolecules: (1) by increasing algal cell density, (2) by increasing the intracellular accumulation of such products or (3) a combination of both. While the first method may depend, largely, on the type of photobioreactor, the second and third may rely more on the growing conditions and stress to which algae are exposed. Therefore, knowledge about the effects of changes in culture conditions that may, in turn, change the synthesis and quantity of certain molecules would be of great interest for both basic and applied research.

In this study, *Chlorella fusca* (Chlorophyta) cultures were grown during 5 d in outdoor thin-layer cascade (TLC) cultivators under 3 nutrient treatments (full nutrients, –N and –S). The cultures were then exposed to different light conditions, including full solar radiation (PAB) or decreased UV radiation (P(AB–)). To evaluate the combined effect of UVR and nutrient depletion, different functional indicators were used (Figueroa et al. 2013). Based on the rationale previously presented, the working hypothesis was that the expected benefits from nutrient depletion (increase of certain biomolecules, such as lipids, carbohydrates and pigments) would be modulated by negative direct effects (i.e. algal acclimation costs) and decreased biomass productivity provoked by an increased exposure to UVR.

MATERIALS AND METHODS

Experimental set-up

Chlorella fusca (Chlorophyta, from the Spanish Collection of Algae) cultures were grown for 5 d in 3 outdoor TLC systems (4 m^2) (see description in Jerez et al. 2014, this Theme Section) and acclimated to different nutrient conditions, i.e. full nutrients (F), limited nitrogen (–N) and limited sulphur (–S). Full media contained the following (g l^{-1}) according to Sorokin & Krauss (1958): KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2$, 0.0835; $FeSO_4 \cdot 7H_2O$, 0.0498; H_3BO_3, 0.1143; $ZnSO_4 \cdot 7H_2O$, 0.0882; $MnCl_2 \cdot 4H_2O$, 0.0142; MoO_3, 0.0071; $CuSO_4 \cdot 5H_2O$, 0.0157; $Co(NO_3)_2 \cdot 6H_2O$, 0.0049 and EDTA, 0.5. The –N treatment received only 25% of the initial nitrate concentration, while the –S treatment received 50% of the normal sulphate concentrations. After this acclimation period, samples of 1.25 l from each treatment were transferred to 18 UVR-transparent methacrylate cylinders (diameter 10 cm, height 20 cm) (see Aphalo et al. 2012). Strong aeration was applied to keep high hydrodynamic conditions in both the TLC tank (see Jerez et al. 2014) and the cylinders. Cultures in cylinders were maintained for 5 d.

Two different light conditions were set: (1) full solar radiation (natural conditions), i.e. PAR+UVA+UVB (PAB), and (2) decreased natural UVA+UVB (P[AB–]) by using cut-off filters surrounding the methacrylate cylinders (Ultraphan 395) according to Villafañe et al. (2003). The PAR irradiance in PAB was the same as in P(AB–) by use of the cut-off filter Ultraphan 295 (Villafañe et al. 2003, Aphalo et al. 2012). This design avoided any problem caused from having different PAR irradiances, e.g. differences in photoinhibition (Villafañe et al. 2003). As a result, algal cultures in PAB vessels were exposed to 75% of incident UVB and UVA radiation, whereas cultures in P(AB–) vessels were exposed to 8% of UVB and UVA. Daily temperature variations were minimized (25 to 28°C) by placing the cylinders in a thermostatically controlled water bath (Fig. 1). Three replicates were set up for each treatment.

Solar radiation, temperature and pH measurements

Temperature was monitored using a HOBO Pro v2 Water Temperature Logger U22-001. The pH was measured using a portable pH meter (pH 3110, WTW). Incident solar irradiance was measured continuously in air using a UV-PAR multifilter radiometer NILU UV6 (Geminali). The irradiance of UVA (320 to 400 nm) and UVB (280 to 320 nm) was calculated from the data of the different UV filters according to Høiskar et al. (2003). The integrated daily irradiance (kJ m^{-2}) was calculated for the whole duration of the experiment. The NILU UV6 is located on the roof of the building housing the Central Services

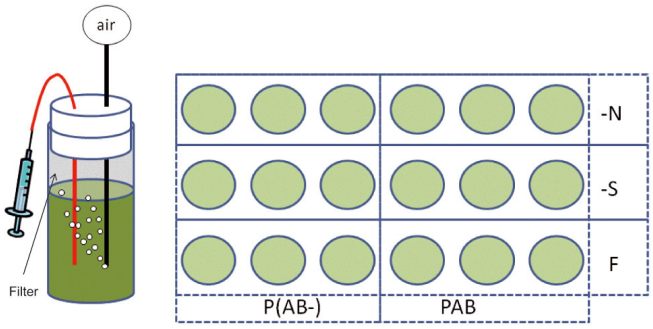

Fig. 1. Left: diagram showing the algal culture vessels equipped with an outlet syringe for sampling algal cultures and surrounded with UV cut-off filters, such as Ultraphan 295 (PAB) and Ultraphan 395 in the P(AB–) treatment. The cultures were kept under agitation by air bubbling. Right: the experimental setup of the vessels in the thermostatic bath. F: full nutrients; –N: nitrogen-limited; –S: sulphur-limited; PAB: full solar radiation and P(AB–) with the same PAR but decreased UVR. The position of the vessels was not randomly selected to facilitate daily measurement protocols. No temperature or light gradients were noticed in the bath

Research of Malaga University, where the experiments were conducted (36° 40′ N, 4° 28′ W).

Bio-optical variables: PAR and UVR extinction

The irradiance of UVR (295 to 400 nm) reaching different depths in the algal cultures was measured using a UV203 radiometer (MACAM, Scotland) connected to UV-B (λ_{max} = 295 ± 2 nm, bandpass FWHM = 19 ± 2 nm) or UV-A (λ_{max} = 365 ± 2 nm, bandpass FWHM = 35 ± 2 nm) sensors, following the procedures described in Navarro et al. (2014). In short, the algal suspensions were added to the upper part of a 50 ml Uthermöl chamber fixed over the sensor; this part is a tube 95 mm in length and 25 mm diameter made with plastic that is opaque to UVR wavelengths. A bit of silicon was used around the bottom of the tube, just making contact with the glass surface of the sensors, in order to avoid leaching of the cell suspension. The natural sunlight UVA and UVB were measured before adding 5 ml suspension aliquots to completely fill the column. Each aliquot increased the height of the suspension column by 1 cm, allowing UVA and UVB intensity data to be plotted as a function of depth (see details in Navarro et al. 2014). The UVR irradiance was calculated using the following equation: ($UVA_{irradiance}$ × 2.94) + ($UVA_{irradiance}$ × 1.17); these constants were used to correct for the sensor's underestimation under the optic conditions of the measuring set-up. The PAR extinction was measured at different depths (0.4 and 3.5 cm) of algal suspensions using a spherical quantum sensor (US-SQS/L, Walz). The extinction coefficients $K_{d,UVR}$ and $K_{d,PAR}$ were estimated by adjusting the UVR and PAR measured irradiances to the Beer-Lambert equation.

The specific attenuation coefficient K_c was calculated for both PAR and UVR ($K_{c,PAR}$ and $K_{c,UVR}$). This is an apparent optical property of cell cultures since it considers both the effect of cell size and pigment content on light absorption (Figueroa et al. 1997) and is expressed as m^2 mg chl a^{-1}.

UV index

In this study, we assessed the UVR screening capacity of algal cells, by measuring the absorbance of cell pigment extracts in the range of UVR wavelengths. That was done using a spectrophotometer (Shimadzu UV-16-03). The absorbances at 3 different wavelength bands (UVR: 295–400 nm, UVA: 320–400 nm, UVB: 295–320 nm) were measured from the pigment extract. Examination of the whole UVR-absorbance range is expected to integrate and reflect any UVR-induced change in the pigment composition of the algal community (Navarro et al. 2007). This UVR index has been previously tested for algal communities and pure cultures (Navarro et al. 2007). In short, the relative proportion of UVR absorbance to chl *a* was calculated as the ratio of absorbance intensity over the range of UVR to that of chl *a* at 665 nm. The area under the absorbance curve in the range of 295 to 400 nm was calculated by the sum of light absorbance at any wavelength (1 nm step). The resulting UVR ratio is a dimensionless number, representing a ratio between the absorbance capacities of the UVR-absorbing compounds per absorbance-unit of chl *a* (Navarro et al. 2007). The same procedure was used to calculate UVA and UVB ratios.

Algal biomass and photosynthetic pigments

Algal biomass was expressed as cell numbers ml^{-1} assessed using Neubauer chambers according to Utermöhl (1958). Total chlorophyll (*a* and *b*) and carotenoids were estimated spectrophotometrically by adding 2 ml of dimethylformamide (DMF) to 1 mg of freeze-dried sample, which was kept overnight in darkness at 4°C. Then, the sample was centrifuged and analyzed at different wavelengths (750, 664, 647 and 480 nm) with a UV-Vis spectrophotometer (Shimadzu UV-16-03). The concentrations of chl *a*

and *b* as well as total carotenoids were calculated according to Wellburn (1994). The results were expressed as µg mg^{-1} of biomass.

Functional variables: photosynthetic activity as *in vivo* chl *a* fluorescence

Photosynthetic performance was measured using pulse-amplitude-modulated (PAM) chl *a* fluorescence of photosystem II (Schreiber et al. 1995). The recommendations of Kromkamp & Forster (2003) were followed for nomenclature. Effective quantum yield ($\Delta F/F_m'$), as defined by Genty et al. (1989), was measured *in situ* from outside the culture using a Pocket-PAM fluorometer (Gademann Instruments) by placing the optical fiber directly into the wall of the experimental vessel, as reported by Figueroa et al. (2013). At the same time, the photon fluence rate of PAR inside the cylinders was measured with a spherical quantum sensor (US-SQS; Walz). Both measurements were performed on the third and fifth day of the experiment at 3 cm depth from the culture surface 3 times a day: morning (09:00 h), noon (13:00 h) and evening (18:00 h).

Rapid light curves (RLCs) were constructed using a Junior-PAM fluorometer (Walz) twice a day (12:00 and 18:00 h) by sampling 10 ml of cultures and transferring them to light-protected chambers for dark adaptation (15 min) to obtain optimal quantum efficiency (F_v/F_m). Samples were exposed for 20 s to 12 increasing E_{PAR} levels between 0 and 1500 µmol photons m^{-2} s^{-1}, which were provided by the internal blue LED of the fluorometer. Relative electron transport rates (rETR) were determined as follows:

$$rETR = \Delta F/F_m' \times E_{PAR} \quad (1)$$

where $\Delta F/F_m'$ is the effective yield where $\Delta F = F_m' - F_t$, F_m' is the maximal fluorescence after saturation light pulse (<4000 µmol photons m^{-2} s^{-1}), and F_t is the intrinsic fluorescence of light-exposed algae. E_{PAR} (µmol photons m^{-2} s^{-1}) is the photon fluence rate of PAR determined by a US-SQS spherical quantum sensor. Unless the number of absorbed quanta is known, it is not possible to give absolute ETR values as an estimation of production. However, RLCs presented as rETR values vs. irradiance can provide data about the relative change of photosynthetic activity under experimental conditions. Therefore, rETR values were fitted according to Eilers & Peeters (1988), using least square error calculation and the Solver function of Excel (Microsoft) to obtain photosynthetic parameters, i.e. photon-capturing efficiency of PSII in the light-limited range (α_{ETR}), $rETR_{max}$ and the light saturation coefficient (E_k).

Biochemical composition

Total C and total N were determined from dry biomass, using a CNH Perkin-Elmer 2400 elemental analyzer in which C was oxidized at 600°C, and resulting peaks were compared with a known mass of an acetanilide standard to determine mass. Acetanilide has a composition of 71.09% C and 10.36% N. The C and N values were expressed as a percentage of dry weight biomass.

Soluble proteins were analyzed using the Bradford method (Bradford 1976): 20 µl of sample supernatant from the cellular extracts and 235 µl of Bradford reagent were added into each well of a 96 well plate and given 45 min to react. The protein levels were quantified in a plate reader (Multiskan FC, Thermo Fisher Scientific) with absorption readings at 595 nm. The total protein concentration in samples was calculated from a standard curve (0 to 250 µg ml^{-1}) made with bovine serum albumin and expressed as mg of protein per ml of extract.

Lipid content (% of dry wt) was measured using the sulpho-phospho-vanillin method (Knight et al. 1972, Izard & Limberger 2003). Concentrated sulphuric acid (2 ml H_2SO_4) was added to a blank in a tube containing 100 µl of 80% methanol, to tubes with a triolein standard (100 µl) and to tubes with 100 µl of sample supernatant. Each tube was incubated for 30 min at 100°C and then cooled to room temperature in a water bath. After the addition of 5 ml of phospho-vanillin reagent, the tubes were incubated at room temperature for 15 min. Absorbance was read on a spectrophotometer at 530 nm (Shimadzu UV-16-03).

Lipid peroxidation was calculated using the thiobarbituric acid reactive substances (TBARS) method after Heath & Packer (1968). Samples for lipid peroxidation were collected on the first and fifth day of the experiment. From each cylinder, 15 ml of algal suspension were collected and centrifuged. The supernatant was discarded, and the cellular pellet was frozen at −80°C. Each sample was resuspended in 2 ml of cold extraction buffer (50 mM KH_2PO_4; 0.1 mM EDTA; 0.1% Triton X-100, pH = 7.4) with butylated hydroxytoluene (BHT) (40 µl ml^{-1}). Extraction was done by sonication (3 cycles of 30 s, with 30 µm amplitude, on ice) on a U200S control sonicator (IKA-Werke, Staufen). Then, 2 ml of 0.5% thiobarbituric acid in 20% trichloroacetic acid were added to cell extracts. The mixture was heated for 30 min at

90°C and immediately put on ice, followed by centrifugation. TBARS absorption peak and unspecific turbidity were read at 532 and 600 nm, respectively. Absorption readings were done on a dual-beam spectrophotometer (HALO DB-20, Dynamica). TBARS concentration was calculated from a tetraethoxypropane standard curve (0 to 250 µM) and expressed as nmol cell^{-1}.

In all cases, at least 1 sample per cylinder was analyzed (i.e. minimum 3 replicates per treatment).

Statistical analysis

Most of the statistical analyses were performed with R statistical computing software (www.r-project.org). Unless otherwise indicated, errors are expressed as standard deviation (SD). A combination of parametric and nonparametric statistics was used. Normality was tested with the Shapiro-Wilkinson test and the Fligner-Killeen test to determine homocedasticity. When variances were homogeneous, the Fisher test was used for comparisons. The Welch 2-sample *t*-test was performed to compare the means when the normality assumption was satisfied, and the Wilcoxon range test was used when normality was not achieved. One-way, 2-way or 3-way ANOVAs were used to compare the treatments when normality and homocedasticity were satisfied, while the Kruskal-Wallis test was applied when they were not. Tukey HSD or Duncan's MRT post-hoc tests were applied to evaluate differences between treatments.

Light extinction curves were fitted to a 2-parameter exponential decay model, using R and the drc package to obtain the corresponding K_d values. The compPAR function was used to compare K_d, using *t*-tests with p-values adjusted using Bonferroni correction for multiple tests. The null hypothesis was that the ratio equals 1. The ratio was obtained by dividing K_d values (i.e. $K_{d,UVR}$ PAR –N /$K_{d,UVR}$ PAR –S from Day 1). If the ratio significantly differed from 1, the null hypothesis was rejected, meaning those values were significantly different ($p < 0.05$).

Pearson's correlation coefficient (r) was determined to define the extent of a linear correlation between the studied variables and was calculated using the Statistica software (v.7.0, Statsoft).

RESULTS

Physico-chemical and bio-optical variables

Daily integrated irradiance

The daily PAR, UVA and UVB integrated irradiance during the 5 experimental days is shown in Fig. 2. On the first day of the experiment (18 September 2012) the daily integrated irradiance of PAR and UVR was much lower than that on the remaining days as a result of cloudy conditions (4065, 472 and 24 kJ m^{-2} of PAR, UVA and UVB daily integrated irradiance, respectively). During the acclimation period (13 to 17 September 2012), PAR daily integrated irradiance ranged between 9330 and 9870 kJ m^{-2}, and this value ranged between 7859 and 9074 kJ m^{-2} in the experimental period (19 to 23 September 2012, see Fig. 2). The UVA daily integrated irradiance during the acclimation period ranged between 1066 and 1124 kJ m^{-2} and between 899 and 1004 kJ m^{-2}, while the UVB ranged between 50–55 kJ m^{-2} and 44–48 kJ m^{-2}, respectively.

pH

The pH of all cultures transferred to the cylinders showed similar values around 7.15 ± 0.15, essentially because pH was controlled by CO_2 injection in the TLC systems. Once in the cylinders, the pH was not controlled. The pH was measured on the last day at 18:40 h. The –S deficiency provoked a significant

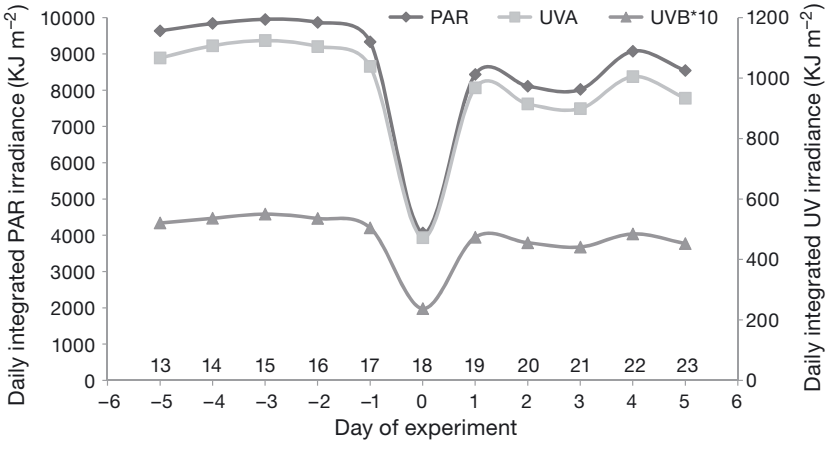

Fig. 2. Daily integrated irradiance of PAR (400 to 700 nm), UVA (320 to 400 nm) and UVB (280 to 320 nm) during the experiment: preacclimation of cultures in thin-layer cascades (from 13 to 17 Sep); Day 0 (18 Sep), transfer from TLC to methacrylate cylindrical vessels; Day 1 to 5 (19 to 23rd Sep), experimental period. UVB irradiance values are multiplied by a factor of 10 for inclusion in the same scale as UVA. Numbers above the *x*-axis are dates in September 2012

increase of pH in the algal culture with values around 7.8 to 8.0, and this effect was significantly enhanced by UVR (data not shown). The pH in the other treatments was around 7.4, a value slightly higher than the initial one.

Extinction coefficients

After 3 d of growing in cylinders, $K_{d,UVR}$ and $K_{d,PAR}$ of the algal cultures increased (Table 1). Later on, $K_{d,UVR}$ and $K_{d,PAR}$ slightly decreased or increased until the end of the experiment, always showing values higher than the initial ones. Statistical analysis was performed each day to compare different nutrient conditions, and no statistical differences were found in $K_{d,UVR}$ values. However, significant differences were shown along the time course such that Day 0 presented lower values than Day 5. Although intermediate values were shown for Day 3, these values were not significantly different from either Day 0 or 5. In the P(AB–) treatment, $K_{d,UVR}$ values increased in all nutrient conditions except under F, where they decreased at Day 5. The highest value was found in –S at Day 5. Similarly, $K_{d,UVR}$ values in the PAB cultures increased throughout the experiment; P(AB–) cultures also increased $K_{d,UVR}$, but to a lesser extent. In this case, the F conditions also showed a slight decrease at the end of the experiment.

$K_{d,PAR}$ was affected by changes in both light and nutrient conditions. P(AB–) light conditions resulted in higher $K_{d,PAR}$. N-deprived cultures presented significantly lower $K_{d,PAR}$ values than the F treatments, while S-deprived algae presented intermediate values, which were not significantly different from the others.

Table 1. Number of cells, bio-optical properties and algal pigments during the experiment. Analyses were performed on Days 0, 3 and 5 (see Figs. 1 & 2 for details and abbreviations). Parameters shown are biomass, expressed as number of cells ($\times 10^6$ ml^{-1}); UVR and PAR extinction coefficients expressed in m^{-1}; K_c expressed as $\times 10^7$ m^2 mg chl^{-1}; chl *a* and *b* and total carotenoids expressed as µg of pigment per mg dry biomass. Two- (light × nutrients) or 3-way (time × light × nutrient) ANOVAs have been performed per each parameter, depending on whether time was relevant or not and on the availability of replicates. Letters (a, b, c...) are used to denote differences (Tukey HSD tests, $p < 0.05$); treatments presenting values with same letter are not significantly different. Error terms are available only for those values obtained using replicates. Differences between extinction coefficients (K_d) have been tested by *t*-tests, and the results are detailed in the corresponding section. nd: not determined

		TLC	Light regime in cylinders			
			P(AB–)		PAB	
		Day 0	Day 3	Day 5	Day 3	Day 5
No. of cells	F	4.55	nd	8.5 ± 4.1^{a}	nd	7.9 ± 3.2^{a}
	–S	11.5		10.1 ± 1.3^{a}		7.3 ± 2.9^{a}
	–N	9.54		5.9 ± 2.3^{a}		5.5 ± 1.8^{a}
$K_{d,UVR}$	F	129	161	130	167	163
	–S	116	146	170	162	179
	–N	109	125	143	128	176
$K_{d,PAR}$	F	42	45	46^{a}	45	47
	–S	40	46	50^{a}	38	33
	–N	33	42	45^{a}	40	39
$K_{c,UVR}$	F	0.028 ± 0.006^{b}	0.120 ± 0.03^{ab}	0.135 ± 0.01^{ab}	0.128 ± 0.02^{ab}	0.164 ± 0.02^{a}
	–S	0.030 ± 0.004^{b}	0.119 ± 0.03^{ab}	0.165 ± 0.01^{a}	0.124 ± 0.01^{ab}	0.158 ± 0.02^{ab}
	–N	0.029 ± 0.004^{b}	0.092 ± 0.04^{ab}	0.097 ± 0.01^{ab}	0.069 ± 0.01^{ab}	0.166 ± 0.03^{a}
$K_{c,PAR}$	F	0.009 ± 0.002	0.033 ± 0.008	0.048 ± 0.003	0.035 ± 0.004	0.047 ± 0.006
	–S	0.011 ± 0.001	0.038 ± 0.011	0.048 ± 0.003	0.029 ± 0.003	0.029 ± 0.004
	–N	0.009 ± 0.001	0.031 ± 0.016	0.031 ± 0.002	0.022 ± 0.002	0.037 ± 0.008
Chl *a*	F	8.18 ± 0^{a}	2.69 ± 0.81^{cd}	1.92 ± 0.1^{d}	2.59 ± 0.32^{cd}	1.98 ± 0.26^{d}
	–S	5.72 ± 0^{b}	2.59 ± 0.84^{cd}	2.06 ± 0.12^{d}	2.81 ± 0.09^{cd}	2.27 ± 0.26^{cd}
	–N	5.83 ± 0^{b}	2.73 ± 1.62^{cd}	2.93 ± 0.2^{cd}	3.7 ± 0.35^{c}	2.13 ± 0.38^{d}
Chl *b*	F	4.36 ± 0^{a}	2.06 ± 0.26^{ab}	0.96 ± 0.07^{b}	1.86 ± 0.31^{b}	0.99 ± 0.02^{b}
	–S	2.7 ± 0^{ab}	1.75 ± 0.51^{b}	1.32 ± 0.05^{b}	2.05 ± 0.17^{ab}	1.63 ± 0.48^{b}
	–N	2.66 ± 0^{ab}	0.53 ± 3.25^{b}	1.77 ± 0.07^{b}	2.36 ± 0.2^{ab}	1.81 ± 0.41^{b}
Carotenoids	F	3.86 ± 0^{s}	0.45 ± 0.37^{de}	0.42 ± 0.04^{de}	0.61 ± 0.08^{cde}	0.43 ± 0.07^{de}
	–S	3.09 ± 0^{b}	0.5 ± 0.18^{de}	0.38 ± 0.04^{c}	0.65 ± 0.19^{cde}	0.45 ± 0.05^{de}
	–N	3.06 ± 0$_{b}$	0.76 ± 0.06^{cd}	0.6 ± 0.01^{cde}	0.9 ± 0.16^{c}	0.39 ± 0.07^{e}

For $K_{c,UVR}$, both time and the interaction of time and light provoked significant differences between treatments. Day 0 presented significantly lower values of $K_{c,UVR}$ than Days 3 or 5; consequently, the results show the same general trend as with K_d, i.e. increasing values until the end of experiment for all the treatments. In detail, $K_{c,UVR}$ showed the highest values in PAB F and –N. Although also higher, PAB –S values were closer to those of P(AB–) –S. Higher values of $K_{c,PAR}$ were found in PAB –N and P(AB–) –S. No differences between light conditions were found in the F treatments.

UV index

The different light conditions did not yield significant differences between treatments. Hence, values were grouped and analyzed according the nutrient condition prevailing during the experiment. Different nutrient conditions resulted in changes in the UVR index (Fig. 3). N depletion resulted in a reduction of UVR-screening capacity, caused by the reduction in the absorbance capacity in the UVA range. In contrast, no differences in UVR-screening capacity were detected, regardless of nutrient conditions.

Pigment content

During the acclimation period, chlorophyll and carotenoid content decreased in the nutrient-depleted treatments (see Day 0 in Table 1). The average chl *a* content under F was about 8 µg mg^{-1}, decreasing to 5.77 µg mg^{-1} in both the –N and –S treatments. A similar trend was found for chl *b* and carotenoids. These trends were even more marked after 3 d in the cylinders (exposed to different light conditions in addition to the nutrient treatment). Chl *a* decreased in all treatments, irrespective of nutrient or light conditions, except for in the PAB –N treatment which showed significantly higher values, although lower than in Day 0. Chl *b* decreased in all treatments, and carotenoids showed very low concentrations. After 5 d, chl *a* content still decreased, but when different treatments were compared, P(AB–) –N presented significantly higher chl *a* content than the others. In contrast, chl *b* was affected by nutrient conditions: –N treatments presented the highest values and F the lowest, while –S presented intermediate values, although not statistically different. Although carotenoid content was generally very low, it was higher under P(AB–) –N conditions.

Biomass and growth

After the acclimation period in the TLC, the number of cells showed no statistical differences among nutrient treatments. However, –N and –S cultures presented higher values than F. After transferring cultures to the cylinders, the average cell number increased under F conditions but decreased under –S and –N, although this difference was not statistically different. This effect was more marked in this last treatment, irrespective of light conditions (Table 1).

Photosynthetic activity

Maximal quantum yield (F_v/F_m) presented similar values, both initially (0.61 to 0.62) and after 5 d of culture in the TLC (Table 2), but significant differences were found after 5 d in cylinders. Under full nutrient conditions, F_v/F_m did not change after 5 d in either light regime. However, significant differences were found by the end of the experiment under –N and –S. For –S, F_v/F_m values did not vary under P(AB–), but a decrease was observed under PAB after 5 d. On the contrary, F_v/F_m increased when N-depleted cultures were exposed to P(AB–), although no changes were observed in PAB.

At the beginning of the experiment, $rETR_{max}$ values obtained from the RLCs were lower under F than in –S and –N conditions. Although $rETR_{max}$ under F

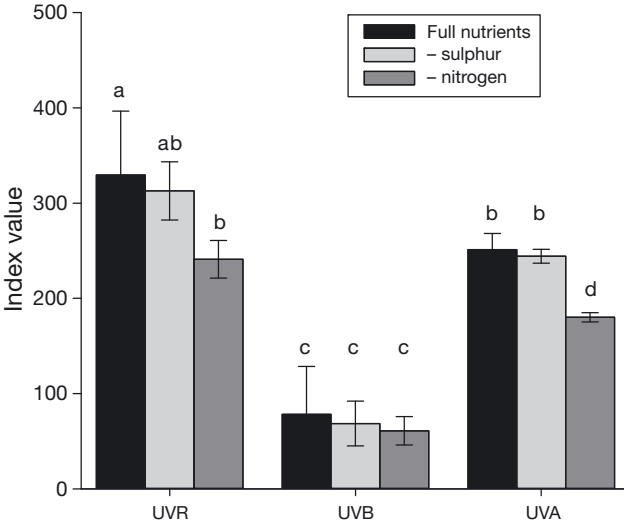

Fig. 3. UVR indexes as UVR (280 to 400 nm), UVB (280 to 320 nm) and UVA (320 to 400 nm) for the different nutrient treatments at Day 5 combining both light treatments, PAB and P(AB–), since no significant differences were found. Columns showing different letters indicate that differences are statistically significant, whereas the use of the same letter indicates no differences

Table 2. Maximal quantum yield (F_v/F_m) and the ETR parameters obtained from the rapid light curves as maximal relative ETR ($rETR_{max}$, µmol e^- m^{-2} s^{-1}), photosynthetic efficiency (α_{ETR}), saturated irradiance (E_k, µmol photons m^{-2} s^{-1}) and rETR at noon i.e. 13:00 h ($rETR_{noon}$, µmol e^- m^{-2} s^{-1}) at the initial time (values of cells after 6 d culture in thin-layer cascades [TLC]) and after 3 and 5 d under the different nutrient regimes, including full nutrients (F), sulphur (–S) and nitrogen (–N) starvation, as well as different light treatments, including natural solar radiation with a 295 nm cut-off filter (PAB) and reduced UVR using a 395 nm cut-off filter, i.e. P(AB–). Three-way ANOVA (time × light × nutrient) has been performed for each parameter. Letters (a, b, c...) are used to denote differences (Tukey HSD tests, $p < 0.05$); treatments presenting values with same letter are not significantly different. nd: not determined

		TLC	Light regime in cylinders			
			P(AB–)		PAB	
		Day 0	Day 3	Day 5	Day 3	Day 5
F_v/F_m	F	0.61 ± 0.00^{abc}	0.64 ± 0.01^{cdf}	0.61 ± 0.02^{abcd}	0.62 ± 0.01^{bcd}	0.60 ± 0.01^{abc}
	–S	0.62 ± 0.01^{bc}	0.66 ± 0.02^{f}	0.60 ± 0.02^{ab}	0.59 ± 0.05^{ab}	0.58 ± 0.01^{a}
	–N	0.61 ± 0.03^{abc}	0.66 ± 0.05^{ef}	0.66 ± 0.03^{f}	0.67 ± 0.01^{f}	0.65 ± 0.01^{def}
$rETR_{max}$	F	87.04 ± 0.90^{bc}	109.46 ± 13.5^{d}	109.24 ± 1.30^{d}	81.50 ± 7.84^{bc}	70.28 ± 4.78^{ab}
	–S	110.58 ± 2.66^{d}	88.42 ± 3.34^{bc}	84.48 ± 0.30^{dc}	150.44 ± 22.60^{e}	86.78 ± 5.56^{bc}
	–N	116.02 ± 6.88^{d}	181.96 ± 13.42^{b}	57.12 ± 6.70^{a}	87.40 ± 15.40^{bc}	98.28 ± 4.88^{cd}
α_{ETR}	F	0.090 ± 0.004^{e}	0.092 ± 0.002^{e}	0.066 ± 0.004^{bcd}	0.100 ± 0.008^{e}	0.076 ± 0.002^{d}
	–S	0.060 ± 0.006^{abc}	0.090 ± 0.002^{e}	0.116 ± 0.012^{f}	0.088 ± 0.008^{e}	0.124 ± 0.006^{f}
	–N	0.058 ± 0.01^{ab}	0.050 ± 0.002^{e}	0.058 ± 0.002^{ab}	0.052 ± 0.002^{a}	0.072 ± 0.002^{cd}
E_k	F	169.77 ± 6.5^{a}	213.63 ± 26.05^{bc}	215.17 ± 20.38^{bc}	147.04 ± 25.54^{a}	120.68 ± 1.79^{a}
	–S	260.55 ± 14.7^{bc}	326.72 ± 4.00^{d}	160.93 ± 24.52^{a}	185.49 ± 77.57^{bc}	170.01 ± 8.46^{a}
	–N	266.36 ± 65.4^{bcd}	149.84 ± 18.86^{a}	164.49 ± 6.36^{a}	162.97 ± 32.49^{a}	116.64 ± 15.54^{a}
$rETR_{noon}$	F	nd	132.68 ± 13.44^{b}	99.90 ± 14.92^{b}	60.74 ± 9.70^{a}	89.60 ± 19.32^{ab}
	–S		88.82 ± 17.48^{ab}	57.56 ± 7.52^{a}	63.22 ± 6.96^{a}	56.78 ± 4.42^{a}
	–N		61.10 ± 11.22^{a}	100.84 ± 5.58^{b}	68.74 ± 14.60^{a}	96.90 ± 2.12^{b}

conditions increased when the cultures were exposed to P(AB–) light, it decreased in –S and –N treatments. On the contrary, $rETR_{max}$ did not vary after 5 d in PAB conditions, although a peak was observed after 3 d in –S cultures. By the end of the experiment, cultures under F conditions showed the highest values under P(AB–) light, whereas the lowest values were reached in –N treatment. The opposite was observed in PAB conditions, where the highest values corresponded to –N cultures and the lowest values were observed in full nutrient conditions.

Photosynthetic efficiency (α_{ETR}) was affected by the initial nutrient conditions; the F treatment presented higher efficiency than the nutrient-deprived ones (Table 2). After being transferred to cylinders for 5 d, efficiency in the –S treatment was significantly higher than in the others regardless of the light conditions, whereas in the F treatment efficiency was reduced. In contrast, PAB light conditions caused an increase over time in efficiency in the –N treatment.

Saturated irradiance (E_k) from the RLCs decreased after 5 d in both –S and –N cultures, irrespective of light regime. However, in full nutrient conditions, E_k increased in P(AB–), whereas it did not vary under PAB. By the end of the experiment, E_k did not show significant differences between treatments, except cultures under full nutrient and P(AB–) conditions, which showed the highest values.

Under outdoor conditions, after 3 d, the photon fluence of PAR followed a similar daily cycle with maximum values at midday in all cases (Fig. 4). Daily maximal values were higher in F and –N than in –S cultures under both light regimes. Common daily $\Delta F/F_m'$ cycles with midday decrease were observed on Day 3 in P(AB–) conditions. After 5 d under these light conditions and throughout the whole experimental period, such a decrease was not observed in PAB treatments, except for cultures in full nutrient conditions. *In situ* rETR reached a peak at midday in all cases. Maximal values tended to decrease in P(AB–) treatments, except for in –N cultures, whereas an increasing trend was observed during the experiment under PAB light conditions.

Biochemical composition

The elemental composition expressed as % of C, N and S varied depending on the treatment and the day of the experiment (Table 3). At Day 0, F cultures presented the highest content of C, N and S, whereas the

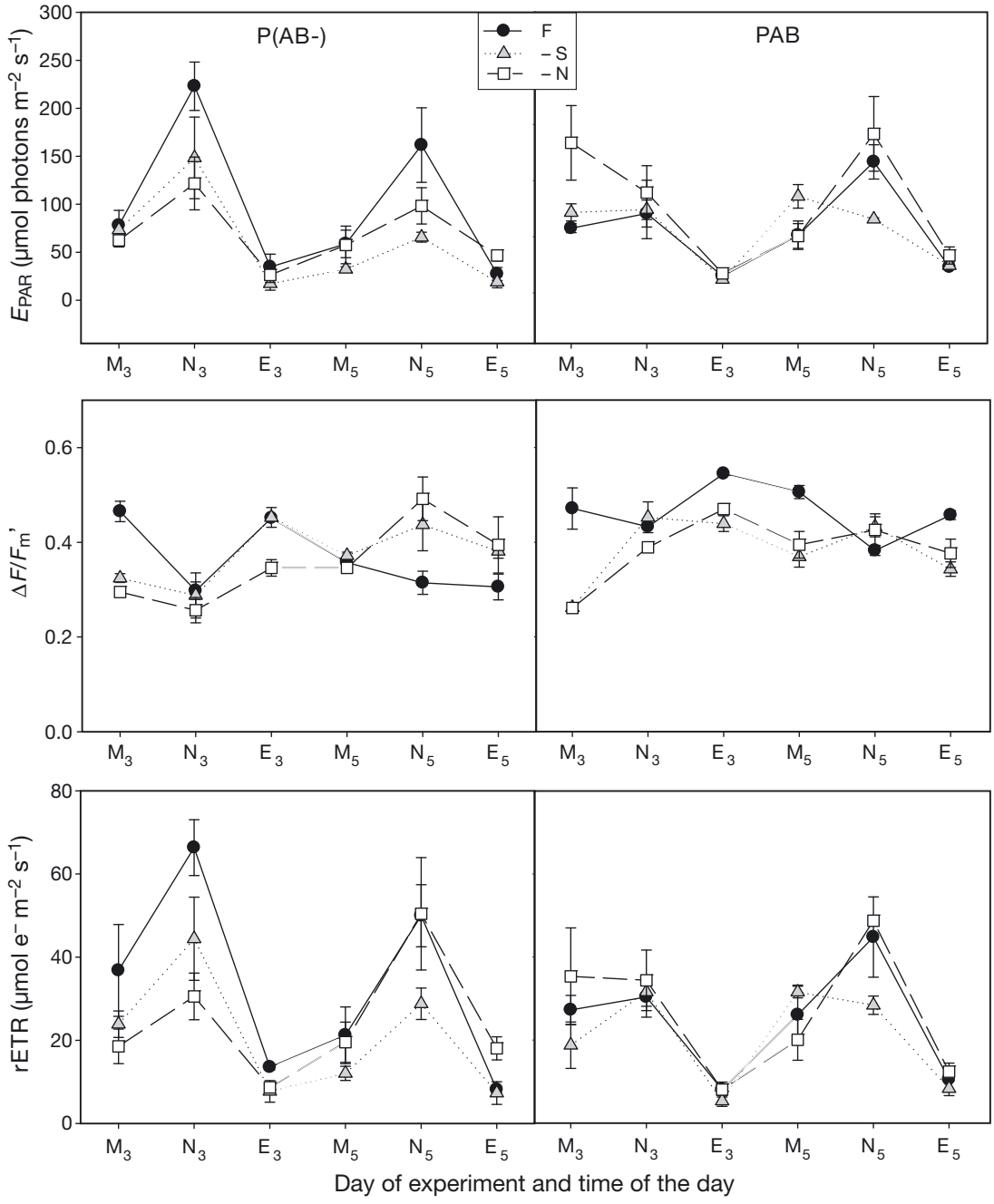

Fig. 4. Photon fluence rate of PAR (E_{PAR}), effective quantum yield of PSII ($\Delta F/F_m'$) and relative electron transport rate (rETR) in the morning (M), noon (N) and evening (E) for 2 different days during the experimental period of culture in methacrylate cylindrical vessels: Day 3 (21 September 2012) and Day 5 (23 September 2012) for the different nutrient treatments, F: full nutrients; –N: nitrogen limited; –S: sulphur limited; and light treatments, including full solar radiation (PAB) or the same PAR but decreased UVR (P(AB–))

–N treatment had the lowest, and –S cultures showed intermediate values for all components. At Day 5, PAB F treatment presented the highest C content, whereas –S had the lowest. The other treatments showed intermediate values that were not statistically different (Table 1). PAB F and P(AB–) –N treatments presented the highest N content, whereas PAB –S presented the lowest, with the remaining treatments presenting intermediate values, although not significantly different from the highest and lowest ones. The S content was slightly affected by all nutrient conditions, with –N presenting the highest

content. A general trend of increasing content of C, N and S occurred over time for the –N treatment, while they decreased or remained the same in F cultures. Along the time course, the –S treatment was more affected by light conditions such that C and N decreased in the PAB –S treatment, while C increased in P(AB–) –S; the S content showed a slight increase in both light conditions.

The nutrient conditions prevailing in the outdoor TLC caused differences in protein content in the vessels at Day 0: –S presented the highest protein content, F the lowest, and –N showed intermediate values, although no statistical differences were found between these initial values. However, after 5 d of culture in the cylinders, these differences disappeared, and all treatments presented similar protein content, regardless of the nutrient and/or light condition. It should be noted that the final protein content was nearly double the initial one. Day 3, not shown, presented intermediate values between Day 0 and 5 (Table 3).

Lipid content (% of dry wt) for the different light and nutrient depletion treatments are presented in Fig. 5. N deprivation in the outdoor TLC resulted in higher lipid content. After 3 d of culture in the cylinders, the lipid content increased in all samples. The greatest increase of lipids was observed in those cylinders combining reduced UVR and nutrient depletion: P(AB–) –S and P(AB–) –N. Of particular interest is the case of –N, with values around 35%. Lipid content reached its lowest values in the F treatment. After 5 d, light conditions continued affecting the lipid content, with cultures exposed to PAB presenting the highest percentages of lipids. Both F and PAB –S conditions increased the lipid content compared to Day 3. Accordingly, P(AB–) –S and –N presented the lowest lipid content, although these treatments showed the highest lipid percentage at Day 3.

Table 3. Total internal C, N and S expressed as percentage (% of dry wt) and soluble protein content (SP, µg mg^{-1} dry wt biomass) from Day 0 (inocula from thin-layer cascade, so no replicates are available, except for SP analysis) and after 5 d in vessels (Day 5) (n = 3). Two-way (treatment × light) or 3-way (treatment × light × time) ANOVAs were performed depending on the availability of samples and replicates. If required, a letter (a, b, c...) is used to denote differences (HSD tests, $p < 0.05$); treatments presenting values with the same letter are not significantly different. F: full nutrients; –S (–N): sulphur (nitrogen) starvation

		TLC	Light regime	
			P(AB–)	PAB
		Day 0	Day 5	Day 5
%C	F	37.5	35.5 ± 0.9^{ab}	37.3 ± 0.8^{a}
	–S	35.9	36.3 ± 1.5^{ab}	33.3 ± 1.6^{b}
	–N	33.7	36.9 ± 1.1^{a}	35.5 ± 0.5^{ab}
%N	F	7	6.2 ± 0.3^{a}	6.5 ± 0.2^{a}
	–S	6.3	6.1 ± 0.3^{a}	5.6 ± 0.3^{b}
	–N	6.1	6.4 ± 0.3^{a}	6.2 ± 0.1^{a}
%S	F	0.4	0.4 ± 0.0^{a}	0.3 ± 0.0^{c}
	–S	0.3	0.4 ± 0.0^{a}	0.4 ± 0.0^{a}
	–N	0.2	0.5 ± 0.0^{b}	0.5 ± 0.0^{b}
SP	F	124 ± 29^{a}	318 ± 28^{c}	320 ± 36^{c}
	–S	193 ± 18^{a}	340 ± 98^{c}	324 ± 67^{c}
	–N	147 ± 22^{a}	282 ± 34^{bc}	319 ± 56^{c}

Lipid peroxidation

After 5 d in cylinders, all algal cultures, with the exception of controls (full nutrients), showed a doubling of TBARS content (Fig. 6). Both nutrient conditions ($p < 0.01$) and time ($p < 0.001$) caused changes in lipid peroxidation (TBARS per cell). The TBARS concentration shown a time-related increase, and both –S and –N resulted in greater increases of TBARS compared to the full nutrient condition. Nutrient limitation, even when the cultures were exposed to a lower UVR intensity, was enough to significantly increase TBARS per cell (101% and 168%, respectively). The synergistic effect of nutrient limitation and UVR increased the TBARS content, especially in the case of the –N cultures (34 and 18% for –N and –S, respectively).

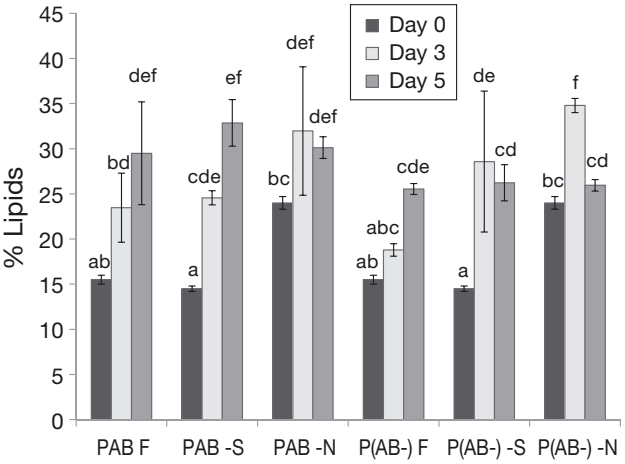

Fig. 5. Lipid content (% of dry wt) in the different nutrient and light treatments (see Fig. 1) at the initial time (algae just transferred from TLC, Day 0), on Day 3 (21 September 2012), and on Day 5 (23 September 2012). Columns showing different letters indicate that differences are statistically significant ($p < 0.05$), whereas the use of the same letter indicates no differences

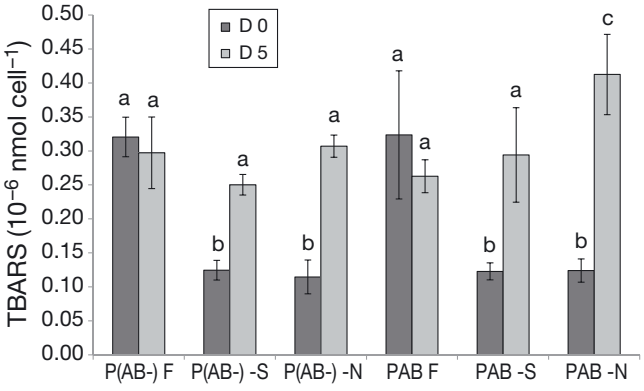

Fig. 6. Lipid peroxidation expressed as nmol MDA equivalent by total cell number in the different nutrient and light treatments (see Fig. 1) at the initial time (algae just transferred from TLC; Day 0) and on Day 5, the last day of the experiment (23 September 2012). Columns showing different letters indicate that differences are statistically significant ($p < 0.05$), whereas the use of the same letter indicates no difference. TBARS: thiobarbituric acid reactive substances

Correlation analysis

As expected, both $K_{c,UVR}$ and $K_{c,PAR}$ were negatively correlated with pigments and cell numbers, whereas a positive correlation was found with proteins and lipids (Table 4). Pigments were positively correlated with cell density. In contrast, cell density was negatively correlated with $rETR_{noon}$ (rETR measured *in situ* at 13:00 h) and with internal compounds, such as proteins and lipids, as well as percentages of C, N, and S. The $rETR_{noon}$ and $rETR_{max}$ (obtained from RLCs) were positively correlated with other RLC parameters, i.e. α_{ETR} and E_k, and also with the UV index. Proteins and lipids were positively correlated, both presenting a negative correlation with pigments. The rest of the analyzed internal compounds, i.e. C, N, S were positively correlated.

DISCUSSION

Initially, cell cultures were grown in TLC to acclimate to the nutrient conditions (N and S deprivation) under complete sunlight (including UVA and UVB). In the TLC, approximately two-thirds of the time, the cells were under dark conditions (due to the pipes and tank of the TLC system), whereas once they were transferred to cylinders, algae were exposed to different light conditions both in quality and quantity (see Jerez et al. 2014). This difference may explain some of the changes during the first moments of the culture in cylinders. The stationary bottle incubation technique for estimating rates of primary productivity has mainly been criticized because of 'bottle effects' related to the elimination of natural turbulence and the presence of photoinhibition. However, these growing conditions have 2 separate, but synergistic, effects. On the one hand, phytoplankton cells move through a light/dark cycle. On the other hand, the boundary layer decreases, which increases the rate of exchange of nutrients and metabolites through the cell wall. Hence, more nutrients are available, and light could be utilized more efficiently, resulting in increased productivity (Grobbelaar 1989). In our study, vigorous aeration was applied to achieve greater hydrodynamics in the culture.

Algae under PAB showed photosynthetic parameters similar to the sun-type pattern, i.e. algae acclimated to high irradiances presented high capacity for energy dissipation and photoprotection (Krause & Jahns 2004). Accordingly, these algae presented an increase of $rETR_{max}$ and E_k but a slight decrease of α_{ETR}, as observed under F nutrient conditions on Day 3. The ETR_{max} was higher on Day 3 under –S and PAB compared to P(AB–), but E_k decreased without any variation in α_{ETR}; no differences were observed after 5 d of culture. However, under –N, no differences in the photosynthetic parameters were observed on Day 3, whereas after 5 d, $rETR_{max}$ was higher under PAB than P(AB–), although E_k and α_{ETR} were not significantly different (Table 2). Higher photosynthetic capacity and recovery after damage under PAB compared to P(AB–) has been previously reported in algae growing under high natural solar irradiance (Flores-Moya et al. 1999, Helbling et al. 2003, Hanelt et al. 2006).

The depletion of nutrients influences many biochemical processes, such as nutrient uptake, pigment synthesis, photosynthesis, cellular growth and organism composition (Dean et al. 2010). The level of proteins in cultures was higher in all nutrient treatments in cylinders than in those under TLCs, even though the total internal N content was similar in the 2 culture systems. No differences in the level of proteins were found due to nutrient treatments. In contrast, the content of chlorophyll and carotenoids was reduced under nutrient-deprived treatments on Day 1. As reported by Young & Beardall (2003), photosynthetic capacity and, consequently, pigment content decrease in microalgae under limitation of N. Overall, pigment concentration was heavily impacted by the N concentration of the medium (Li et al. 2008, I. Malpartida et al. unpubl. data). Moreover, since chlorophyll is a N-rich compound, it can be used as an internal supply of N for algae metabolism (Smart

Table 4. Pearson correlation for the studied variables ($^{\star}p < 0.05$). (1) Bio-optics: UVR extinction coefficient, $K_{d,UVR}$ (m^{-1}); PAR extinction coefficient, $K_{d,PAR}$ (m^{-1}); UVR specific attenuation coefficient, $K_{c,UVR}$ (m^2 mg chl a^{-1}); PAR-specific attenuation coefficient, $K_{c,PAR}$ (m^2 mg chl a^{-1}); chl *a* concentration (µg ml^{-1}); chl *b* concentration (µg ml^{-1}); total carotenoids (Car.) (µg ml^{-1}) and UV index (r.u.). (2) Growth: cell numbers (cells ml^{-1}). (3) Photosynthetic performance: effective quantum yield ($\Delta F_v/F_m'$); photosynthetic active radiation (PAR) (µmol photons m^{-2} s^{-1}); *in situ* rETR measured at noon (µmol e^- m^{-2} s^{-1}); maximum rETR ($rETR_{max}$) (µmol e^- m^{-2} s^{-1}); photosynthetic efficiency (α_{ETR}) and light saturation (E_k) (µmol photons m^{-2} s^{-1}). (4) Internal compounds: total proteins (PRT) (µg ml^{-1}); lipid (% of dry wt), carbon, nitrogen and sulphur content (% of dry wt)

	Bio-optics						Growth	Photosynthetic performance					Internal compounds			
	$K_{d,UVR}$	$K_{d,PAR}$	$K_{c,UVR}$	$K_{c,PAR}$	Chl *a*	Chl *b*	Car.	UV index	No. cells	$\Delta F/F_m'$ PAR	$rETR_{noon}$	$rETR_{max}$	α_{ETR}	E_k	PRT	Lipids	%C	%N	%S
$K_{d,UVR}$	1																		
$K_{d,PAR}$	0.27	1																	
$K_{c,UVR}$	0.87*	0.20	1																
$K_{c,PAR}$	0.70*	0.09	0.94*	1															
Chl *a*	−0.65*	−0.18	−0.89*	−0.88*	1														
Chl *b*	−0.31	0.01	−0.69*	−0.76*	0.84*	1													
Car.	−0.47	−0.13	−0.72*	−0.70*	0.87*	0.75*	1												
UV index	0.07	−0.05	0.14	0.21	−0.17	−0.07	−0.15	1											
No. cells	−0.61*	−0.19	−0.83*	−0.82*	0.94*	0.75*	0.94*	−0.17	1										
$\Delta F/F_m'$	0.47	0.18	0.27	0.24	−0.24	0.11	0	0.20	−0.20	1									
PAR	0	−0.26	0.20	0.25	−0.41	−0.46	−0.56*	−0.12	−0.45	−0.33	1								
$rETR_{noon}$	0.20	−0.21	0.32	0.37	−0.52*	−0.44	−0.57*	−0.04	−0.54*	0.04	0.92*	1							
$rETR_{max}$	−0.21	−0.15	−0.20	−0.17	0.07	0.03	−0.05	0.70*	0.09	0.20	−0.07	0.02	1						
α_{ETR}	0.78*	0.15	0.56*	0.40	−0.22	0.06	−0.11	0.11	−0.21	0.17	−0.17	−0.11	−0.28	1					
E_k	−0.33	−0.09	−0.34	−0.35	0.23	0.14	0.09	0.57*	0.28	0.06	−0.17	−0.15	0.95*	−0.30	1				
PRT	0.55*	−0.03	0.75*	0.73*	−0.69*	−0.65*	−0.51*	−0.13	−0.56*	−0.04	0.28	0.32	−0.26	0.29	−0.33	1			
Lipids	0.35	0.01	0.59*	0.56*	−0.70*	−0.69*	−0.76*	0.18	−0.80*	−0.07	0.31	0.30	−0.02	−0.03	−0.19	0.50*	1		
%C	0.26	0.35	0.25	0.26	−0.41	−0.16	−0.35	0.34	−0.50*	0.59*	−0.12	0.05	0.09	0	−0.04	−0.29	0.32	1	
%N	0.26	0.35	0.25	0.26	−0.42	−0.17	−0.37	0.34	−0.51*	0.58*	−0.11	0.05	0.09	0	−0.04	−0.29	0.33	1.00*	1
%S	0.27	0.35	0.26	0.26	−0.43	−0.18	−0.37	0.34	−0.51*	0.58*	−0.11	0.06	0.09	0	−0.04	−0.28	0.34	1.00*	1.00* 1

1994, Díaz et al. 2006). After 5 d, all treatments showed a decrease in pigment contents. Niyogi et al. (1997) described how promotion of the production of carotenoids allows adaptation to possible photo-oxidation when irradiance decreases the synthesis of chlorophyll structures. Low N nutrition reduces the levels of chlorophyll and soluble proteins, such as RUBISCO, in different algae (Beardall 1991, Wulff et al. 2000). Biliprotein contents decreased in both cyanobacteria (Boussiba & Richmond 1979, Schenk et al. 1983) and red algae (Talarico & Maranzana 2000); in contrast, high nutrient supply produced a rapid increase in phycobiliproteins, reaching about 30 to 40% of the soluble proteins in cyanobacteria (Tandeau de Marsac & Houmardd 1993). Our results show a decrease in chlorophyll but not an increase in carotenoids content, which was very low. This pattern can be explained by the possible cellular acclimation to photo-oxidation of pigments under high irradiance, as suggested by Rosales-Loaiza et al. (2008). In fact, these cultures were first acclimated to complete sunlight, as they were cultivated in TLCs.

Because of the potential commercial interest in lipids, lipid accumulation was carefully assessed. The variation in biomass productivity provoked by treatments affected the biochemical composition of the cultures, showing a clear bioaccumulation of lipids under starvation conditions (–S and –N treatments). Lipid metabolism is a good example of the synergistic effect of nutrients and UVR, showing 2 differentiated stages. That is, during the first 3 d, the nutrient condition factor controlled the accumulation of lipids, while during the last 2 d, light was the controlling factor. After 3 d in cylinders, the –N treatment, irrespective of light conditions, showed the highest lipid content, reaching nearly 35% of dry wt. At the same time, the low ETR data indicated that this increase was realized under low-production conditions, in agreement with other studies (Illman et al. 2000, Yeh & Chang 2012). In other studies, the addition of N and Fe increased the lipid production in *Dunaliella salina* based on the increase of biomass productivity (Mata et al. 2013). In our case, the decrease in the cell number by N limitation was

not compensated by the increase of lipid content per cell, as in the case of –S cultures. This effect may have been a response to nutrient stress; the responses to stress may include a decrease of cellular growth along with the simultaneous increase of energy storage molecules (Meng et al. 2009).

The UVR effects presented a strong time dependency, suggesting the importance of the UVR dose accumulated by the algae. After 5 d, cultures under PAB presented the greatest lipid content. Particularly, –S showed the greatest enhancement in lipid production, possibly because microalgal requirements of S are quite low, between 0.15 and 1.96% (Barsanti & Gualtieri 2006). S deficiency provoked a significantly higher pH in the algal culture, with values around 7.8 to 8.0. The pH increase was significantly enhanced by UVR. Kosourov et al. (2003) reported that S deprivation of cultures of *Chlamydomonas reinhardtii* resulted in the photoproduction of H_2, which could alter pH equilibrium. However, a significant change in CO_2 availability is not expected due to pH differences among treatments (in the range of pH shown, most of the C is available as HCO_3^-). Several studies have shown that negative effects on productivity of marine or freshwater planktonic algae appear only at pH > 8.8 (Azov 1982, Chen & Durbin 1994).

The effect of UVR depends on maintaining a dynamic equilibrium between damage and repair (Lesser et al. 1994, Heraud & Beardall 2000, Litchman et al. 2002). Any imbalance in these processes affects PSII dynamics and leads to photoinhibition. The decrease in chlorophyll fluorescence under UVR was related to the decrease in pigment content observed under both of the light treatments. The fluorescence decrease was probably also related to damage to PSII (see review by Vincent & Neale 2000). Over both short and long terms, Carrillo et al. (2008) showed the lack of harmful UVR effects on primary production, chl *a* and biomass, suggesting that the loss of C, which results in low sestonic C:P ratios, might be part of an adaptive strategy of phytoplankton to high UVR and extreme nutrient limitation. It is also known that nutrient enrichment (P) may reduce the negative effect of UVB radiation on the growth of other microalgae (Germ et al. 2002).

High PAR irradiance can also provoke photoinhibition (Villafañe et al. 2003). In our study, PAR irradiance was the same under both light treatments, and in P(AB–), both UVA and UVB were decreased by the cut-off filter used. UVA has been reported to have both negative and positive effects in phytoplankton. A decrease in primary production (measured as C incorporation) is among the negative effects (Villafañe et al. 2003), while among the positive effects, UVA can act to enhance C fixation (Helbling et al. 2003), allow photorepair (Buma et al. 2003), increase biomass (Wu et al. 2005) and favour primary productivity by means of utilization of UVA as an energy supply for CO_2 fixation (Gao et al. 2007). The impact of UVR on the cells depends on the bio-optical characteristics related to cell size and pigment composition (Figueroa et al. 1997).

Evidence of photoacclimation can also be seen in our study. *Chlorella fusca* is a relatively large species, and therefore, it is expected to present higher resistance to UVR than species with smaller cells. The increase in cell size diminishes UVB penetration in the nucleus and chloroplasts, reducing the potential damage to DNA and photosystems. It is commonly accepted that small cells (nanoplankton) are more vulnerable to UVR than large cells (microplankton) because the latter have slower kinetics of photoinhibition and can therefore resist greater UVR-related damage to photosynthesis (Figueroa et al. 1997, Villafañe et al. 2003). In this study, both $K_{c,PAR}$ and $K_{c,UVR}$ were correlated with photosynthetic efficiency (α_{ETR}) but not with photosynthetic capacity (rETR). Figueroa et al. (1997) showed that a specific attenuation coefficient (K_c) ranging from 0.01 to 0.03 m^2 mg^{-1} chl *a* explained the acclimation to increased irradiance, demonstrating that increases of K_c were related to increased photoinhibition. In our experiment, $K_{c,UVR}$ showed the highest values for PAB F and –N, which is consistent because UVR was complete in these treatments, allowing for a certain level of photoinhibition. In the case of –S, the value is higher in P(AB–) conditions but still close to the value in PAB. It is possible that PAB conditions provoked more damage and less recovery in cells and that this effect was also important for the –S treatment after 5 d, even under lower UVR conditions.

Finally, the UVR effect on lipids was determined because UVR is a known source of reactive oxygen species, which increase oxidative stress in photosynthetic organisms (Lesser et al. 1994, Foyer & Shigeoka 2011). However, oxidative stress may also be increased if the antioxidant mechanisms of cells are stopped or diminished. One of the consequences of oxidative stress is lipid peroxidation, as a result of the oxidation of unsaturated lipids; this process has been reported in most algal groups (Malanga & Puntarulo 1995, Lesser 1996, Malanga et al. 1997, Rijstenbil 2001, 2002). However, it is noteworthy that lipid peroxidation did not happen in the full nutrient treatment, which can be attributed to the full effec-

tiveness of repair mechanisms that had no limitation from nutrient availability. Nutrient limitation is known to induce ROS production and decrease the repair capability of a cell (Berges & Falkowski 1998, Logan et al. 1999, Bucciarelli & Sunda 2003, Menon et al. 2013). Here, the combined effect of limitation of essential nutrients like N and S needed for oxidative repair mechanisms under PAB conditions resulted in increased lipid peroxidation (Lesser et al. 1994, Litchman et al. 2002, Van De Poll et al. 2005).

Based on appropriate control of the nutrient and light growing conditions, our data showed that it would be feasible to control productivity, growth and UVR acclimation of *Chlorella fusca* cultures. These processes would lead to changes in the biochemical composition of the algal cells, which may result in the bioaccumulation of molecules at rates that make its commercial exploitation feasible.

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