# Photosynthetic characterization of two *Nannochloropsis* species and its relevance to outdoor cultivation

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## Abstract

Despite the increased interest in exploring the potential of algal biomass production for food stock and renewable energy, very little work has been done in developing reliable screening protocols to enable the identification of species that are best suited to mass cultivation outdoors. *Nannochloropsis* is an algal genus identified as a potential source of lipids due to its ability to accumulate large quantities of these compounds, especially under nutrient-limiting conditions. The objective of the current work was to use two species of this genus, *Nannochloropsis oceanica* and *N. oculata*, as model organisms to develop a protocol that will allow the evaluation of their capacity to yield high biomass productivity under outdoor conditions. Growing the alga under different light intensities and measuring growth rate as well as a range of photosynthetic parameters based on light response curves and variable fluorescence highlighted significant differences between the two species. Our data show that *N. oceanica* cells have a better capacity to respond to higher light intensities, as reflected by growth measurements, photosynthetic electron transport rates, and oxygen evolution as well as their response to the very high photon flux densities expected in outdoor culture. On the other hand, *N. oculata* showed a higher tolerance to oxidative stress as reflected in its resistance to the reactive oxygen species generating compounds Rose Bengal (RB) and methyl viologen (MV). Based on the above evidence, we suggest that *N. oceanica* may perform better than *N. oculata* when grown under high light conditions typically found outdoors in summer, while *N. oculata* may perform better than *N. oceanica* under oxidative stress conditions usually found in outdoor cultures exposed to a combination of high light and low temperature commonly occurring in winter time.

**Keywords** Photosynthetic characterization · *Nannochloropsis* · Outdoor cultivation · Rose bengal · Methyl viologen · Light stress · Oxidative stress

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## Introduction

Algal biotechnology has been a topic of intensive research for the last 50 years and aims to exploit the potential of algal biomass as a source for the production of a range of commercially valuable bioproducts, as well as using algae for other applications such as wastewater treatment and environmentally friendly biofuels. However, while production of useful products is relatively well characterized at the laboratory scale, translation to large-scale cultivation of algae for biotechnology faces an array of challenges. Recently, Borowitzka and Vonshak (2017) pointed out the main limitations of, and obstacles to, the process of scaling up from laboratory scale to outdoor mass production of microalgae.

Importantly, studies dealing with up-scaling can be divided into three major topics of concern. The first topic deals with the "outdoor physiology" of the cultures and involves attempts to understand the role of the major limitations to algal

growth and productivity. The second involves the development of down-stream processing implicated in the harvesting, extraction, and formulation of the desired product. The third topic is the design of suitable reactors in order to develop and make the best fit between the need to produce large amounts of biomass in an efficient way while reducing the initial investment cost and eventually achieving a sustainable large scale production facility.

There have been significant breakthroughs in the last two topics, which may be related to the fact that many technologies have been developed in the food and fermentation industries that are easily modified and adjusted to the needs of processing algal biomass at the large scale. On the other hand, in relation to the first topic, there is still a large knowledge gap between our understanding of algal physiology in the laboratory, with controlled conditions for growth, and the response of algal culture to the diverse and extreme conditions prevailing in large-scale outdoor cultures (Sandnes et al. 2005).

The two major factors, which need to be considered in upscaling of outdoor cultures as affecting productivity, are light availability and temperature. These show diurnal as well as seasonal changes (Vonshak et al. 2014; Borowitzka and Vonshak 2017), in addition to the light/dark regime affected by irradiance changes in the depth of the mixed growth medium. Accordingly, an important role in the scaling-up process is selecting species that rapidly acclimate to these variable conditions, including surviving dilution from a concentrated inoculum culture that may cause a rapid increase in the average light intensity in the culture and thus potentially lead to photoinhibition.

The re-discovery of the potential use of microalgae for sustainable production of fuels of the future has led to a burst of publications using algal species capable of accumulating lipids at high concentrations. In this connection, green microalgae of the genus *Nannochloropsis* (Class Eustigmatophyceae) have emerged as leading candidates for this application from work that commenced some 30 years ago (Boussiba et al. 1987) and has been continuing to the present day (Solovchenko et al. 2014; Ma et al. 2016). According to recent data from the ISI Web of Knowledge, out of more than 2500 publications using this genus, most of which were published in the last 10 years, more than 50% related to maximizing the ability of species to accumulate lipids for their potential use as biodiesel. Despite this, very little information has been presented which deals with the justification for, or the rationalization of, the use of the selected species from among all the other species of *Nannochloropsis* (Simionato et al. 2013). In addition, only a very small number of publications actually deal with the photosynthetic apparatus of *Nannochloropsis* and its response to light or temperature changes (Fisher et al. 1996; Sandnes et al. 2005; Simionato et al. 2011; Palacios et al. 2018). Moreover, even fewer publications deal with the photoacclimation process under outdoor conditions (Sukenik et al. 2009).

All photosynthetic organisms demonstrate an ability to acclimate to varying light conditions in order to maintain a balance between capturing a sufficient number of photons to sustain the photosynthetic machinery in an active state and avoiding the possible damaging effects of over-reduction of the photosynthetic electron transport components. Generally, following exposure to stress conditions, the photosynthetic apparatus is subject to photo-oxidative stress due to generation of reactive oxygen species (ROS). In order to protect the organism, various strategies have evolved. Among these is the process known as non-photochemical quenching (NPQ) which allows rapid dissipation of excess absorbed energy as heat when the electron transport chain becomes saturated by excessive light (Weis and Berry 1987; Ruban 2014).

In a range of environmental conditions, such as the presence of photoinhibitory light or low temperature, mitochondrial respiration also plays a significant role in protecting the chloroplasts from photoinhibition. This protection mechanism is accomplished through energy dissipation involving the photorespiratory reactions (Padmasree et al. 2002) which are controlled by the activity of Rubisco and affect the rate of net photosynthetic $O_2$ evolution, particularly in C3 vascular plants. Measurement of dark respiration can thus be an additional tool in evaluating the mechanisms and efficiency by which algal species are capable of acclimating to light stress.

Rose Bengal (4,5,6,7-tetrachloro-2′,4′,5′,7′-tetraiodofluorescein, RB) interacts with oxygen in the presence of light to produce reactive oxygen species (see Methods) and thus sensitivity to RB might be used as a measure of an algal species' sensitivity to oxidative stress. Through a different process, methyl viologen (1,1′-dimethyl-4,4′-bipyridinium, MV) catalyzes $O_2$ photoreduction thus producing $O_2^-$ and $H_2O_2$ in the chloroplast. In addition, MV has often been used as an inducer of photooxidative stress. Using both chemicals as a selection tool may therefore be used as a useful indicator to evaluate the ability of cells to deal with oxidative stress (Ben Sheleg et al. 2019).

The final effect on acclimation by these photoprotective mechanisms translates into alterations and adjustment of the photosynthesis versus irradiance (P versus I) curve parameters of cultures grown under different light conditions, including photoinhibition at high light. The achievement of photoacclimation is brought about by the modification in one or more of the parameters of the P vs I curve, namely dark respiration ($R_d$), the light-saturated photosynthetic rate ($P_{max}$), and the initial slope of the P versus I curve, $\alpha$, that represents the light utilization efficiency. These in turn lead to changes in other parameters such as the point of saturating irradiance ($I_k$) and the compensation light intensity ($I_c$) (Palacios et al. 2018).

The objective of the current study was to develop a tool based on a set of measurements, related to the response of growth and photosynthesis to light intensity, in order to be able to select algal species more suitable for large-scale

outdoor cultivation. We have chosen two species of *Nannochloropsis* and evaluated their ability to respond to a variety of light conditions. We followed the changes in photosynthetic performance occurring during acclimation to high light as changes in chlorophyll fluorescence quenching and oxygen evolution measurements, which were compared to the growth rates attained by the two species.

## Materials and methods

### Species

Two species of the unicellular marine green alga *Nannochloropsis* (Eustigmatophyceae) were used in this study. *Nannochloropsis oceanica* strain CCALA 804 [ZMORA/1995] was obtained from the Culture Collection of Autotrophic Organisms (CCALA), Institute of Botany, and Academy of Sciences of the Czech Republic. *Nannochloropsis oculata*, strain CS-179, was obtained from the CSIRO Australian National Algae Culture Collection.

### Growth condition

**Medium** Both species were cultured in Reef Salt Enriched (RSE) medium which comprised: 34 g $L^{-1}$ Reef Salt (Seachem, Madison, GA, USA), 2 g $L^{-1}$ $KNO_3$, 70 mg $L^{-1}$ $KH_2PO_4$, with the addition of 1 mL $L^{-1}$ $A_5$+Co microelements solution (0.77 μM $ZnSO_4 \cdot 7H_2O$, 0.31 μM $CuSO_4 \cdot 5H_2O$, 1.61 μM $Na_2MoO_4 \cdot 2H_2O$, 1.61 μM $Na_2MoO_4 \cdot 2H_2O$, 46.3 μM $H_3BO_3$, 9.15 μM $MnCl_2 \cdot 4H_2O$, 0.172 μM $Co(NO_3)_2 \cdot 6H_2O$) and 1 mL $L^{-1}$ ferric citrate mixture (26.7 μM ferric citrate and 42.8 μM citric acid), and 500 μL $L^{-1}$ vitamin solution (1 g $L^{-1}$ vitamin $B_{12}$ and 1 g $L^{-1}$ biotin).

**Inoculum preparation** Stocks of the cultures were maintained in a rotary shaker agitated continuously at 120 rpm. Continuous light at a photon flux density of 70–75 μmol photons $m^{-2}$ $s^{-1}$ was provided by cool-white fluorescent lamps (Aqua Relle, TL-D 30W, Philips). Cultures were grown in flasks and bubbled with air enriched with 2% $CO_2$. Constant temperature was maintained at the optimal value of 25 °C. The chlorophyll concentration of cultures was kept within the range of 10–20 μg $mL^{-1}$ by dilution once a week with fresh medium.

**Growth in a multi-cultivator (MC)** For all experiments, cultures were incubated in the 80-mL culture cylindrical vessels (i.d., 4 cm) of a Photon Systems Multi-Cultivator (MC1000-OD, Photon Systems Instruments, Drasov, Czech Republic, www.psi.cz). The cultivation vessels were immersed in a temperature-controlled water bath at the optimal temperature

of 25 °C. Each cultivation vessel was monitored for growth by automatic measurement of optical density at 680 nm and 720 nm though, since the light path is 5 cm, readings become saturated at a very early stage of the growth phase and thus only a very short stage of real logarithmic growth rate can be monitored. For this reason, specific growth rate was calculated on the basis of other parameters (see below). Cultures were acclimated to the multi-cultivator (MC) incident light of 100 μmol photons $m^{-2}$ $s^{-1}$ for at least 1 week, along with dilution every 3 to 4 days to a chlorophyll concentration of 2 μg $mL^{-1}$ to maintain growth in the exponential phase. Subsequently, each cultivation vessel was independently adjusted to the required light (Cool White LED illumination) for growth at 50, 100, 200 or 300 μmol photons $m^{-2}$ $s^{-1}$ and bubbled with air enriched with 2% $CO_2$. All experiments were started with a chlorophyll concentration of 2 μg $mL^{-1}$.

**High light experiment** Acclimated cultures, in the 80-mL cultivation vessels of the multi-cultivator, were diluted to 2 μg chlorophyll $mL^{-1}$ and maintained at the same optimal temperature of 25 °C and bubbled with 2% $CO_2$ enriched air. The same light intensity for growth was maintained in each cultivation vessel (50, 100, 200, or 300 μmol photons $m^{-2}$ $s^{-1}$). After 3 days, the culture in each vessel was diluted to a chlorophyll concentration of 5 μg $mL^{-1}$, and the light was increased to 1600 μmol photons $m^{-2}$ $s^{-1}$ for 24 h (high photon flux density, HPFD). Growth parameters (chlorophyll *a* and cell number), photosynthetic activity ($O_2$ evolution), and chlorophyll fluorescence measurements were carried at the initial time (d0) and after 24 h (d1).

### Growth measurements

Chlorophyll *a*: Samples (1–2 mL) were harvested by centrifugation for 5 min at 12,000× *g*. Chlorophyll was extracted with dimethyl sulfoxide (DMSO) (Burnison 1980) at 70 °C for 5 min. The absorbance of the supernatant at 665 nm was subsequently measured by spectrophotometry (Cary 50 Bio spectrophotometer, Varian, USA), and chlorophyll *a* concentration was determined according to Seely et al. (1972).

**Cell number** Cells were counted using a Luna Dual Fluorescence Cell Counter, Logos Biosystems, Korea (www.logosbio.com). A sample of culture (10 μL) of was loaded into a Luna Cell Counting Slide (C.N L12001), and cells were counted manually.

**Growth parameters** The specific growth rate was calculated using Eq. 1:

$$\mu = (\ln x_2 - \ln x_1)/(t_2 - t_1) \tag{1}$$

where $x_1$ and $x_2$ are biomass concentrations (as Chl mL$^{-1}$ or cell number mL$^{-1}$) at time intervals $t_1$ and $t_2$ within the exponential phase of growth.

## Photosynthesis vs. irradiance curve (P vs I curve)

Rates of photosynthetic oxygen evolution as a function of irradiance (P vs I curve) and of dark respiration ($R_d$) were measured with a Clark-type oxygen electrode (DW2/2 Oxygen Electrode, Hansatech Instrument Ltd., UK) connected to a Hansatech Oxylab control box. Algal samples (2 mL) of known chlorophyll concentration (5 µg mL$^{-1}$) were inserted into the water-jacketed, temperature-controlled chamber (25 °C), and mixed by magnetic stirrer. Using the Oxygraph Plus software or O$_2$ViewXP software (Hansatech Instrument Ltd.), the oxygen evolution rate was recorded. Oxygen evolution rates (average of at least three repetitions) are reported in units of µmoles of O$_2$ evolved per unit of time per unit of biomass, the latter expressed as cell number or chlorophyll concentration.

The irradiance was controlled and modified by intensity-controllable white LED light, which was programmed to increase in intensity according to a specific, pre-determined light regime (10–1000 µmol photons m$^{-2}$ s$^{-1}$), which allowed for the production of photosynthetic response curves (P vs I curves). Each P vs I curve was analyzed according to Eq. 2 (Eilers and Peeters 1988):

$$P = (I-I_c)/a(I-I_c)^2 + b(I-I_c) + C \qquad (2)$$

and allowed the determination of several photosynthetic parameters, such as the dark respiration ($R_d$); photosynthetic compensation point ($I_c$); when the photosynthetic rate is just balanced by respiration, maximum rate of light-saturated photosynthesis ($P_{max}$); irradiance at the onset of light saturation ($I_k$); and the light utilization efficiency as determined by the initial slope ($\alpha$). By using the equations of Eilers and Peeters (1988), the three latter parameters can be estimated as given in Eq. 3:

$$P_{max} = 1/(b + 2\sqrt{ac}), \alpha = 1/c \text{ and } I_k = c/b + 2\sqrt{ac} \quad (3)$$

## Measurement of specific chlorophyll optical absorption cross-section

The optical absorption cross-section ($a^*$) of the cells (m$^2$ mg$^{-1}$ Chl $a$) was determined from *in vivo* absorption spectra (in the range from 400 to 750 nm) according to Kromkamp and Limbeek (1993) using a spectrophotometer (Varian Cary50 UV-visible). To minimize the impact of the light scattering effect from the cell surface, the sample cuvette was placed close to the detector window with standard white A4 copy paper as a light diffuser placed on the opposite face of the 1-cm cuvette.

The maximum quantum yield $\theta_{max}$, i.e., the ratio of the rate of oxygen evolution to that of light absorption under limiting light, was calculated according to Eq. 4 (Kromkamp and Limbeek 1993):

$$\theta_{max} = \alpha/a^* \qquad (4)$$

where $\alpha$ and $a^*$ are expressed per Chl $a$ (see Table 1 for abbreviations) (Fisher et al. 1996).

## Fluorescence measurements

The maximum quantum yield of PSII ($F_v/F_m$) was measured in dark adapted cells (10 min). Rapid light-response curves: (RLCs) of *Nannochloropsis* cultures were measured using a 2-mL cell sample (4 µg mL$^{-1}$ Chl $a$) placed in a liquid-phase oxygen electrode chamber (Hansatech, DW3) thermoregulated at 25 °C cuvette using a pulse-amplitude-modulation fluorimeter (PAM-2100, H. Walz, Germany). A series of stepwise increasing irradiance intensities (LEDs, 0–636 µmol photons m$^{-2}$ s$^{-1}$) provided by a PAM-2100 were automatically applied at 20-s intervals to obtain the light-adapted fluorescence level F' (steady-state fluorescence yield in the light), and at the end of each step a saturating pulse (> 6000 µmol photons m$^{-2}$ s$^{-1}$, 0.6 s duration) was triggered to reach the maximum fluorescence level $F_m'$ (steady-state maximum fluorescence in the light). The effective PSII photochemical quantum yield in the light, $Y_{II}$, was determined as $(F_m'-F')/F_m'$ in the light-adapted state at the respective irradiance level. The electron transport rate was calculated according to Kromkamp and Forster (2003) (Eq. 5):

$$ETR = PFD \times (F_m'-F')/F_m' \times 0.5 \times a^*$$
$$\times 60 \left( \mu mol\ e^- \ mg^{-1}\ Chl\ min^{-1} \right) \qquad (5)$$

Although measurement of $a^*$ is based on the extinction coefficient of chlorophyll $a$ and therefore cannot be considered specific for PSII (Schreiber et al. 2012; Szabó et al. 2014), it allows for a more accurate measure of ETR in microalgal suspensions compared to what directly extracted from the PAM Software which was developed to perform measurement in leaves. Moreover, the results are easier to compare with those obtained with the P/I curve measurements which are also normalized on a Chl $a$ basis.

Analysis of RLCs was used to calculate changes in important parameters, namely the maximum relative electron transport rate through PSII, ETR$_{max}$; the initial slope, $\alpha$, of the ETR vs. PFD curve; the saturation irradiance, $I_k$, given as the intercept between $\alpha$ and ETR$_{max}$; and the maximum quantum yield

**Table 1** Symbols and abbreviations of the photosynthesis and fluorescence parameters.

| Symbols | Abbreviations (units) |
| --- | --- |
| PFD | Photon flux density (400–700 nm) ($\mu$mol photons m$^{-2}$ s$^{-1}$) |
| Chl $a$ | Chlorophyll $a$ (mg L$^{-1}$) |
| a* | *In vivo* optical absorption cross-section (m$^2$ mg$^{-1}$Chl) |
| $\alpha$ per chl $a$ | Light utilization efficiency per chl $a$ ($\mu$mol O$_2$ mg$^{-1}$ Chl $\mu$mol$^{-1}$ photons m$_{-2}$) |
| $\Phi_{max}$ | Maximum quantum yield (mol O$_2$ evolved mol$^{-1}$ photons absorbed) |
| $\Phi_{max}^{-1}$ | Minimum quantum requirement (mol photons mol$^{-1}$ O$_2$ evolved) |
| $I_k$ | Irradiance at the onset of light saturation ($\mu$mol photons m$^{-2}$ s$^{-1}$) |
| $P_{max}$ per cell | Maximum photosynthesis rate per cell ($\mu$mol O$_2$ cell$^{-1}$h$^{-1}$) |
| $P_{max}$ per chl $a$ | Maximum photosynthesis rate per chl $a$ ($\mu$mol O$_2$ mg$^{-1}$Chl h$^{-1}$) |
| $R_d$ per cell | Dark respiration rate per cell ($\mu$mol O$_2$ cell$^{-1}$h$^{-1}$) |
| $R_d$ per chl $a$ | Dark respiration per chl $a$ ($\mu$mol O$_2$ mg$^{-1}$Chl h$^{-1}$) |
| $I_c$ per chl | Compensation point. Light intensity at which photosynthesis rate is zero (i.e. photosynthesis = respiration) |
| $F_v/F_m$ | Maximum photochemical quantum yield of PSII |
| ETR | Electron transfer rate ($\mu$mol e$^-$ mg$^{-1}$Chl min$^{-1}$) |
| NPQ | Non-photochemical quenching (relative units) |
| $\mu$ | Growth rate (h$^{-1}$) |
| DT | Doubling time (h) |

of photosynthesis $\Phi_{max}$. The curves were fitted to the non-linear least-squares regression model of Eilers and Peeters (1988) using PamWin 3 software.

Non-photochemical quenching was calculated as shown in Eq. 6:

$$NPQ = (F_m - F_m')/F_m' \qquad (6)$$

It is inversely related to photochemistry ($Y_{II}$) and is considered a safety valve protecting PSII reaction centers from damage by excess irradiance (Bilger and Björkman 1990).

## Light measurements

Values of photosynthetically active radiation (PAR), for both fluorescence and photosynthesis measurements, were measured as 15-s average values using a LI-250A light meter (Li-Cor, USA) equipped with a spherical sensor, model SQSA0191 (H·Walz, Germany) placed in the cuvette, or with a flat LI-190SA quantum sensor (cosine-corrected up to 80° angle of incidence) for cultures grown in the Photon Systems Multi-Cultivator PSI cultivator.

Definitions and abbreviations used are reported in Table 1.

## Exposure to ROS

The rose bengal (RB) molecule (Sigma R3877) produces singlet oxygen ($^1O_2$) when it is exposed to photons in the 450–580-nm range. The reactions of singlet oxygen produced by the photosensitizer are generally limited to the subcellular region where the photosensitizer molecule is located. This is due to the fact that singlet oxygen is highly reactive with a very short half-life (Kochevar and Redmond 2000; Kovács et al. 2014). RB has been shown to localize in the chloroplast and is capable, due to the creation of $^1O_2$, of decreasing photosystem II (PSII) electron transport and of damaging the PSII reaction center complex (Padmasree et al. 2002).

The bipyridilium herbicide paraquat (1,1-dimethyl-4,4-bipyridilium) or methyl viologen (MV) (Sigma M2254) is a highly potent herbicide. Its mode of action is to mediate the generation of superoxide by re-routing electrons from photosystem I, subsequently initiating the production of other ROS, leading to oxidative damage and cell death (Hawkes 2014). Thus, MV toxicity may mimic the mechanisms of photooxidative damage. Consequently, tolerance to photooxidation may correlate well with resistance to MV (Iturbe-Ormaetxe et al. 1998; Murgia et al. 2004).

Cultures of the two *Nannochloropsis* species, *N. oceanica* and *N. oculata*, at a concentration of 10 $\mu$g Chl mL$^{-1}$ were dispended into 2-mL aliquots in each well of a 24-well plate, and exposed to a gradient of concentrations of RB (0-25 $\mu$M) or MV (0–400 $\mu$M). Plates were incubated under cool white fluorescent light of 70 $\mu$mol photons m$^{-2}$ s$^{-1}$ for 4 days, and mixed every day and before photographing the plate to provide a record of relative growth.

## Statistical analysis

Sigma Plot 11.0 was used to determine significant differences between treatments. One-way ANOVA and a Holm-Sidak test

were conducted for every binary combination of systems. $P$ values less than 0.05 were considered significantly different.

## Results

### Growth

For any algal species that is evaluated as a potential candidate for outdoor cultivation, its ability to respond and acclimate to a wide range of light intensities should be carefully assessed. To this end, cells were acclimated to light intensities from 50 to 300 µmol photons m$^{-2}$ s$^{-1}$ and growth was monitored by following both the daily increase in chlorophyll $a$ and cell number. In both species, a positive response of growth to increases in light intensity was observed. However, different trends were obtained in the response to light intensity when using either chlorophyll (Fig. 1a) or cell number (Fig. 1b) to calculate the specific growth rate (µ). Indeed, significant differences ($P < 0.05$) between the two methodologies of calculating growth rate were observed at 200 and 300 µmol photons m$^{-2}$ s$^{-1}$ for *N. oceanica*, and at 300 µmol photons m$^{-2}$ s$^{-1}$ for *N. oculata*. On the whole, *N. oceanica* appears to respond better to a wider range of light intensities than *N. oculata*, i.e., the growth rate of *N. oculata* becomes saturated at 200 µmol photons m$^{-2}$ s$^{-1}$ when evaluated on the basis of cell number (Fig. 1b).

### Electron transport rate

Cells grown at 50, 100, 200, 300 µmol photons m$^{-2}$ s$^{-1}$ were used to measure the ETR vs light relationship, and the results are presented in Fig. 2. Although in both species a trend of increase in the ETR with growth light is observed, in cultures grown at higher light intensities (300 µmol photons m$^{-2}$ s$^{-1}$)

as compared to those grown at 50 µmol photons m$^{-2}$ s$^{-1}$, it is worth noting that in the *N. oceanica* cultures (Fig. 2 (a1)) the increase is in the range of twofold, while in the *N. oculata* cells (Fig. 2 (a2)) (SD is provided in Table S1 in the supplementary section), this increase is no more than 30%. This different behavior supports the idea that each species has a different ability to respond to variations in light. More detailed analysis of the calculated parameters is provided in Table S1 in the supplementary data section. Calculation of the quantum requirement at the different light intensities showed only small increases with increase of the light intensity (Table S1).

### Maximum photochemical quantum yield, NPQ, and optical absorption cross-section changes

The maximal photochemical efficiency $F_v/F_m$ is commonly used as an indicator of the potential for photosynthetic activity. It showed no significant changes in both species (Table 2).

Non-photochemical quenching (NPQ) is an additional parameter extracted from measurements of variable fluorescence of chlorophyll and may serve as an indicator of the ability of cells to dissipate excess energy and thus better acclimate to high light stress. As expected, measuring the NPQ of the two species grown in different light intensities indicated that the capacity of cells to dissipate light energy via NPQ increased as the growth irradiance increased (Table 2). However, as for ETR (Fig. 2), in *N. oceanica* cultures grown at higher light intensities (300 µmol photons m$^{-2}$ s$^{-1}$) as compared to those grown at 50 µmol photons m$^{-2}$ s$^{-1}$, the NPQ increased in the range of threefold while in *N. oculata*, this increase was no more than 30%, with no difference between cells grown at 200 or 300 µmol photons m$^{-2}$ s$^{-1}$.

Changes in the *in vivo* optical cross-section of cells a* (Table 2) may also serve as an indication of the ability of cells to acclimate to a variety of light intensities. These

**Fig. 1** Specific growth rate of the two *Nannochloropsis* species: *N. oceanica* (solid) and *N. oculata* (white) grown at different light intensities. Data were calculated from consequent measurements of chlorophyll content per mL (**a**) or a cell number per mL (**b**). Data are presented as the mean ± SD ($n$ = 3)
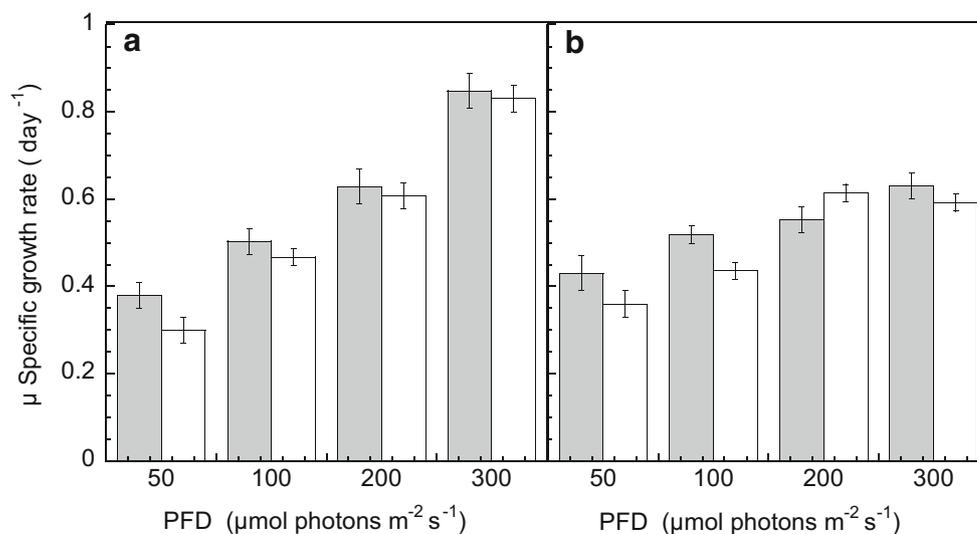
**Fig. 2** The ETR Curves of both *Nannochloropsis* species: *N. oceanica* (a1) and *N. oculata* (a2) grown at the different light intensities: 50 (black circles), 100 (red squares), 200 (blue diamonds), and 300 µmol photons m$^{-2}$ s$^{-1}$ (green triangles). Data are presented for chlorophyll content per mL. Data are presented as the mean ± SD ($n = 3, 4$)
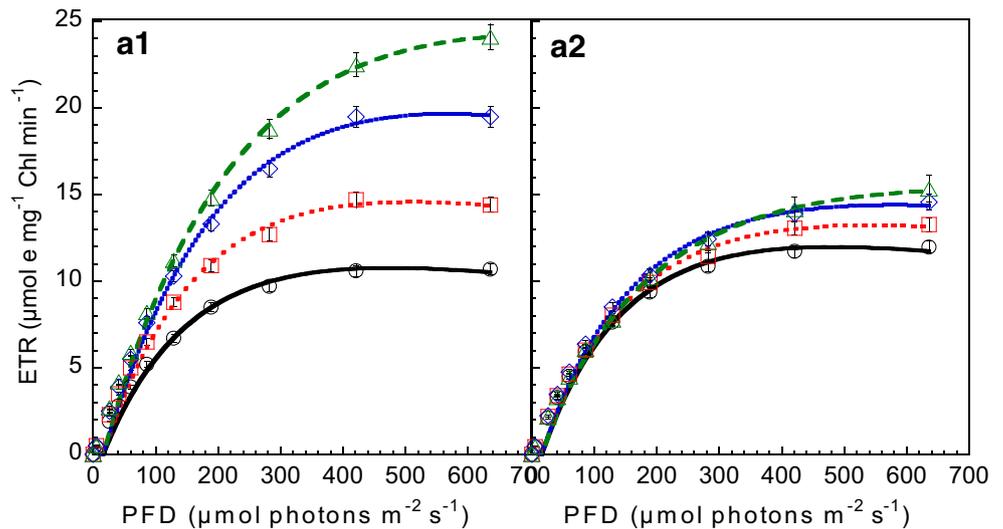
measurements were also used in converting the ETR values from relative values, as they come from the PAM fluorimeter expressed as µmol e$^-$ m$^{-2}$ s$^{-1}$ to ETR values expressed as µmol e$^-$ mg$^{-1}$ Chl min$^{-1}$. In *N. oceanica*, the optical absorption cross-section increased about 30% from the lowest to the highest growth irradiance tested, both normalized in terms of chlorophyll (m$^2$ mg$^{-1}$Chl) and cell (m$^2$ cell$^{-1}$), while with *N. oculata* changes in cross-section were not significant (5%, $P > 0.05$) (Table 2).

## Oxygen evolution

Although measurement of ETR is a common tool in the study of photosynthesis, it does not give a direct measurement of photosynthetic activity. It uses changes in variable chlorophyll florescence and converts this to a measure of photosynthetic "activity." Another drawback in the use of the fluorescence methodology is that it does not provide information on the dark respiration activity that may play an important role in the light acclimation process. Consequently, another parameter that is not available when using the ETR methodology is the "light compensation point" I$_c$, a value indicating the light

required to drive a sufficient rate of light-dependent oxygen evolution to compensate for respiratory oxygen uptake. We have thus complimented the ETR measurements with a more direct measure of photosynthesis, i.e., the oxygen evolution rate. Cells grown and acclimated to different light intensities were treated as described in the "Materials and methods" and the oxygen evolution rate was measured at different light intensities (P vs I curves). The results are presented in Fig. 3, and the major parameters are summarized in the inserted table in each figure. SD is provided in Table S2 in the supplementary section. When using P-I curves to evaluate light acclimation, there is a continuing debate as to what is the best basis for normalizing the activity. MacIntyre et al. (2002) discussed the issue in detail, pointing out the limitation of each methodology. However, the use of both procedures to evaluate photosynthesis capacity should be considered when chlorophyll per cell varies between cultures. We therefore provide the results with rates calculated on the basis of (i) chlorophyll concentration (Fig. 3 (a1 and a2)) as well as (ii) cell counts (Fig. 3 (b1 and b2)). *N. oceanica* demonstrated, particularly when acclimated at light intensities above 50 µmol photons m$^{-2}$ s$^{-1}$, a much higher response to the light growth conditions than *N. oculata* when activities were expressed on the basis of

**Table 2** F$_v$/F$_m$, NPQ, a* *in vivo* optical absorption cross-section (m$^2$ mg$^{-1}$ Chl) and Φmax maximum quantum yield (mol O$_2$ evolved mol$^{-1}$ photons absorbed) in *N. oceanica* and *N. oculata* acclimated to different light intensities. Data are presented as the mean ± SD ($n = 3, 4$).

| PFD | *N. oceanica* | | | | *N. oculata* | | | |
|---|---|---|---|---|---|---|---|---|
| | F$_v$/F$_m$ | NPQ | a* (10$^{-3}$) | Φ$_{max}$ | F$_v$/F$_m$ | NPQ | a* (10$^{-3}$) | Φ$_{max}$ |
| 50 | 0.663 ± 0.03 | 0.23 ± 0.014 | 4.2 ± 0.25 | 0.088 ± 0.004 | 0.677 ± 0.033 | 0.35 ± 0.012 | 4.6 ± 0.23 | 0.087 ± 0.0026 |
| 100 | 0.680 ± 0.05 | 0.41 ± 0.030 | 5.1 ± 0.31 | 0.079 ± 0.004 | 0.675 ± 0.032 | 0.29 ± 0.006 | 4.9 ± 0.20 | 0.082 ± 0.0028 |
| 200 | 0.668 ± 0.03 | 0.82 ± 0.004 | 5.5 ± 0.27 | 0.076 ± 0.005 | 0.646 ± 0.028 | 0.45 ± 0.018 | 5.0 ± 0.31 | 0.082 ± 0.0033 |
| 300 | 0.661 ± 0.03 | 0.76 ± 0.053 | 5.8 ± 0.37 | 0.076 ± 0.004 | 0.687 ± 0.021 | 0.46 ± 0.015 | 4.8 ± 0.26 | 0.078 ± 0.0025 |

chlorophyll ($P < 0.05$). However, when photosynthetic performance was expressed on the basis of cell number, an opposite pattern was observed, that is, *N. oculata* performed better than *N oceanica* between 50 and 200 μmol photons m$^{-2}$ s$^{-1}$ light irradiance, while at 300 μmol photons m$^{-2}$ s$^{-1}$, there was a reduction in P$_{max}$ and the associated parameters, indicating it may already be above the saturation level of its growth response capacity and in a region of photoinhibition. It is conceivable that the better photosynthesis performance per cell showed by *N. oculata* is the result of its higher chlorophyll content per cell (Table 3). As a matter of fact, the decline in P$_{max}$ per cell (Table 5) in *N. oculata* was observed when its chlorophyll content was comparable to that found in *N. oceanica* (0.33 vs 0.30 μg (10$^6$ cells)$^{-1}$ (Table 3).
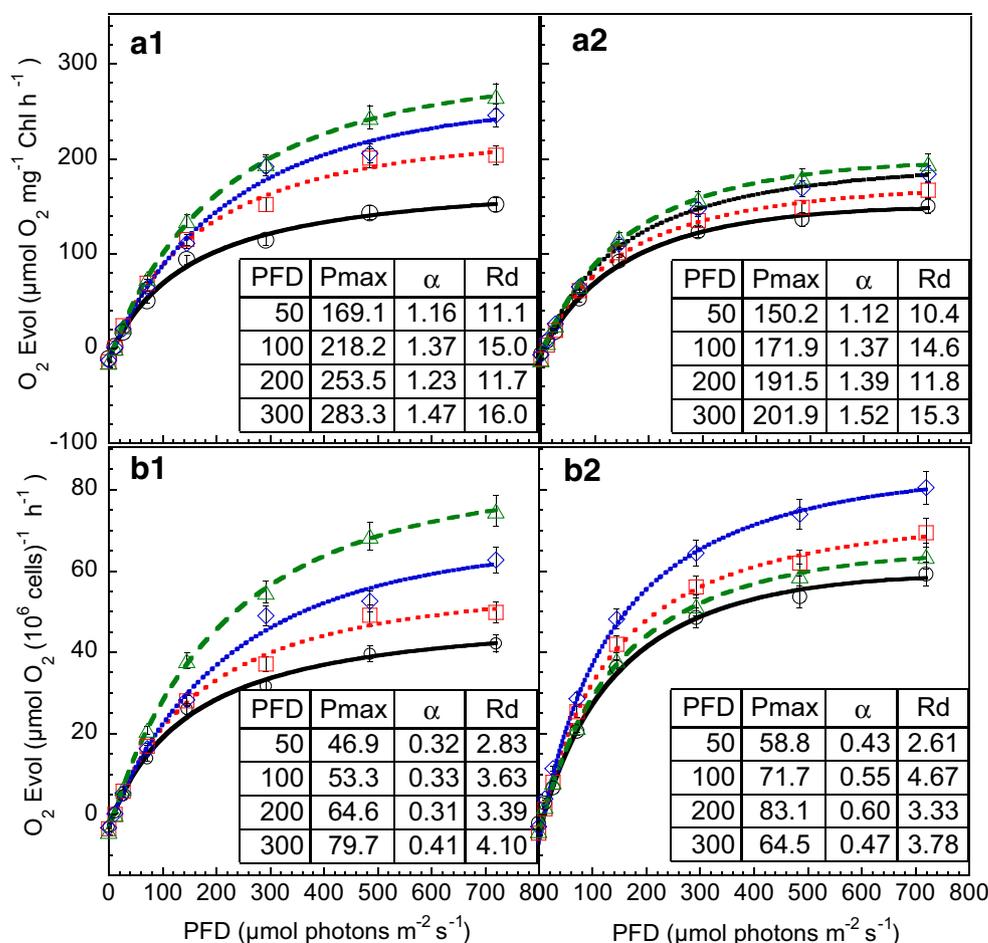
### Response to high light

Outdoor algal cultures are frequently exposed to excess light, particularly around midday. We thus decided to evaluate and compare the response of the two species to a high light stress by measuring their P-I curves before and after a 24-h exposure to 1600 μmol photons m$^{-2}$ s$^{-1}$. We chose a relatively lower level of light stress to avoid lethal conditions which may occur

in a relatively diluted culture. In order to make sure that this was indeed the situation, we measured the changes in F$_v$/F$_m$ of each culture before and after 24 h of exposure to the high light stress and found a relatively small, although significant ($P < 0.05$), reduction of less than 15% in both species (compare Table 2 with Table 4). *In vivo* optical cross-section (a*) showed the opposite behavior; it increased in both species by 60% indicating a strong effect of acclimation to high light by the cultures (compare Table 2 with Table 4). Following exposure to high light, NPQ reached 1.5 in *N. oceanica* and 1.24 in *N. oculata* (compare Table 2 with Table 4) ($P < 0.05$).

The responses of photosynthesis of the two species to a HPFD stress are shown in Fig. 4. In general, a significant decline in the photosynthesis parameters of the cultures after light stress was observed (see the inserted table on each figure; SD is provided in Table S3 in the supplementary section). In order to facilitate a quantitative analysis and comparison between the two species before and after exposure to the HPFD, we have summarized the data in Table 5. For many of the parameters, expressed on a cell basis, it is clear that *N. oculata* demonstrated a much greater sensitivity to light stress. Indeed, the maximal photosynthetic activity evaluated in terms of P$_{max}$, and the initial slope ($\alpha$), were significantly

**Fig. 3** P-I curves of the two *Nannochloropsis* species: *N. oceanica* (a1, b1) and *N. oculata* (a2, b2) as a function of light intensity for growth: 50 (black circles), 100 (red squares), 200 (blue diamonds), and 300 μmol photons m$^{-2}$ s$^{-1}$ (green triangles). Photosynthetic activity is expressed on the basis of Chl content per mL (a1, a2) or cell number per mL (b1, b2). In each figure, the insert is a table of the parameters derived from the curves. Data are presented as the mean ± SD ($n = 3$)



Panel a1 table:

| PFD | Pmax | $\alpha$ | Rd |
|-----|------|----------|-----|
| 50 | 169.1 | 1.16 | 11.1 |
| 100 | 218.2 | 1.37 | 15.0 |
| 200 | 253.5 | 1.23 | 11.7 |
| 300 | 283.3 | 1.47 | 16.0 |

Panel a2 table:

| PFD | Pmax | $\alpha$ | Rd |
|-----|------|----------|-----|
| 50 | 150.2 | 1.12 | 10.4 |
| 100 | 171.9 | 1.37 | 14.6 |
| 200 | 191.5 | 1.39 | 11.8 |
| 300 | 201.9 | 1.52 | 15.3 |

Panel b1 table:

| PFD | Pmax | $\alpha$ | Rd |
|-----|------|----------|-----|
| 50 | 46.9 | 0.32 | 2.83 |
| 100 | 53.3 | 0.33 | 3.63 |
| 200 | 64.6 | 0.31 | 3.39 |
| 300 | 79.7 | 0.41 | 4.10 |

Panel b2 table:

| PFD | Pmax | $\alpha$ | Rd |
|-----|------|----------|-----|
| 50 | 58.8 | 0.43 | 2.61 |
| 100 | 71.7 | 0.55 | 4.67 |
| 200 | 83.1 | 0.60 | 3.33 |
| 300 | 64.5 | 0.47 | 3.78 |

**Table 3** Chlorophyll content ($\mu$g mL$^{-1}$) cell number ($10^6$) mL$^{-1}$, chlorophyll per $10^6$cells for *N. oceanica* (A) and *N. oculata* (B) of cultures before (d0), and after 24 h (d1) of exposure to HPFD (1600 $\mu$mol photons m$^{-2}$ s$^{-1}$). Data are presented as the mean $\pm$ SD ($n = 3$)

**A. *N. oceanica***

| PFD | Chlorophyll | | Number of cells | | Chl cell$^{-1}$ | |
|---|---|---|---|---|---|---|
| | d0 | d1 | d0 | d1 | d0 | d1 |
| 50 | 5.03 $\pm$ 0.21 | 4.75 $\pm$ 0.19 | 18.2 $\pm$ 0.55 | 29.7 $\pm$ 0.89 | 0.28 | 0.16 |
| 100 | 4.97 $\pm$ 0.17 | 4.53 $\pm$ 0.13 | 20.4 $\pm$ 0.61 | 36.9 $\pm$ 1.11 | 0.24 | 0.12 |
| 200 | 4.76 $\pm$ 0.14 | 4.59 $\pm$ 0.14 | 18.7 $\pm$ 0.56 | 29.3 $\pm$ 0.88 | 0.26 | 0.16 |
| 300 | 4.62 $\pm$ 0.15 | 4.59 $\pm$ 0.14 | 15.3 $\pm$ 0.46 | 45.8 $\pm$ 1.37 | 0.30 | 0.10 |

**B. *N. oculata***

| PFD | Chlorophyll | | Number of cells | | Chl cell$^{-1}$ | |
|---|---|---|---|---|---|---|
| | d0 | d1 | d0 | d1 | d0 | d1 |
| 50 | 4.93 $\pm$ 0.15 | 5.01 $\pm$ 0.15 | 12.5 $\pm$ 0.38 | 36.0 $\pm$ 1.08 | 0.39 | 0.14 |
| 100 | 4.87 $\pm$ 0.15 | 5.15 $\pm$ 0.15 | 11.7 $\pm$ 0.35 | 46.2 $\pm$ 1.38 | 0.42 | 0.11 |
| 200 | 4.56 $\pm$ 0.14 | 4.25 $\pm$ 0.13 | 10.4 $\pm$ 0.31 | 29.8 $\pm$ 0.89 | 0.44 | 0.14 |
| 300 | 4.71 $\pm$ 0.14 | 4.80 $\pm$ 0.14 | 14.5 $\pm$ 0.44 | 30.2 $\pm$ 0.91 | 0.33 | 0.16 |

lower in *N. oculata* after the HPFD treatment, except in the culture previously acclimated at 300 $\mu$mol photons m$^{-2}$ s$^{-1}$ (Table 5). This may be a result of the higher reduction in chlorophyll per cell in *N. oceanica*, which dropped threefold (from 0.3 to 0.1) compared to *N oculata* in which values dropped by twofold (Table 3). It is important to point out that in *N. oceanica*, the residual activity after photoinhibition was, in general, significantly higher compared to that found in *N. oculata*, indicating the ability of the former species to better respond to the changes in growth and environmental stress conditions. Following exposure to high light, the average respiration rates strongly increased (by about 250 %) in both species, but without a clear trend in relation to prior light acclimation.

### Maximum quantum yield of photosynthesis and photochemical quantum yield of PSII changes

*Nannochloropsis* cultures exposed to high light showed a strong reduction in their maximum quantum yield of

photosynthesis ($\Phi_{max}$) measured through chlorophyll fluorescence (compare Table 2 with Table 4). The reduction in $\Phi_{max}$ following exposure to high light was greater in *N. oculata* than in *N. oceanica*.
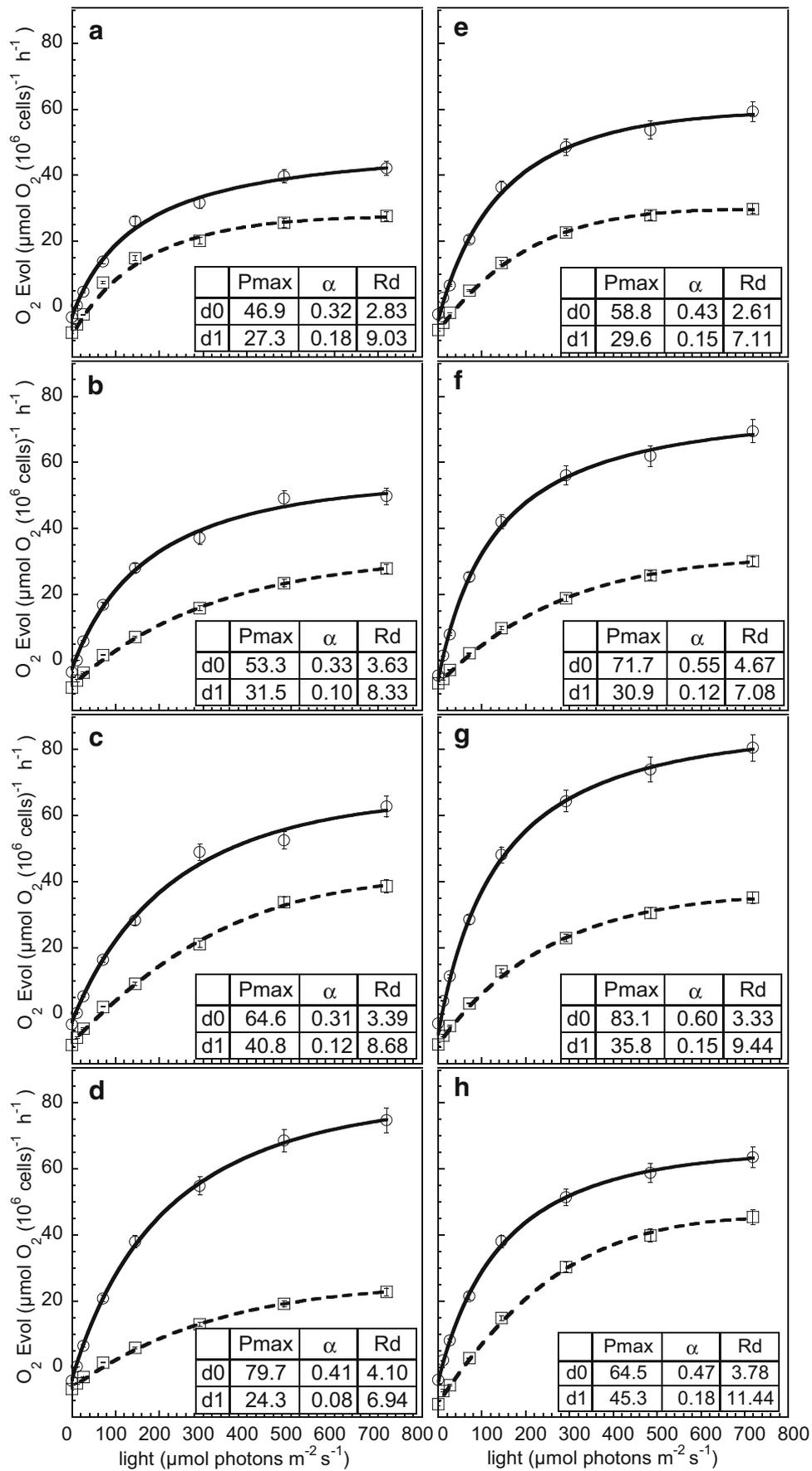
### Response to Rose Bengal or methyl viologen

With the aim of evaluating whether the two *Nannochloropsis* species have a different ability to cope with oxidative stress, they were exposed to a gradient of concentrations of RB (0–25 $\mu$M) or MV (0–400 $\mu$M) and incubated in the light for 4 days. As depicted in Fig. 5. *N. oculata* demonstrated a much higher ability to tolerate the oxidative stress imposed by the exposure to RB.

## Discussion

Although the potential application and uses of algal biomass are well documented, only very few species have reached the state of being a commercially viable and sustainable business operation. *Arthrospira* and *Chlorella*, mainly used for total cell biomass, and *Dunaliella* and *Haematococcus*, used as sources of high value carotenoids, are the few species which have attained this status (Borowitzka and Vonshak 2017). All other species of interest are at a stage of exploration of their potential or struggling with the challenges of scaling up the production facility. Even within the commercially produced species, very little is known about the physiology of the specific species used and the rationale for choosing them. The inherent limitations of the lack of established breeding practices in most of the microalgae limit the genetic tools one can use for species improvement, especially given the current regulatory limitations imposed on GMO organisms used for food and feed. Thus, the two options left are the use of random induced mutagenesis followed by a tedious selection process that hopefully will yield improved species (Ben-Shelg et al. 2019), or relying on mother nature and selecting for existing species isolated from different habitats that may represent a variation induced by a set of environmental selection

**Table 4** $F_v/F_m$, NPQ, and a*, *in vivo* optical absorption cross-section (m$^2$ mg$^{-1}$ Chl) and $\Phi_{max}$ maximum quantum yield (mol O$_2$ evolved mol$^{-1}$ photons absorbed) in *N. oceanica* and *N. oculata* previously acclimated to light intensities between 50 and 300 $\mu$mol photons m$^{-2}$ s$^{-1}$, and thereafter exposed to high light (1600 $\mu$mol photons m$^{-2}$ s$^{-1}$) for 24 h. Data are presented as the mean $\pm$ SD ($n = 3$, 4)

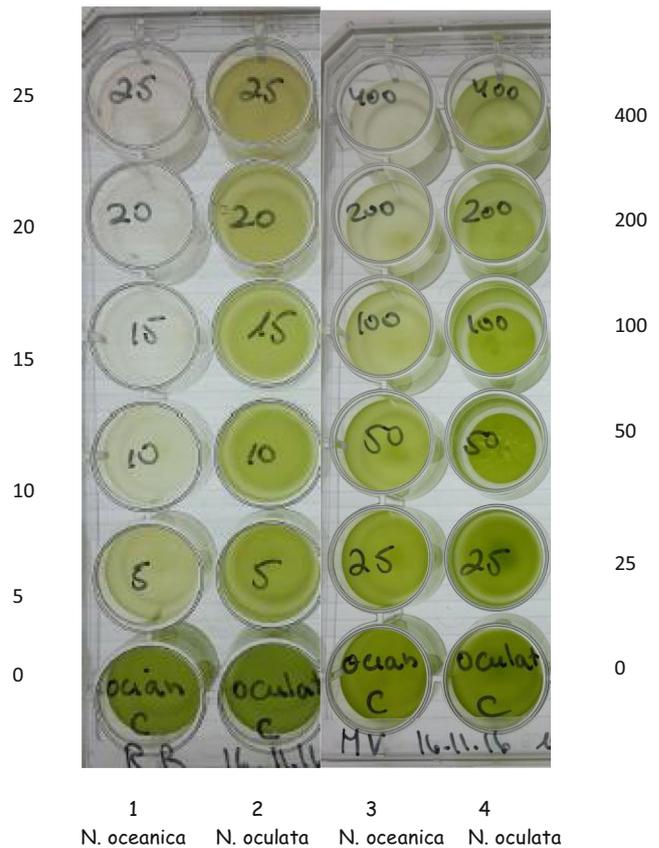| PFD | *N. oceanica* | | | | *N. oculata* | | | |
|---|---|---|---|---|---|---|---|---|
| | $F_v/F_m$ | NPQ | a* ($10^{-3}$) | $\Phi_{max}$ | $F_v/F_m$ | NPQ | a* ($10^{-3}$) | $\Phi_{max}$ |
| 50 | 0.547 $\pm$ 0.02 | 0.99 $\pm$ 0.05 | 8.19 $\pm$ 0.42 | 0.054 $\pm$ 0.0027 | 0.561 $\pm$ 0.02 | 0.83 $\pm$ 0.04 | 7.91 $\pm$ 0.43 | 0.047 $\pm$ 0.0014 |
| 100 | 0.544 $\pm$ 0.03 | 1.19 $\pm$ 0.07 | 8.55 $\pm$ 0.51 | 0.042 $\pm$ 0.0024 | 0.527 $\pm$ 0.03 | 0.91 $\pm$ 0.05 | 7.58 $\pm$ 0.47 | 0.042 $\pm$ 0.0012 |
| 200 | 0.573 $\pm$ 0.05 | 1.23 $\pm$ 0.06 | 8.95 $\pm$ 0.53 | 0.051 $\pm$ 0.0022 | 0.577 $\pm$ 0.03 | 1.14 $\pm$ 0.07 | 9.02 $\pm$ 0.65 | 0.045 $\pm$ 0.0017 |
| 300 | 0.587 $\pm$ 0.04 | 1.50 $\pm$ 0.06 | 9.24 $\pm$ 0.58 | 0.048 $\pm$ 0.0014 | 0.567 $\pm$ 0.02 | 1.24 $\pm$ 0.06 | 8.91 $\pm$ 0.59 | 0.039 $\pm$ 0.0016 |

pressures that will yield improved strains, better fitted for mass culturing of a desired species. Adopting the latter approach requires the development of a set of well-defined, easy-to-perform, and easy-to-interpret measurements that will help in identifying a better fitted species as a candidate for outdoor cultivation.

The objective of the current study was to use *Nannochloropsis*, a promising species for lipid production, as a case study to demonstrate the relevance of the need to develop and apply such a set of criteria to evaluate and expect success in outdoor cultivation.

The term "balanced growth" refers to microbial growth conditions in which the increase in any cell component is in line with increases in cell number. In such a case, one can follow growth by measuring a single-cell component like cell number and assume that a similar change occurs when following the increase in biomass or pigment content. Such a case is relevant when all nutritional requirements are provided in excess, including the source of energy. When dealing with heterotrophic growth, the source of energy is mainly an organic carbon source that allows the above-mentioned conditions to be expressed in parallel. In phototrophic growth, where the source of energy (light) is coming from outside the cell culture, any increase in biomass concentration results in a



**Fig. 5** The effect of RB (rows 1 and 2) or MV (rows 3 and 4) on the growth of the two *Nannochloropsis* species: *N. oceanica* (rows 1, 3) and *N. oculata* (rows 2, 4) after 4 days of incubation in light (70 μmol photons m$^{-2}$ s$^{-1}$). Numbers to the left indicate the concentration of RB and to the right of MV in μM.

**Table 5** Change in $P_{max}$, $\alpha$, and $R_d$ after exposure of *N. oceanica* and *N. oculata* to HPFD expressed as a % of pre-exposure levels. Data are extracted from insert tables in Fig. 4. Meaning of symbols and unit of parameters ($P_{max}$, $\alpha$, and $R_d$) are reported in Table 1. Standard deviations are in parenthesis (*n* = 3, 4). The values for each parameter in the same column followed by different letters (a, b, c), are significantly different (*P* = 0.05), while values of the same rows labeled by the same symbol (+, #, *, $), mean significant differences between two species

| Parameters | PFD | *N. oceanica* | *N. oculata* |
|---|---|---|---|
| $P_{max}$ | 50 | 58(5.7)a | 50(5.4)b |
|  | 100 | 59(5.9)a* | 43( 2.6)c* |
|  | 200 | 63(2.4)a+ | 43(4.7)c+ |
|  | 300 | 30(2.8)b$ | 70(10.5)a$ |
| $\alpha$ | 50 | 56(3.0)a* | 35(3.2)a* |
|  | 100 | 30(4.0)b$ | 22 (1.6)b$ |
|  | 200 | 39(3.8)b+ | 25 (2.6)b+ |
|  | 300 | 20(2.7)c≠ | 38 (2.9)a≠ |
| $R_d$ | 50 | 319(56.0)a* | 272 (37.1)b* |
|  | 100 | 229(39.2)c$ | 152(18.1)c$ |
|  | 200 | 256(28.3) b | 283( 30.6)b |
|  | 300 | 169(31.5)d& | 303 (32.72)a& |

decrease in light (energy) availability due to self-shading. Although both species used in the current study were each acclimated to the specific growth light, no balanced growth was achieved. This is reflected by the fact that at any given irradiance, the rate of increase in cell number was not similar to the rate of increase in chlorophyll concentration, indicating a fast and active mechanism of response of chlorophyll content per cell as a function of light availability in the culture.

From the data presented in the growth experiments, two major observations can be made. The first one is the unique ability of the two *Nannochloropsis* species to respond rapidly to the changes in the light environment, while the second one points to the difference between calculation of specific growth rate on the basis of chlorophyll or on the basis of cell number which results in different values of specific growth rate (μ) (Fig. 1), and suggests that balanced growth is not taking place in *N. oculata* under the high light intensity of 300 μmol photons m$^{-2}$ s$^{-1}$. While at this irradiance, *N. oceanica* maintains approximately the same Chl/cell ratio (Table 3A) and shows increased growth rate also at 300 μmol photons m$^{-2}$ s$^{-1}$; in the *N. oculata* culture, the ratio of chlorophyll per cell decreased at that light intensity for growth (Table 3B) with an influence

on the growth rate (μ) calculated per cell and indicates a higher sensitivity to this level of light.

These findings correlate with the photosynthesis measurements calculated per cell number. Indeed, $P_{max}$ in *N. oculata* grown at 300 μmol photons $m^{-2}$ $s^{-1}$ decreased to a lower value compared to that in cultures grown at 200 μmol photons $m^{-2}$ $s^{-1}$ (Fig. 4 (B2)). Consequently, we recommend that, when evaluating species performance, growth should be measured under a wide range of light intensities in order to mimic the change in Chl/cell ratio in variable light conditions in outdoor cultivation.

Comparing the two species, *N. oceanica* cultures demonstrated a similar response between the photosynthesis parameters calculated per chlorophyll including $P_{max}$ (Fig. 3 (a1 and a2)), ETR measurements (Fig. 2), NPQ (Table 2), and absorption cross-section (a*) (Table 2), which may indicate a better ability of the photosynthetic apparatus of *N. oceanica*, compared to *N. oculata* to respond to a wide range of light intensities and which may be an important characteristic for species that are to be cultivated under outdoor conditions. The observed lack of significant changes in absorption cross-section at different growth intensities in *N. oculata* is consistent with previous studies (e.g., Kandilian et al. 2013; Szabó et al. 2014). In contrast, *N. oceanica* increased its optical cross-sections by about 30% when light irradiance was increased from 50 to 300 μmol photons $m^{-2}$ $s^{-1}$, which corresponded to an appreciable rise in NPQ, while no remarkable changes were observed with *N. oculata*, both in optical cross-section and NPQ. The values of the optical absorption cross-section found in *Nannochloropsis* cells before their exposure to high light (4.2–5.8 × $10^{-3}$ $m^2$ $mg^{-1}$ Chl*a*) were comparable to that found by Kandilian et al. (2013) (4.32 × $10^{-3}$ $m^2$ $mg^{-1}$ Chl*a*), while in cells exposed to high light (1600 μmol photons $m^{-2}$ $s^{-1}$), the values compared to that found by Kromkamp et al. (2009) (6–8 × $10^{-3}$ $m^2$ $mg^{-1}$Chl*a*) in outdoor *Nannochloropsis* cultures. Increased optical absorption cross-section in cells grown under high light may be explained by a reduced effect of pigment packaging and by the synthesis of pigments such as xanthophyll which are deputed to dissipate light instead to transfer it to reaction center thus assigning the light absorbed by any pigment to chlorophyll *a*, thereby virtually increasing cross-sections of Chl *a* (Dubinsky 1992).

We found that even after exposure of cells to high light, NPQ was much lower than that found by other workers (Simionato et al. 2011; Szabó et al. 2014; Chukhutsina et al. 2017). Indeed, they found NPQ values up to 4–5 in cultures exposed to high light, while in our cultures, maximum NPQ in high light-exposed cells of *N. oceanica* reached 1.5 (Table 4). In contrast, other studies indicate that NPQ in *Nannochloropsis* was close to that found by us (max value 1.5) (Kotabová et al. 2011; Cao et al. 2013). Higher values of NPQ have been found in cultures grown without additional $CO_2$ supply (Simionato et al. 2011; Szabó et al. 2014; Chukhutsina et al. 2017). It is conceivable that limitation of $CO_2$ supply may have limited the dissipation of the absorbed energy via photochemical quenching and increased the complementary mechanism of dissipation of energy, via NPQ.

Under excess light, the photosynthetic electron transport chain becomes increasingly reduced as a result of limitation of reductant and ATP use by the Calvin Benson cycle. Thus, energy supply potentially becomes greater than its dissipation, leading, in the absence of protective mechanisms, to ROS formation. One of these protection mechanisms is the activation of excess energy dissipation via the xanthophyll cycle, evidenced by non-photochemical quenching of chlorophyll fluorescence (see Goss and Jakob 2010 for details of the mechanisms involved). We observed an increase in values of NPQ in both cultures following exposure to higher light (Table 4). This behavior can be explained by the synthesis of xanthophyll cycle pigments during the exposure to high light. The lower values of NPQ observed in cultures before their exposure to high light may be attributed to energy quenching promoted by ΔpH, particularly for the cultures grown under the lower light intensities of 50–100 μmol photons $m^{-2}$ $s^{-1}$.

An additional physiological parameter that tends to be overlooked in outdoor cultivation is dark respiration. It is clearly demonstrated that light environment significantly affects the dark respiration activity, and high respiration rates may help in the ability to deal with excess light by being a sink for extra reducing power as seen in *N. oculata* after exposure to high light (calculated per cell) (Table 5).

Last but not least, we would like to suggest that the ability of *N. oculata* to withstand increased concentrations of RB and MV, which generate free radicals, may indicate a different protective mechanism such as upregulation of enzymes like SOD and catalase to environmental stress that result in oxidative stress (Fig. 5). Thus, this may serve as an additional tool of screening and evaluating the potential of specific algal species to be grown under outdoor conditions.

## Conclusion

*Nannochloropsis* species, grown under optimal conditions, are able to acclimate to high light by reducing their cellular pigment content, thereby increasing cellular optical cross-section which entails an increased ETR and photosynthesis rates with consequent little dissipation of energy via NPQ. An interesting question concerns the evaluation of the performance of the cultures when exposed to high light irradiance, not only for photosynthesis studies but also for outdoor mass culture and species phenotyping. Photosynthesis parameters elaborated on a chlorophyll basis showed an increase in the photosynthetic activity measured either via chlorophyll fluorescence (ETR) or oxygen evolution. Presentation of data on a cell basis showed the opposite behavior, that is, cells exposed to high light showed a strong decline in their photosynthesis rate. This is not surprising since the chlorophyll

content per cell was lower, and the increase in chlorophyll optical cross section was not sufficient to balance the chlorophyll reduction. As a result, two cultures having the same chlorophyll concentration but a different acclimation state may behave very differently when inoculated outdoors under high light. High chlorophyll content per cell (low light acclimation state) may cause excessive light absorption coupled with a lower capacity to dissipate excess light via non-photochemical quenching, with a high risk of photoinhibition.

This study has demonstrated that a simple set of experiments may reveal differences between species that are systematically closely related. For this study, we applied both photosynthesis and chlorophyll fluorescence coupled to growth measurements. Obviously, for a definitive evaluation of the two species, a further validation of the data gathered in the lab is required under outdoor conditions.

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