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Photosynthetic and growth responses of *Nannochloropsis oculata* (Eustigmatophyceae) during batch cultures in relation to light intensity

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Abstract: Light is one of the most important factors affecting photosynthesis and growth of microalgal cultures. Photosynthetic responses to light change with the stage of the culture; however, little is known about how changes in the photosynthetic capacity with the duration of batch culture affect growth at the end (final harvest day) of the culture. Accordingly, we studied the photosynthetic performance and growth of *Nannochloropsis oculata* in the exponential phase (day 4), the linear phase of growth (day 8) and stationary phase (day 15) of batch cultures grown under a range of light intensities from 20 to 200 μmol photons m^{-2} s^{-1}. *Nannochloropsis oculata* showed a high ability to acclimate to changing light conditions; cells almost doubled their cellular Chl *a* concentration under low light conditions and increased their photosynthetic oxygen evolution capacity, which allowed this alga to maintain a stable photosynthetic efficiency in the three stages of the culture studied. Changes in the Chl *a* per cell under different light conditions were not paralleled by changes in antenna size, which suggests that *N. oculata* uses an alternative strategy to acclimate to changing light levels. Finally, increasing photosynthetic capacity in cells growing under 20 μmol photons m^{-2} s^{-1} did not avoid light limitation and decreasing exponential growth rate. However, the lack of differences in final cell density achieved between 20 and 200 μmol photons m^{-2} s^{-1} treatments suggests that bicarbonate limitation started to affect photosynthesis and growth at the higher light treatments, from the linear phase of growth onwards. Understanding of the photosynthetic and growth responses of *N. oculata* under changing light conditions, where the heterogeneity of light distribution in the cultures substantially affects growth, is crucial to optimisation of the photosynthetic efficiency and growth of microalgal cultures.

Key Words: Antenna size, Chlorophyll *a*, Final cell density, Light acclimation and photosynthesis

INTRODUCTION

Development of mass culture of microalgae for biotechnological purposes is still challenged by fundamental constraints on productivity. Of these constraints, light is one of the most important factors affecting algal productivity in outdoor cultures; the efficiency of energy conversion of light into biomass has a significant effect on production cost. The highest efficiencies of energy conversion into biomass found for algae are ~ 10% based on photosynthetically active radiation (PAR) and ~ 3% based on total solar radiation (Williams & Laurens 2010) but this substantially decreases when the amount of light that cells are exposed to is unfavourable (Simionato *et al*. 2013a; Vonshak *et al*. 2014). In this regard, large scale cultures of microalgae are normally challenged by a heterogeneous light distribution; at the surface, cells absorb around 90% of incident photons (Beardall & Raven 2013), potentially exposing algae to photoinhibition, and just 10% are absorbed in deeper layers, where growth is severely limited by light availability (Beardall & Raven 2013).

The negative effect of heterogeneous light distribution on mass algal cultures has led to a search for solutions, such as determining the optimum depth of outdoor cultures for light penetration. For example, 63, 71 and 87 mm have been calculated as optimum depths for growing *Phaeodactylum*, *Dunaliella salina* (Dunal) Teodoresco and *Chlorella*, respectively, in open ponds, based on net photosynthesis optimization, where shallower depth decreases the light harvesting efficiency of cells (Ritchie & Larkum 2012). However, *Nannochloropsis* sp. grown in an open pond system showed at least 50% lower photosynthetic activity based on $rETR_{max}$ and 60% lower biomass production than in cells grown in a flat photobioreactor, when the cultures were exposed to high light conditions (Kromkamp *et al*. 2009). These results indicate that optimizing culture systems for better use of light for photosynthesis and growth is still a challenge in mass microalgal cultures. It is believed that a better understanding of the photosynthetic strategies that microalgae can develop under changing light conditions and how this can affect growth responses at exponential phase, as well as at late log and stationary phase, are key to maximizing the use of light by microalgae.

Microalgae have evolved different strategies to efficiently convert sunlight into chemical energy for growth (Formighieri *et al*. 2012). The study of the antenna system of photosynthetic organisms and the performance of photosynthesis are crucial to understanding the photo-acclimation strategy that these organisms have evolved for coping with changing light conditions. The antenna system is a connection of chlorophyll molecules (plus other secondary pigments) held in the thylakoid membrane of the chloroplast by a matrix of proteins that harvest photons (Raven & Johnson 2002). Under high light conditions, a decrease in the antenna size has been observed in microalgae by measuring the content of chlorophyll molecules per photosynthetic unit

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(Melis *et al*. 1998) and more recently by photosystem quantification from oxidized chlorophyll in the presence of DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and a saturating light of 2050 μmol photons m^{-2} s^{-1} (Meneghesso *et al*. 2016). For instance, Shin *et al*. (2016) observed that a truncated antenna of *Chlorella vulgaris* Beyerinck (56.5% decrease in its size) improved the biomass productivity by 44.5% as a result of increasing light use efficiency, indicating that changes in antenna size have a significant effect on microalgal growth. Measuring the effective absorption cross-sectional area of PSII α centres (σPSIIα) from a Fast Fluorescence Induction (FFI) curve is also a well-known technique to determine antenna size of photosystem II in microalgae (Koblížek *et al*. 2001; Pierangelini *et al*. 2014b). Another strategy used by microalgae under high light conditions is decreasing the number of photosynthetic units (PSU), i.e. the number of reaction centres in the photosystems (Fisher *et al*. 1996), causing cells to decrease their light absorption efficiency under high light conditions. As an example, Quigg *et al*. (2006) observed a decrease in PSU number per cell from 1100 to 730 (PSU number $cell^{-1}$) in cells of *Phaeodactylum tricornutum* Bohlin grown under 6 and 100 μmol photons m^{-2} s^{-1}, respectively, where an increase in light exposure also increased the cell division rate from 0.16 d^{-1} in cells grown under 6 μmol photons m^{-2} s^{-1} to 0.5 d^{-1} in cells grown under 100 μmol photons m^{-2} s^{-1}. Changes in either antenna size or PSU number have great impact on the photosynthetic performance of microalgae. Richardson *et al*. (1983) discussed the *P* vs *I* curves of microalgae acclimated to low and high light in relation to changes either in antenna size or number of photosynthetic units, showing that cells with increasing PSU numbers exhibit increasing maximum photosynthetic rate (P_{max}) per cell and light absorption efficiency (α).

The effects of light on photosynthetic parameters such as antenna size, PSU numbers and P_{max} and on growth have been studied mainly during the exponential phase of microalgal cultures. For instance, in *Dunaliella salina* at day 3 of the culture (exponential phase), cells showed an increase in both P_{max} from 31.6 to 148.9 μmol O_2 (mmol $Chl)^{-1}$ s^{-1} and biomass production from 1.87 to 2.52 g l^{-1} in cultures grown under constant illumination and flashing illumination of 250 μmol photons m^{-1} s^{-2}, respectively (Abu-Ghosh *et al*. 2015). In batch cultures, cells in exponential phase are more likely to experience optimal environmental conditions to grow at their fastest rate; therefore, the effect of light on photosynthesis can be studied without being affected by other variables such as nutrient or CO_2 limitation. In contrast, at late log and stationary phases, changes in nutrient availability, increasing cell density and self-shading, CO_2 limitation and pH changes, for example, start interacting with light utilization for photosynthesis and growth, and this can have a great impact on biomass production of mass cultures at time of harvest (Fidalgo *et al*. 1998; Boelen *et al*. 2017). A study of seven marine microalgal species showed a clear decrease in maximum quantum efficiency of photosystem II (F_v/F_m) from at least 0.65 during exponential phase of the cultures to 0.55 at stationary phase in all the species studied (Boelen *et al*. 2017). Identifying the photosynthetic strategy developed by *Nannochloropsis oculata* (Droop) D.J. Hibberd at exponential phase under light changing conditions, and for how long this strategy continues as the cultures reach stationary phase and how this strategy affects growth, are crucial to understanding the photoacclimation strategy that microalgae develop under changing light conditions in batch cultures. Beardall and Morris (1976) studied the light acclimation strategy of *Phaeodactylum tricornutum* adapted to either low light (0.7 Klux, ~ 12 μmol photons m^{-2} s^{-1}) or high light (12 Klux, ~ 200 μmol photons m^{-2} s^{-1}) during the course of batch cultures, observing a maximum photosynthetic rate on day 4 of cells grown under low light. Maximum photosynthetic rate in cells grown under high light was on day 2; therefore, a comparison of the photosynthetic responses between low and high light treatment resulted in notably different findings depending on the day of observation. The ability of a species to maintain high photosynthetic capacity continuously from exponential to stationary phase will impact their biomass at the end of the culture period.

The genus *Nannochloropsis* (Eustigmatophyceae) is a promising algal candidate for industry, with biomass productivity of up to ~ 0.20 g l^{-1} d^{-1} (Rodolfi *et al*. 2009). *Nannochloropsis* and its products are widely used in human and aquaculture nutrition as a source of carotenoids (Nobre *et al*. 2013), eicosapentaenoic acid (EPA) (Renaud & Parry 1994) and triacylglycerol (TAG) (Pal *et al*. 2011). The photoacclimation of *Nannochloropsis* has been studied (Sforza *et al*. 2012; Kandilian *et al*. 2013; Szabó *et al*. 2014). In addition, the effects on photoacclimation of nutrient status (Sobrino *et al*. 2004; Simionato *et al*. 2013b) and temperature (Sukenik *et al*. 2009), among other factors, have also been examined. For example, a study performed on *Nannochloropsis* sp. in a flat panel photobioreactor showed a decreased effective quantum efficiency of photosystem II (F'_v/F'_m) from 0.80 to 0.45 during the exponential phase of the culture when cells were growing under 30°C and 20°C, respectively (Sukenik *et al*. 2009).

Regarding photoacclimation responses, Sukenik *et al*. (2009) showed that *Nannochloropsis* can rapidly recover from photodamage when cells are exposed to high light and are able to resist high irradiance levels by maintaining a stable photosynthetic efficiency. The acclimation potential of *Nannochloropsis* to changing light conditions, at exponential phase of the cultures, has been studied (Kromkamp *et al*. 2009; Sforza *et al*. 2012; Szabó *et al*. 2014). For instance, at exponential phase, no differences in antenna size were found in this genus when cells were exposed to different light intensities, but instead they acclimated by undergoing changes in PSU number (Fisher *et al*. 1996); thus, increasing light exposure from 30 to 650 μmol photons m^{-2} s^{-1} resulted in a decrease in PSU number from 36608 to 7730 PSU $cell^{-1}$ (Fisher *et al*. 1996). However, changes in photoacclimation strategy under changing light conditions with time in batch culture and effect on growth have not been adequately studied in species such as *Nannochloropsis oculata*.

Our research aimed to monitor the effect of light on photosynthesis at exponential, lag and stationary phases during batch culture of *Nannochloropsis oculata* and the corresponding effects on growth. Sforza *et al*. (2012) observed that in *Nannochloropsis salina* D.J. Hibberd, from 5 to 150 μmol photons m^{-2} s^{-1} cells were growing under low light conditions, as an increasing cell division rate from 0.30

to 0.55 d^{-1} was observed as light increased, while above this level cells were exposed to supra-optimal light conditions, as a decrease from 0.55 to 0.35 d^{-1} was observed from 150 to 1000 μmol photons m^{-2} s^{-1} exposure. The growth curve observed in *N. salina* grown under eight increasing light intensities from 5 to 1000 μmol photons m^{-2} s^{-1} also showed that days 4 and 8 correspond to exponential and lag phase, respectively (Sforza *et al*. 2012). To this end, we studied the effect of light intensity from 20 to 200 μmol photons m^{-2} s^{-1} on parameters such as cellular Chl *a* concentration, antenna size, maximum rate of oxygen evolution, dark respiration and maximum quantum efficiency of PSII, as well as on growth, at days 4, 8 and 15 of *N. oculata* cultures. Our aims in so doing were to investigate how light acclimation strategies might change as cultures moved from exponential, through linear and finally into stationary phases of growth.

MATERIAL AND METHODS

The unicellular green marine alga *Nannochloropsis oculata* (Eustigmatophyceae) (CS-179) was obtained from the CSIRO Australian National Algae Culture Collection, Hobart, Tasmania. A volume of 150 ml of culture was grown in each of 12 Erlenmeyer flasks (250 ml) using PHK medium (Rukminasari 2013) and incubated under a 16:8 h light:dark cycle at 20°C. Flasks were exposed in triplicate to one of four levels of photosynthetically active radiation (PAR): 20, 50, 100 and 200 μmol photons m^{-2} s^{-1}, illuminated with Cool White fluorescent lights (Phillips, Eindhoven, Netherlands) providing a broad spectrum of PAR (400 nm to 700 nm). This wide spectral range covers the pigment absorption spectrum of *Nannochloropsis,* with the main pigment being primarily Chl *a*. For each light treatment, the distance of the flasks from the light source was set by measuring the light intensity of each spot with a Li-Cor 1000 light meter (Li-Cor, Lincoln, Nebraska, USA). This measurement was done at least three times during the experiment to make sure the incident light in each flask was stable during the entire experiment. After three generations of acclimation (given a total of 12 d), each flask was used as a stock culture to start the experiment by inoculating 2×10^5 cells ml^{-1} into three independent replicates of 1 litre of batch culture, grown in 2-litre Erlenmeyer flasks under the same conditions previously set in the stock cultures. Sterile tubing of 0.5 mm diameter and 50 cm long was placed inside each flask and closed with a clamp. This was used for all sampling by attaching a syringe to the end of the tubing and withdrawing the desired amount of sample. After sampling, the tubing was immediately clamped to ensure a sterile environment inside the flasks. All flasks were shaken manually twice a day to ensure continued suspension of cells in the culture.

For the following measurements, cultures were shaken manually prior to sampling to ensure a homogeneous cell distribution in the culture. Algal growth was monitored during the experiment by taking 1 ml of sample, fixing it with 10 μl of Lugol's iodine and counting the cells with a Neubauer Brightline haemocytometer (Boeco, Hamburg, Germany) using an Olympus CH-2 (Tokyo, Japan) light microscope. The cell division rate (μ_c) was calculated using Equation 1:

$$\mu_c = \ln(N_{t1}) - \ln(N_{t0})/(t_1 - t_0), \quad (1)$$

where N_{t1} is cellular density (cell ml^{-1}) at time $t1$ and N_{t0} is initial cellular density (cell ml^{-1}). Growth was also monitored by sampling 3 ml of culture and measuring optical density (OD) at 750 nm in a Cary-50 UV–visible spectrophotometer (Varian Inc., Palo Alto, California, USA), which was used to calculate specific growth rate at exponential phase of the culture by using Equation 2:

$$\mu_c = \ln(\text{OD } 750_{t1}) - \ln(\text{OD } 750_{t0})/(t_1 - t_0), \quad (2)$$

where OD 750_{t1} is culture OD at 750 nm at time t_1 and OD 750_{t0} is the initial culture OD at 750 nm.

For Chl *a* estimation, cells were analysed at exponential phase (day 4), in the linear growth phase (day 8) and at stationary phase (day 15). An average of 4×10^8 cells of sample were taken to ensure a consistent chlorophyll extraction across the days, centrifuged (10 min, 3500 rpm) at 4°C and resuspended in 96% ethanol for 24 h at 4°C. After extraction, the suspension was centrifuged again, and pigment concentration in the supernatant was determined spectrophotometrically, using a Cary-50 UV–visible spectrophotometer (Varian Inc.) at 630, 647, 664 and 750 nm. Chlorophyll *a* concentration as milligram per millilitre extract was then calculated using Equation 3 and converted to $\mu g/10^6$ cells from the number of cells extracted (Jeffrey & Humphrey 1975).

$$\text{Chl } a = 11.85(A_{664} - A_{750}) - 1.54(A_{647} - A_{750}) - 0.08(A_{630} - A_{750}) \quad (3)$$

For all photosynthetic parameters and respiration rate measurements, samples were taken at exponential phase (day 4), linear growth phase (day 8) and stationary phase (day 15).

Maximum photosynthetic capacity (P_{max}) and dark respiration (R_d) were measured using a Clark-type oxygen electrode (Hansatech, Norfolk, UK). Approximately 1×10^8 cells were harvested from the culture, centrifuged (10 min, $2600 \times g$) and the pellet resuspended in 3 ml of fresh PHK medium containing 2 mM dissolved inorganic carbon. A total of 2 ml of this suspension was placed into an O_2 electrode chamber and stirred with a magnetic stirrer at 70 rpm for measurement of oxygen exchange rates; 200 μl was taken for cell counting and 400 μl in duplicate was used to measure Chl *a* concentration. Prior to R_d and P_{max} measurements, the O_2 concentration in the suspension was reduced to 30% of air saturation by bubbling with N_2, since previous testing showed that high levels of oxygen could potentially inhibit photosynthetic rates. Dark respiration (R_d) was measured after dark incubation of the cell suspension for 10 min. Maximum O_2 evolution rates of the culture were determined by measuring oxygen evolution responses under four levels of light intensities within an estimated range of saturating light intensity, previously determined from rapid light curves performed in a Phyto-PAM phytoplankton analyser system (Heinz Walz, Effeltrich, Germany). The highest evolution rate measured within the four selected light intensities was considered the maximum O_2 evolution rate of the culture.

Chlorophyll fluorescence of PSII was measured to determine maximum quantum yield (F_v/F_m), using the Phyto-PAM. A sample of 3 ml was taken from the culture and incubated in the dark for 15 min prior to measurements. After dark adaptation, the sample was exposed to a minimum intensity measuring light to avoid photochemistry and obtain a stable F_0 value. Maximum fluorescence (F_m) was measured by exposing the sample to a saturating flash of red light to estimate the maximum quantum yield in PSII (F_v/F_m) as

$$F_v/F_m = (F_m - F_0)/F_m. \quad (4)$$

Flash fluorescence induction curves (FFI) were measured to determine the effective absorption cross-sectional area of PSII α centres (σPSIIα), using a double-modulation fluorometer (Photon Systems Instrument, Brno, Czech Republic). Approximately 1×10^8 cells were collected from the culture, resuspended in fresh PHK medium and incubated in the dark for 5 min prior to measurement. For measurements, the sample was exposed to a single 50 µs turnover flash at a light intensity setting of 50% (1×10^6 µmol photons m^{-2} s^{-1}), using 5% of detection sensitivity. The fluorescence rise from F_0 to F_m in the FFI curve was measured to calculate the area under the curve, which corresponded to the cross-sectional area of PSII α centres (σPSIIα). All calculations were done using the methods described by Melis and Homann (1975) and Nedbal *et al.* (1999).

pH and temperature of the sample were measured using a PH31 pH-meter (Hach, Loveland, Colorado, USA) and salinity determined using a RHS-10ATC refractometer (John Morris Group, Chatswood, New South Wales, Australia). Dissolved inorganic carbon (DIC) was measured in the medium by filtering 30 ml of culture with a 0.2 µm syringe filter (PALL Life Sciences Acrodisc® Suport® Membrane, Port Washington, New York, USA). DIC level was determined after conversion of DIC to CO_2 by acidification with 1 N hydrochloric acid (HCl) in a glass chamber. The amount of CO_2 was measured by a LI-840A CO_2/H_2O gas analyser (Licor), and data were plotted and saved automatically as CO_2 (g) vs time by the software (LI-840A v2.0.0). A standard curve constructed from 0 to 6 mM of sodium bicarbonate ($NaHCO_3$) was used to calculate the DIC concentration in the sample. Temperature, pH, salinity and DIC concentration were used to calculate the level of carbon dioxide (CO_2), bicarbonate (HCO_3^-) and carbonate (CO_3^{2-}) in the samples according to Weiss (1974), Dickson and Riley (1979) and Millero (2010).

All analyses were performed using the software GraphPad Prism 6.07 (La Jolla, California, USA). Significant differences between treatments were tested by one-way ANOVA (significance level $P < 0.05$) and Tukey test as a posterior multiple comparison test (significance level $P < 0.05$). Linear regression and Pearson correlation were also performed between cellular chlorophyll *a* concentration and P_{max} (pmol O_2 $cell^{-1}$ min^{-1}).

RESULTS

Growth curves based on cell numbers are given in Fig. 1. Growth rates obtained from cell counting and OD measurements showed no difference between the two techniques (Fig. 2). In the range of light intensity between 20 and 100 µmol photons m^{-2} s^{-1}, specific growth rate µ increased from 0.55 to 0.90 d^{-1} (Fig. 2). Above 100 µmol photons m^{-2} s^{-1}, no further increase was observed, which suggests that 100 µmol photons m^{-2} s^{-1} was the optimum light intensity for *Nannochloropsis oculata* growth and that cells grown above this were in conditions of saturating light (Fig. 2). A light saturation level above 100 µmol photons m^{-2} s^{-1} was also confirmed by the I_k values obtained from the rapid light curves (RLC) performed on cells from all light treatments, which showed a range of light saturation from 84 to 162 µmol photons m^{-2} s^{-1}. Statistically significant differences were found between the maximum cell numbers at the end of batch cultures under each treatment (one-way ANOVA $P = 0.005$); further Tukey tests revealed no differences in this parameter between 20 and 200 µmol photons m^{-2} s^{-1} treatments and between values for cultures grown at 50 and 100 µmol photons m^{-2} s^{-1} (Fig. 3).

Nannochloropsis oculata grown under limiting light conditions (20 and 50 µmol photons m^{-2} s^{-1}) showed greater chlorophyll *a* concentration per cell (Fig. 4) compared with cells grown at 100 and 200 µmol photons m^{-2} s^{-1}. Decreasing cellular chlorophyll concentration with increasing light intensities was observed at all three measuring times during the course of batch culture. However, the largest effect of light limitation on cellular chlorophyll *a* concentration was observed under 20 µmol photons m^{-2} s^{-1} after day 8 of the culture (Fig. 4). There was no effect of light intensity observed on antenna size (σPSIIα) on any of the measuring days (Fig. 5).

Increasing chlorophyll *a* per cell under light-limited conditions was paralleled by increasing light-saturated rates of oxygen evolution (P_{max} based on cell number) (Fig. 6), while a substantially decreased maximum photosynthetic capacity was observed in cells grown under 200 µmol photons m^{-2} s^{-1} (Fig. 6), reflecting the organism's strategy of light acclimation (see discussion). A positive correlation was observed between changes in P_{max} and cellular chlorophyll *a* concentration at the three stages of the cultures (Fig. 7), where increasing Chl *a* per cell and P_{max} concentrated with decreasing light intensity treatments. Fig. 6 also showed that under 20 µmol photons m^{-2} s^{-1}, no differences in P_{max} were observed between days 4, 8 and 15, while under 50 µmol photons m^{-2} s^{-1}, decreased P_{max} was observed on day 15. At 100 and 200 µmol photons m^{-2} s^{-1}, decreasing P_{max} values were observed from day 8 of the culture (Fig. 6). A comparison of P_{max} based on Chl *a* between light treatments showed no effect of light on P_{max} per Chl *a* at day 4 (one way ANOVA $P = 0.023$, $P > 0.05$ between pairs analysed by Tukey's multiple comparisons test), or at day 8 (one way ANOVA $P = 0.073$) or day 15 (one way ANOVA $P = 0.84$; Fig. 8).

On day 4 of the culture (Fig. 9), higher R_d values were observed under both 20 and 50 µmol photons m^{-2} s^{-1}, while no clear effect of light on R_d was observed after day 8 of any of the cultures (Fig. 9). Fig. 10 showed no effect of light on R_d per Chl *a* at day 4 (one-way ANOVA $P = 0.3$), while differences between light treatments were observed at day 8 (one-way ANOVA $P = 0.02$) and day 15 (one way ANOVA $P = 0.004$).

Fig. 1

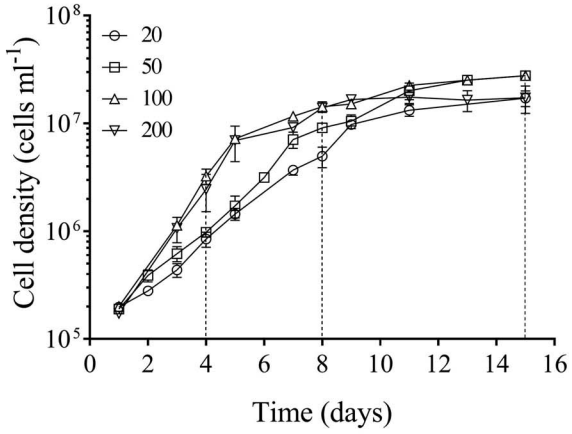


Fig. 2

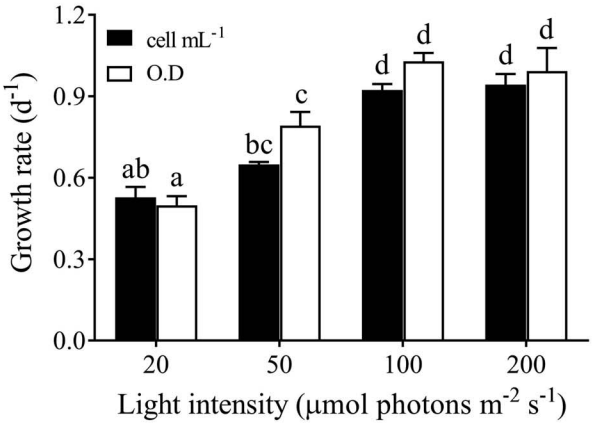


Fig. 3

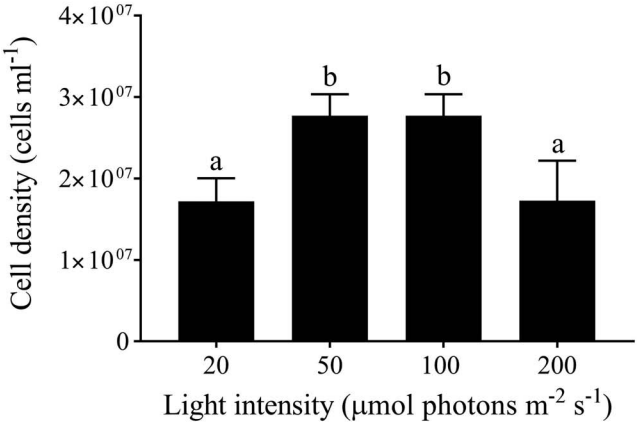


Fig. 4

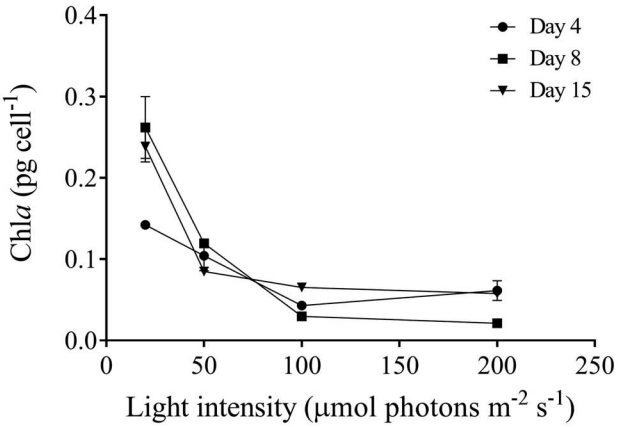


Fig. 5

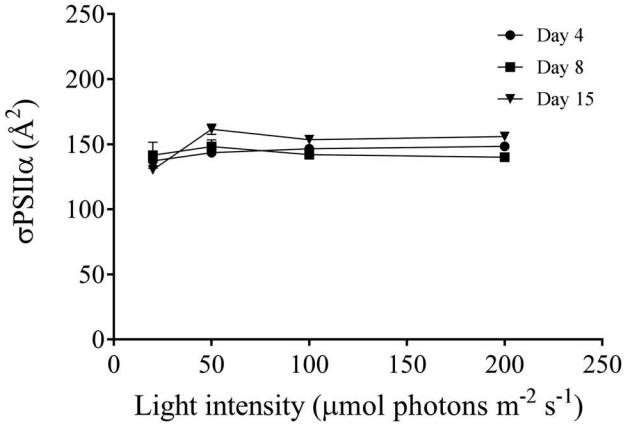
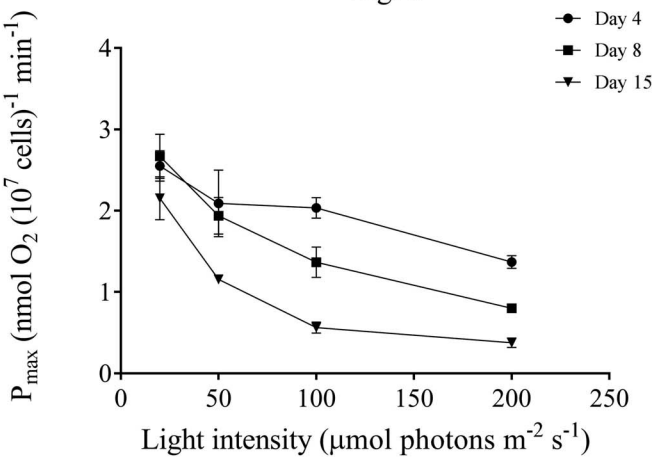


Fig. 6



Figs 1–6. Growth and photo-physiological responses of *Nannochloropsis oculata* grown under 20, 50, 100 and 200 µmol photons m^{-2} s^{-1}. Data are means ± *s*, $n = 3$.

Table 1. pH and inorganic carbon composition of the medium at the days 4, 8 and 15 of *Nannochloropsis oculata* cultures grown under 20, 50, 100 and 200 μmol photons m^{-2} s^{-1}. Data are means ± SD, $n = 3$. Different letters with each value indicate statistically significant differences (Tukey's multiple comparisons test $P < 0.05$).

	Day 4			
	20	50	100	200
pH	8.13 ± 0.09^{a}	8.43 ± 0.06^{a}	8.48 ± 0.06^{a}	8.50 ± 0.06^{a}
DIC (μM)	1759.85 ± 70.45^{a}	1658.56 ± 51.63^{a}	1407.44 ± 59.12^{a}	1502.84 ± 127.89^{a}
$[CO_2]$ (μM)	10.87 ± 3.00^{a}	4.10 ± 0.70^{b}	2.80 ± 0.62^{b}	3.00 ± 0.83^{b}
$[HCO_3^-]$ (μM)	1592.12 ± 90.05^{b}	1367.16 ± 63.10^{ab}	1108.24 ± 78.03^{bc}	1191.15 ± 137.05^{c}
$[CO_3^{2-}]$ (μM)	156.0 ± 25.00^{a}	287.0 ± 25.00^{b}	296.0 ± 28.00^{b}	307.0 ± 14^{b}
	Day 8			
	20	50	100	200
pH	8.64 ± 0.05^{ab}	9.46 ± 0.13^{cde}	9.79 ± 0.02^{de}	10.05 ± 0.05^{de}
DIC (μM)	1271.69 ± 22.36^{a}	390.96 ± 142.36^{b}	192.77 ± 11.01^{b}	265.79 ± 19.59^{b}
$[CO_2]$ (μM)	1.67 ± 0.30^{a}	0.02 ± 0.01^{b}	0.03 ± 0.01^{b}	0.00 ± 0.00^{b}
$[HCO_3^-]$ (μM)	937.15 ± 44.23^{a}	108.23 ± 51.25^{b}	29.16 ± 2.18^{b}	26.48 ± 4.14^{b}
$[CO_3^{2-}]$ (μM)	332.03 ± 24.15^{a}	282.12 ± 25.23^{a}	163.56 ± 8.05^{bc}	239.68 ± 16.35^{ac}
	Day 15			
	20	50	100	200
pH	8.91 ± 0.07^{abc}	9.27 ± 0.20^{bd}	9.72 ± 0.44^{d}	9.59 ± 0.48^{d}
DIC (μM)	1551.97 ± 118.93^{a}	443.27 ± 59.28^{b}	139.75 ± 40.13^{b}	816.48 ± 161.08^{b}
$[CO_2]$ (μM)	1.75 ± 0.30^{a}	0.05 ± 0.03^{b}	0.16 ± 0.03^{b}	0.12 ± 0.03^{b}
$[HCO_3^-]$ (μM)	959.56 ± 134.23^{a}	159.52 ± 45.08^{b}	18.12 ± 11.89^{c}	311.03 ± 20.25^{b}
$[CO_3^{2-}]$ (μM)	591.05 ± 16.56^{a}	283.13 ± 13.22^{bc}	99.15 ± 1.70^{b}	504.78 ± 141.03^{c}

The maximum quantum yield of PSII (F_v/F_m) of the cultures remained stable at all light treatments from day 4 to day 8 of the cultures (Fig. 11). Under 20 and 50 μmol photons m^{-2} s^{-1}, F_v/F_m remained stable at the three growth stages of the culture (one way ANOVA $P = 0.17$ and $P = 0.35$, respectively). However, a decreasing F_v/F_m was observed with increasing light intensities on day 15 of the cultures in cells grown under 100 μmol photons m^{-2} s^{-1} (comparison of days 4, 8 and 15, one way ANOVA $P = 0.0035$) and 200 μmol photons m^{-2} s^{-1} (comparison between day 4, 8 and 15, one way ANOVA $P < 0.0001$) (Fig. 11); thus at late stationary phase (day 15) of the cultures, increasing light intensity from 100 to 200 μmol photons m^{-2} s^{-1} severely impacted the photo-physiology of the cells.

In cells growing at 20 μmol photons m^{-2} s^{-1}, a lower culture pH was observed than in all the other light treatments on day 4 of the cultures (Table 1). At later stages, pH increased in all treatments and under high light reached values > 9.6 by day 8. On day 4 of the cultures, DIC levels were slightly higher in cells grown under 20 and 50 μmol m^{-2} s^{-1} than under 100 and 200 μmol photons m^{-2} s^{-1}, respectively (Table 1). At 20 μmol m^{-2} s^{-1}, DIC level remained stable across all days, while it decreased as cultures became older in the other treatments, and by day 8 bicarbonate and CO_2 levels were extremely low in cultures growing at the two highest light levels (Table 1).

DISCUSSION

The effect of a range of light from 20 to 200 μmol photons m^{-2} s^{-1} on the growth rate of *N. oculata* indicates that at 20 and 50 μmol photons m^{-2} s^{-1} cells were growing under light limitation, while 100 μmol photons m^{-2} s^{-1} appeared to be a saturating intensity for growth. Thus 200 μmol photons m^{-2} s^{-1} represents a condition where at least half of the available light energy is wasted. Sforza *et al.* (2012) observed a similar effect of light on the growth rate of *Nannochloropsis salina*, where the highest specific growth rate obtained under around 170 μmol photons m^{-2} s^{-1} was 0.55 d^{-1}. Our results for *N. oculata* showed the highest specific growth rate (1.02 d^{-1}) under 100 μmol photons m^{-2} s^{-1}.

←

Fig. 1. Growth curves of *Nannochloropsis oculata* grown under 20, 50, 100 and 200 μmol photons m^{-2} s^{-1}. The three vertical dashed lines show the sampling days for analysis at day 4 (exponential phase), day 8 (late log phase) and day 15 (stationary phase).

Fig. 2. The growth rate (d^{-1}) of *Nannochloropsis oculata* calculated during exponential phase of cultures (day 4). Black bars represent calculations based on cell number and white bars based on optical density measurements. Different letters above each column indicate statistically significant differences (Tukey's multiple comparisons test $P < 0.05$).

Fig. 3. The final cell density (day 15) of *Nannochloropsis oculata* achieved under 20, 50, 100 and 200 μmol photons m^{-2} s^{-1}. Means are presented as a single-sided bar. Different letters above each mean indicate statistically significant differences (Tukey's multiple comparisons test $P < 0.05$).

Fig. 4. Chlorophyll *a* concentration (pg $cell^{-1}$) in *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

Fig. 5. Effective absorption cross-sectional area of PSII α centres (σPSIIα) of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

Fig. 6. Maximum rate of oxygen evolution (P_{max}) [nmol O_2 (10^7 cells)$^{-1}$ min^{-1}] of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

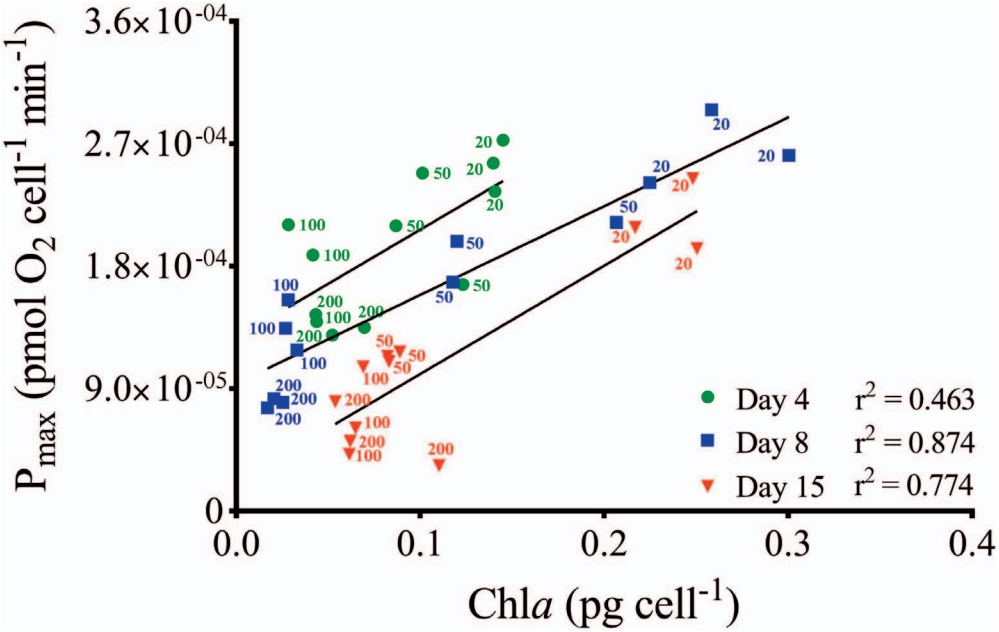


Fig. 7. Linear regression of cellular chlorophyll *a* concentration and maximum rate of oxygen evolution (P_{max}) (pmol O_2 $cell^{-1}$ min^{-1}) of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures. Each symbol represents an independent replicate; the number next to each symbol represents its light intensity treatment. Solid lines (± 95% confidence intervals) show the linear regression.

Nannochloropsis oculata has a high capacity for light acclimation. Under low light conditions, cells at least doubled their chlorophyll *a* concentration over the first 8 d of culture. Higher chlorophyll concentration per cell under low light allowed the cells to increase their capacity to absorb available photons in order to maintain photosynthetic rates (Beer *et al*. 2014). As a photoacclimation strategy, increasing cellular chlorophyll concentration is paralleled by either increasing the size of the antenna serving each reaction centre or increasing PSU number, each with a similar size antenna (Richardson *et al*. 1983). In our results, the analysis of the antenna size revealed that increasing chlorophyll *a* per cell under light limitation was not followed by an increase in antenna size of the photosynthetic apparatus, and this was observed at the three stages of culture studied. Fisher *et al*. (1996) compared the effect of low light (30 µmol photons m^{-2} s^{-1}) and high light (650 µmol photons m^{-2} s^{-1}) on the photosynthesis of *Nannochloropsis* sp. and concluded that there were no changes in PSU size between low and high light treatments but rather a substantial increase of PSU number per cell in cultures grown under low light. Even though the PSU number was not measured in the experiments reported here, previous research on this genus suggests that *N. oculata* might employ the same photoacclimation strategy.

Observations of increased chlorophyll *a* per cell in cells growing under light limitation (20 µmol photons m^{-2} s^{-1}) on day 4, and an even higher increase on days 8 and 15, indicates that the photoacclimation strategy of *N. oculata* under light limitation remains stable, independent of culture stage. Beardall and Morris (1976) observed a similar trend in cultures of *P. tricornutum* grown under low light (~ 12 µmol photons m^{-2} s^{-1}), when the cellular chlorophyll concentration almost doubled from day 2 to day 7 of the culture. It has already been mentioned that the increasing cell density with the days of microalgal cultures can create a shelf-shading effect between the cells, leading to a decrease in light availability to the deeper layers of the culture (Jain *et al*. 2015).

Knowing that low light conditions lead to an increase in cellular chlorophyll concentration in microalgal species (Falkowski & Owens 1980; Fisher *et al*. 1996), we suggest that increasing cell density from day 8 to 15 decreases light availability by a self-shading effect. This in turn led to increases in cellular chlorophyll content toward the end of the culture growth period, and the consequently stable P_{max} and F_v/F_m from day 4 to 15, which is believed to reduce the impact of light limitation on final cell density achieved by the 20 µmol photons m^{-2} s^{-1} treatment. It is also possible that by day 8 in the two highest light intensities used here, inorganic carbon contributed to the slowing down and/or cessation of growth, as bicarbonate and CO_2 levels were very low in these cultures at day 8 and day 15. This is discussed in more detail below.

Richardson *et al*. (1983) showed that changes in either antenna size or PSU numbers are directly related to changes in the photosynthetic capacity of the cells. They presented a model showing that species which increase the PSU number at low light intensities normally show higher P_{max} per cell, compared with cells acclimated to high light intensities, while no changes of P_{max} per Chl *a* are expected between cells grown under low and high light conditions. In our results, cells increased their P_{max} per cell under both light-limitation conditions (20 and 50 µmol photons m^{-2} s^{-1}), while the lowest photosynthetic rate per cell was observed under the highest light treatment studied (200 µmol photons m^{-2} s^{-1}). At the same time, no differences in P_{max} per Chl *a* concentration were observed between any of the light

Fig. 8

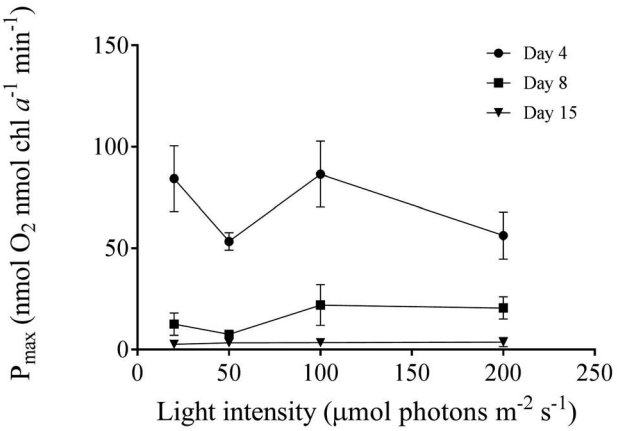


Fig. 9

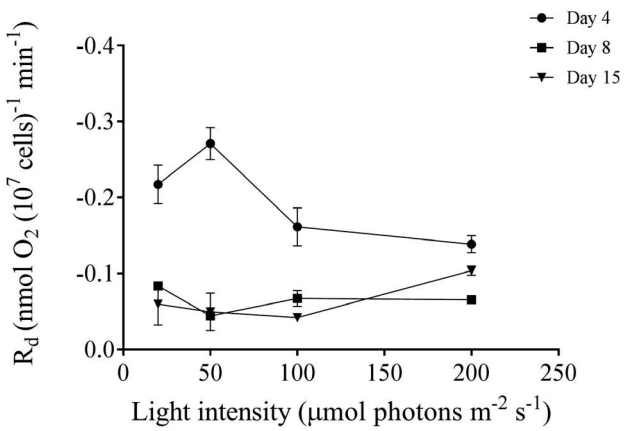


Fig. 10

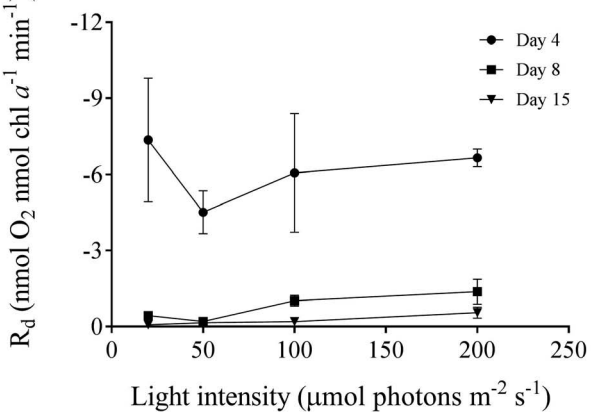
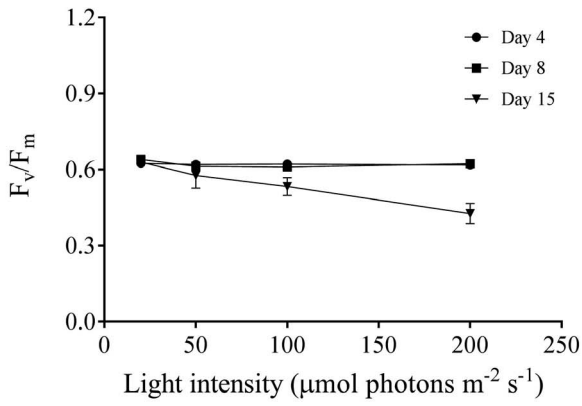


Fig. 11



Figs 8–11. Performance of the photosynthesis of *Nannochloropsis oculata* grown under 20, 50, 100 and 200 μmol photons m^{-2} s^{-1}, measured at days 4, 8 and 15 of the cultures. Data are means ± *s*, $n = 3$.

Fig. 8. Maximum rate of oxygen evolution (P_{max}) (nmol O_2 nmol Chl a^{-1} min^{-1}) of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

Fig. 9. Dark respiration (R_d) (nmol O_2 (10^7 cells)$^{-1}$ min^{-1}) of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

Fig. 10. Dark respiration (R_d) (nmol O_2 nmol Chl a^{-1} min^{-1}) of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

Fig. 11. Maximum quantum efficiency of PSII (F_v/F_m) of *Nannochloropsis oculata* measured at days 4, 8 and 15 of the cultures.

treatments studied, results that remained stable for the duration of the culture. These results suggest that *Nannochloropsis oculata* follows the strategy of increasing PSU number and P_{max} under low light conditions instead of increasing the antenna size. A recent a study on temperature and light interactions on *N. oculata* has shown increasing PSU number per cell with decreasing light intensities in cells of *N. oculata* (Y.M. Palacios, A. Vonshak and J. Beardall, unpublished observations).

It is worth noting that Kandilian *et al.* (2013) presented data showing that, under red light at least, short light-path-length (shallow) cultures exhibited a smaller cross-sectional area of PSII than in cells grown in deeper bioreactors (with presumably a lower average light level), arguing that absorption cross-sectional area increased under lower light. The same effect of light on P_{max} per cell was observed at all three stages of the culture. DIC levels at day 4 of the cultures indicate that it was unlikely that carbon limitation affected the photosynthetic rates in any of the light treatments. This supports our theory that increasing cellular chlorophyll concentration and P_{max} with decreasing light intensities is a clear photoacclimation strategy driven by lower light conditions in *Nannochloropsis*. Linear regression performed on cellular chlorophyll *a* concentration and P_{max} per cell further indicates that the photoacclimation strategy developed by *Nannochloropsis oculata* at the exponential phase remains stable until the stationary phase, regardless of changes in carbon source, shading effects, or nutrient limitation that normally appear at later stages of algal culture.

Few investigations have studied the photosynthetic responses of microalgal cultures over the course of batch culture and compared this with growth responses. Beardall and Morris (1976), studied the effect of low light (0.7 Klux,

$\sim$ 12 μmol photons m^{-2} s^{-1}) and high light (12 Klux, $\sim$ 200 μmol photons m^{-2} s^{-1}) on the photosynthetic capacity of *Phaeodactylum tricornutum* on days 1, 2, 3, 4 and 7 of batch cultures, observing that while at days 1 and 2, higher P_{max} was found in cells grown under high light, and at days 4 and 7 higher P_{max} was observed in cells grown under low light. In our results, photosynthetic characteristics from day 4 to day 15 show that cells grown under higher light intensities (100 and 200 μmol photons m^{-2} s^{-1}) had lower Chl *a* concentration and P_{max} compared with cells grown under low light treatments at day 4. In other words, cells adjusted their light absorption capacity to light availability, thereby maintaining a high photosynthetic efficiency, which was reflected in a higher growth rate during the exponential phase, compared with low light treatments.

Gradual decreases in P_{max} and F_v/F_m with increasing light intensities from 50 to 200 μmol photons m^{-2} s^{-1} at day 15 of the culture agree with the decrease in the level of CO_2 and HCO_3^- in the medium. The various inorganic carbon (C_i) forms in aquatic systems at pH levels from 9.3 to 10.0 comprise $<$ 1% CO_2, $\sim$ 30–3% HCO_3^- and $\sim$ 60–90% CO_3^{2-}, depending on pH (Beer *et al*. 2014), which is important for the photosynthetic and growth responses of *Nannochloropsis oculata*. This species shows a preference for HCO_3^- under alkaline pH (Huertas & Lubián 1998) and possesses a bicarbonate uptake system as a component of its CO_2 concentrating mechanism (CCM) (Huertas *et al*. 2000), which increases the intracellular CO_2 level for photosynthesis and growth. Recently, Gee and Niyogi (2017) proposed a model for the CCM of *Nannochloropsis oceanica* Suda & Miyashita, which shows that the carbonic anhydrase CAH1 catalyses the formation of CO_2 in the lumen of the epiplastid ER by using bicarbonate (pumped by transporters from the medium). The relationship between the use of bicarbonate by the CCM and its effect on photosynthesis was shown when mutation of the CAH1 gene resulted in an almost 35% drop of photosynthetic efficiency under DIC limitation (Gee & Niyogi 2017). Huertas and Lubián (1998) found a value for the half-saturation constant ($K_{0.5}$) in DIC-dependent photosynthesis in *Nannochloropsis oculata* of 167.2 μM for HCO_3^- of photosynthesis under alkaline pH (8.2). This suggests that, in our experiments, by days 8 and 15, cells grown under 50, 100 and 200 μmol photons m^{-2} s^{-1} might be carbon limited since at this time HCO_3^- concentrations in the medium were very much less than the $K_{0.5}$ for HCO_3^-. A clear decrease in bicarbonate availability in cells at days 8 and 15 of 100 and 200 μmol photons m^{-2} s^{-1} treatments is linked with a dramatic decrease in P_{max}. On day 15, the photosynthetic rate of cultures grown at 200 μmol m^{-2} s^{-1} was so low that bicarbonate and CO_2 levels had begun to rise, presumably because by this stage photosynthetic drawdown was less than CO_2 diffusion into the culture. The relationship between bicarbonate limitation and photosynthesis observed from day 8 in cells grown under 100 and 200 μmol photons m^{-2} s^{-1} is also linked to the lack of increase in final cell density compared with cell density in the lower light treatments, suggesting that bicarbonate limitation rather than light started to affect the growth at higher light treatments during linear and stationary phases. Pierangelini *et al*. (2014a) showed that DIC and CO_2 availability can induce major changes to antenna size and other photo-physiological parameters in the cyanobacterium *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya & Subba Raju, but whether similar changes in photo-physiology also occur in *N. oculata* under changing CO_2 is yet to be investigated.

In summary:

1. *Nannochloropsis oculata* showed high capability to acclimate to changing light conditions, adjusting its photosynthetic apparatus and photosynthetic performance according to light availability.
2. *Nannochloropsis oculata* decreased its cellular chlorophyll concentration with increasing light intensities, which was not followed by a decrease in antenna size of the photosynthetic apparatus. Therefore, we believe that *N. oculata* uses an alternative acclimation strategy under changing light conditions.

Light limitation (at $<$ 100 μmol photons m^{-2} s^{-1}) negatively affected the growth rate of *Nannochloropsis oculata* culture. However, the lack of change in final cell density observed between cultures grown at 20 and 200 μmol photons m^{-2} s^{-1} suggests that carbon limitation may have affected photosynthesis and growth at the later stages of the higher light treatments.

This research provides a better understanding of the relationship between the photosynthetic and growth responses of *Nannochloropsis oculata* under changing light conditions in batch cultures, which is crucial to understand the light utilization process by microalgae, and thus maximize the use of light in microalgal cultures.

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