MONASH University

THE STUDY OF LIGHT AS PREREQUISITE FOR THE OPTIMIZATION OF *NANNOCHLOROPSIS* CULTURES

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ABSTRACT

Nannochloropsis oculata and *Nannochloropsis oceanica* are unicellular eustigmatophyte microalgae (Heterokonta) occurring in the oceans. Due to their high lipid content, particular interest on growing these species at a large scale has arisen as new sources of lipid are being sought by the biotechnology sector. However, controlling the environmental factors to optimise the growth or macromolecular composition in microalgal cultures is still a challenge. Light is the main factor affecting growth and macromolecular composition of microalgal cells, due to the effect of light on photosynthesis and therefore, on the energy available for growth.

In this work, I aimed to study how light affects the photosynthetic performance, composition of the photosynthetic apparatus, growth and macromolecular composition of *N. oculata* and *N. oceanica*. For this purpose, in chapter 2 of this thesis, I focused on the effect of increasing light intensities on the photosynthesis and growth of *N. oculata* in the exponential, linear and stationary phase of batch culture. The next two chapters of this thesis focused on the mechanisms of light utilization for photosynthesis and growth under changes in temperature, pH and inoculum size of the culture, which led this thesis to the last chapter which reports on the effect of excess light on photosynthesis, growth and macromolecular composition of *N. oculata* and *N. oceanica* under optimum levels of pH, inoculum size and a pre-acclimation range of light intensity covering low, optimum and high light conditions.

The results of this research demonstrated that both *N. oculata* and *N. oceanica* have great acclimation capability to changing light conditions, increasing cellular chlorophyll content and photosynthetic unit (PSU) numbers under light limitation, which led to increases in their photosynthetic capacity to optimize their light harvesting efficiency. A temperature of 20 ˚C was found to be a better growth condition than 25 ˚C for cells grown either under low or high light intensity, and cells grown under 2% CO_2 preferred a pH of 7.5 pH rather than an unbuffered value of 6.9, where the performance of photosynthesis was highly affected by the pH decrease in an inoculum size independent fashion.

Finally, the study of exposure to excess light intensity revealed a decreasing effect of photoinhibition on cellular chlorophyll concentration, PSU number and maximum photosynthetic rate as cells were pre-acclimated to higher light conditions. On the other hand, a drop in F_v/F_m in both *N. oculata* and *N. oceanica* after excess light exposure was followed by a dramatic increase in lipid content in cells pre-acclimated to 50, 100 and 200 µmol photons m^{-2} s^{-1}. These findings suggest, firstly that the physiological state of cells as reflected in F_v/F_m values can be used as an indicator of the level of stress and hence lipid content in both *N. oculata* and *N. oceanica* and, secondly, that a treatment of 24 h exposure to excess light can be a promising treatment to increase lipid content in cells prior to biomass harvesting.

We can conclude that studying the interaction between photosynthetic capacity, growth and macromolecular composition, at a cellular level, is crucial to optimize microalgal cultures, which is the foundation of the knowledge that will bring us forward to the optimization of microalgal cultures at an industrial level.

Publications during enrolment

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers submitted in peer reviewed journals and two unpublished publications. The core theme of the thesis is the study of light for the optimization of microalgal cultures. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Science under the supervision of Professor John Beardall and co-supervision of Dr Gerry Rayner and Professor Avigad Vonshak. The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapter 2, 3, 4, and 5, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s)	Co-author(s), Monash student Y/N*
2	Photosynthetic and growth responses of *Nannochloropsis oculata* (Eustigmatophyceae) during batch cultures in relation to light intensity	Submitted (*Phycologia*)	85%. Key ideas, experimental set up, lab work and writing.	1) Prof John Beardall 2) Prof Avigad Vonshak	No No
3	Combined effects of light and temperature on photosynthetic acclimation strategy and growth in *Nannochloropsis oculata*	Submitted (*Algal Research*)	85%. Key ideas, experimental set up, lab work and writing.	1) Prof John Beardall 2) Prof Avigad Vonshak	No No

4	Effect of pH buffering *Nannochloropsis oculata* cultures and culture density on light utilization for photosynthesis and growth	Manuscript	85%. Key ideas, experimental set up, lab work and writing.	1) Prof John Beardall 2) Prof Avigad Vonshak 3) Ricky Vitas Dent	No No No
5	The effect of photoinhibition on growth, photosynthesis and macromolecular composition in *Nannochloropsis oculata* and *Nannochloropsis oceanica* previously acclimated to low and high light conditions	Manuscript	85%. Key ideas, experimental set up, lab work and writing.	1) Prof John Beardall 2) Prof Avigad Vonshak 3) Dr Phill Heraud	No No No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date: 23rd August 2017**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date: 23rd August 2017**

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CHAPTER 1: INTRODUCTION

Microalgae offer enormous benefits to humanity as a natural source of pigments, lipids, carbohydrates and proteins (Guedes *et al.* 2011; Borowitzka 2013). Since the 1970's microalgae have been the focus of attention for industrial commercialization (Soeder & Pabst 1970; Venkataraman *et al.* 1977). Some examples are the genus *Dunaliella*, which started to be studied in the 1980s as a rich source of β-carotene (Ben-Amotz *et al.* 1988), *Haematococcus pluvialis*, investigated as a promising species for the production of astaxanthin followed in the 1990s (Harker *et al.* 1996; Boussiba *et al.* 1997) and more recently genera such as *Phaeodactylum* and *Nannochloropsis* for the production of oil for biodiesel (Belarbi *et al.* 2000; Chisti 2007). However, one of the major obstacles impeding the commercialization of microalgae is the high production cost due to low biomass productivity (Sheehan *et al.* 1998).

The photosynthetic efficiency and growth of microalgae in large-scale cultures is strongly affected by the heterogeneous light distribution through the culture. It has been observed that in deeper layers of such cultures light intensity decreases exponentially (Ritchie & Larkum 2012). Around 90% of photons are absorbed at the surface of the culture and just 10% is absorbed in deeper layers, where cells are severely light limited (Beardall & Raven 2013). Determining the optimum depth of out-door cultures has been suggested to be one of the key solutions to improving light distribution in microalgal cultures. For instance, Ritchie and Larkum (2012) reported a significant decrease in total net photosynthesis in *Chlorella*, *Dunaliella* and *Phaeodactylum* in cells growing deeper than 87 mm, 71 mm and 63 mm in open ponds respectively. Thus, improving the efficiency of use of light by microalgae leads to an enhancement of growth and photosynthesis (Simionato *et al.* 2013). For instance, it was observed in *Dunaliella salina* that providing light as flashing illumination of 250 µmol photons m^{-1} s^{-2} versus constant illumination increased their P_{max} on day 3 of the culture 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} respectively (Abu-Ghosh *et al*. 2015).

The study of photosynthesis in microalgae

Microalgae have evolved a vast range of different strategies in order to efficiently convert sunlight into chemical energy (Formighieri *et al.* 2012). This includes changes in the composition of the photosynthetic apparatus and alterations in photosynthetic performance under different light conditions used for growth (Beardall & Morris 1976; Cosgrove & Borowitzka 2006). The performance of photosynthesis has been studied by determining the ability of microalgae to process captured photons and deliver them to PSII reaction centers as described by the maximum photosynthetic capacity (P_{max}), maximum quantum yield (F_v/F_m) and light harvesting efficiency (α), and is thus a function of both the light harvesting efficiency and photosynthetic energy conversion efficiency (Pierangelini *et al.* 2014). Photosynthetic performance has also been quantified by determining the photoacclimation status of microalgae which is commonly described by the light intensity at which photosynthesis reaches saturation (I_k) (Pierangelini *et al.* 2014). On the other hand, the composition of the photosynthetic apparatus in microalgae has been studied extensively, with early work paying special attention to the cellular chlorophyll concentration. In 1911, Willstätter and Stoll (1911) studied pigment extraction and developed equations to calculate chlorophyll concentration, research used by Emerson and Arnold (1932) to study the effect of changing cellular chlorophyll concentration on the photosynthetic reactions of *Chlorella pyrenoidosa*. Later, several researchers incorporated the study of the antenna system of the photosynthetic apparatus by specifically studying the photosynthetic unit (PSU) size (more recently referred to as antenna size) (Prézelin & Alberte 1978) and the photosynthetic unit number in order to understand the acclimation of the photosynthetic apparatus under changing environmental conditions (Falkowski & Owens 1980). The antenna system is a connected array of chlorophyll molecules (plus other secondary pigments) held in the thylakoid membrane of the chloroplast by a matrix of proteins (Raven & Johnson 2002), where the PSU number correspond to the number of reaction centres in the photosystems (Fisher *et al*. 1996) and increases in their number lead cells to increase their light absorption efficiency under low light conditions (Fisher

et al. 1996). The antenna size had been mostly studied by measuring the content of chlorophyll molecules per photosynthetic unit (Melis *et al*. 1998) and more recently has been studied by measuring the effective absorption cross-sectional area of PSII α-centers (σPSIIα) from a Fast Fluorescence Induction (FFI) curve, which has become a well-known technique used to measure the antenna size of photosystem II in microalgae (Koblížek *et al*. 2001; Pierangelini *et al*. 2014). Fisher *et al.* (1996) studied the photoacclimation of *Nannochloropsis* sp. under low and high light conditions by combining measurements of chlorophyll per cell, PSU size, PSU number and the performance of the photosynthesis, providing a well-defined understanding light acclimation in *Nannochloropsis*. More recent studies have also incorporated the study of the antenna system: important parameters are the effective absorption cross-sectional area of PSII α-centers (σPSIIα) (known as antenna size as mentioned above), the antenna connectivity parameter of PSIIα (J_{con}PSIIα) and non-photochemical quenching (NPQ), involved in dissipation of excess light energy (Pierangelini *et al.* 2014).

Photosynthetic and growth responses to light

Under changing light conditions, the photosynthetic apparatus, performance of photosynthesis and growth are strongly affected. At low light intensity, photosynthesis and growth rate increase linearly with increasing light, corresponding to conditions where light absorption and the light reactions are limiting the overall process of photosynthesis (Formighieri *et al.* 2012; Simionato *et al.* 2013). Under this condition, microalgae evolve strategies of photoacclimation to enhance their light harvesting capacity, such as increasing the antenna size or increasing the PSU number per cell (Fisher *et al.* 1996; Quigg *et al.* 2006). *Dunaliella salina*, for example, has been observed to increase the antenna size under continuous low-light condition (Melis *et al.* 1998), while D. tertiolecta was reported to increase the number of PSUs with decreasing light intensity for growth rather than the size of the individual antenna serving each reaction centre (Quigg *et al.* 2006). Photosynthetic units includes reaction centres, photosystem I (PSI), photosystem II (PSII), light-harvesting pigment molecules, enzymes and

electron transport components (Falkowski *et al.* 1981). Increases in the photosynthetic unit size in contrast involve only antenna size increases (Beer *et al.* 2014). Both responses have consequences for the shape of the P-I curves (Richardson *et al.* 1983).

From the information above, it would be assumed that all available light energy is used in photosynthesis. However, at increasing light intensities, photosynthesis and growth rate increase non-linearly as a function of light (Sforza *et al.* 2012). Sforza *et al.* (2012) showed that beyond a light intensity of 150 µmol photons m^{-2} s^{-1} the growth rate of *Nannochloropsis salina* was approximately constant, suggesting that in this range there is wasteful dissipation of absorbed sunlight. In response to this, photosynthetic organisms have a type of regulation of photosynthetic light harvesting referred to as NPQ (non-photochemical quenching) that dissipates excess absorbed light energy to avoid damage to the photosynthetic apparatus (de Bianchi *et al.* 2010). The predominant, fast and reversible component of NPQ named qE (energy quenching) is triggered by changes in the thylakoid lumen pH when the light reaction rates exceeds those of carbon dioxide fixation (de Bianchi *et al.* 2010). Therefore, it is clear that at increasing light intensities, photosynthesis and growth rate are potentially limited by CO_2 supply, among other factors.

On the other hand, when the light intensity is beyond the saturation limit of photosynthesis, algal growth is no longer stimulated by increasing light. In fact, light levels above the saturation limit for photosynthesis often have an inhibitory effect on microalgal growth, causing reduction in division rate. For example, Sforza *et al.* (2012) found that the growth rate and light use efficiency of *Nannochloropsis* salina rapidly decreased as light increased from 150 to 1000 µmol photons m^{-2} s^{-1}, suggesting that at high light intensities the cells are highly inefficient in converting light into biomass. It is observed that at these irradiance levels, NPQ is also activated to dissipate excess absorbed energy (Li *et al.* 2009).

Microalgal cultures can be exposed daily to a large excess of light that has the greatest effect on the photosynthetic and growth responses, which affects biomass production, but also influences the macromolecular composition of the cells, which affects the quality of the produced biomass.

Photoinhibitory conditions are considered as an excessive exposure of microalgal cells to light that cause an oversaturation of light absorption by the antenna complex of the photosynthetic apparatus, which can lead to photodamage in the principal components of the photosystems such as the proteins of the light harvesting complex of the photosystem II (Vass 2012). The impact of photoinhibition can be found in the cellular chlorophyll concentration, causing a dramatic reduction in the photosynthetic capacity of the cells. Photoinhibition occurs when the photoprotective mechanisms of excess energy dissipation (NPQ) are overtaken by the excess of light , leading to photodamage and the formation of Reactive Oxygen Species (ROS) (Formighieri *et al.* 2012), which can cause damage to pigments, proteins and lipids in the cells.

The increasing inefficiency of microalgae to convert light into biomass with increasing light intensities discussed above clearly support the conclusions made by Williams and Laurens (2010) that in the best cases algae convert 10% of photosynthetic active radiation (PAR) and 3% of solar light into biomass. It suggests that there are significant problems in the efficient use of solar light and PAR by microalgae and their photosynthetic efficiency which need to be further explored and resolved.

The effects of environmental conditions on the utilization of light by microalgae

Apart from the heterogeneous light distribution in microalgal cultures, other environmental factors such as temperature, pH and the inoculum size of the culture can affect the utilization of light by microalgae. High temperatures can affect the membrane fluidity as well as denature proteins (Falk. *et al.* 1996). As a consequence of this, non-optimum temperatures affect the performance of photosynthesis; it has been observed that elevated temperatures can affect the electron transport chain (ETC) (Gray *et al.* 1998) and the components of the P v I curve, such as P_{max}, α and I_k (Maxwell *et al.* 1994; Coles & Jones 2000). Figueroa *et al.* (1997) studied the effect of time of exposure to high light (1500 µmol photons m^{-2} s^{-1}) on the chlorophyll concentration of cells of *Nannochloropsis gaditana*

grown under 25 ˚C and 35 ˚C, observing a decrease in chlorophyll concentration after 120 minutes of high light exposure in cells grown under 25 ˚C (from 120 to 110 pmol/10^6 cells), while an effect of high light on cellular chlorophyll concentration in cells grown under 35 ˚C was observed after 240 minutes (from 130 to 97 pmol/10^6 cells). It is known that changes in the cellular chlorophyll concentration can affect the efficiency of light harvesting by the photosynthetic apparatus and the light intensity needed to achieve maximum photosynthetic rate (Fisher *et al.* 1996; Coles & Jones 2000).

The effect of pH on light utilization for photosynthesis and growth has been less well studied. However, it is known that when CO_2 is bubbled through microalgal cultures, the result is a decreased pH which can impact both the process of photosynthesis and growth. In microalgal cultures, the availability of CO_2 for photosynthesis is pH dependent; at alkaline pH of 8-8.3 the proportions of the different components of dissolved inorganic carbon (C_i) is ~90% HCO_3^-, ~9% CO_3^{2-} and less than 1% CO_2, while at 6.5 pH the C_i proportion changes to ~80% HCO_3^-, less than 1% CO_3^{2-} and ~20% CO_2 (Beer *et al.* 2014). As such, the affinity of the species for either CO_2 or HCO_3^- becomes crucial for the carbon fixation process by RubisCO in the Calvin-Benson cycle. Huertas and Lubián (1998) observed that *Nannochloris atomus* and *Nannochloris maculata* had low affinity for HCO_3^- at pH 8.2, while *Nannochloropsis gaditana* and *Nannochloropsis oculata* showed high affinity for HCO_3^- at the same pH. The same research conducted by Huertas and Lubián (1998) showed that the light harvesting efficiency (α) and maximum photosynthetic capacity (P_{max}) was almost three times higher in *Nannochloropsis oculata* than in *Nannochloris atomus* at pH 8.2. In addition, a bicarbonate uptake system was found in *Nannochloropsis* sp. (Huertas *et al.* 2000), which lead to an increased C_i level inside the cells, together with an internal carbonic anhydrase (Huertas *et al.* 2000) that allow the cells to maintain the internal equilibrium between CO_2 and HCO_3^- and therefore maintain high carbon assimilation rates for photosynthesis and growth.

Light penetration in microalgal cultures is highly affected by the cell density of the culture, which has a significant impact on the light availability for photosynthesis and growth of the culture. Therefore,

the inoculum size will determine the cell density, and hence light availability, of the culture in the following days. It has been previously shown that the cell density in the culture can affect the cellular chlorophyll concentration due to changes in the light availability; at low cell densities light availability increases and the chlorophyll concentration per cell decreased (Huang *et al.* 2016).

These studies show that a better understanding of the effect of environmental condition on the light utilization for photosynthesis and growth of microalgal cultures is crucial to improve the use of light for biomass production in such cultures.

Selecting microalgae

Microalgae are photosynthetic organisms with a large biological variability, and are capable of responding efficiently to changes of light availability for growth by photosynthetic acclimation, which makes them promising candidates for industrial commercialization. For instance, the diatom *Phaeodactylum tricornutum*, which is reported to be a high biomass producer (0.24 g L^{-1} d^{-1}) (Rodolfi *et al*. 2009), acclimates to high light by reducing its PS II functional cross-section (σPS II) from 128 $Å^2$ to 60 $Å^2$ and can reverse this change nearly fully after only 30 min of darkness (Koblížek et al. 2001). The genus *Chlorella* (Trebouxiophyceae) is also a promising for biotechnology applications as it has shown high biomass productivity (~0.22 g L^{-1} d^{-1}) and has a high capacity for light acclimation; thus under low light, *Chlorella fusca* was reported to be able to enhance its cellular chlorophyll content from 4.3 to 7.2 µg $µl^{-1}$, but decrease its photosynthetic capacity so as to decrease requirements for the energetic costly synthesis of enzymes involved in CO_2 assimilation, as a strategy to capture more light for growth (Senge & Senger 1990).

Among all the species of microalgae with biotechnological interest, the genus *Nannochloropsis* is a suitable feedstock for macromolecular production because of their ability to accumulate lipids. Within a screening of thirty microalgal strains made for their biomass productivity and lipid content,

Nannochloropsis sp. was highly productive, with a biomass and lipid productivity of 0.17 g L^{-1} day^{-1} and 60.9 mg L^{-1} day^{-1} respectively and 35.7% of biomass accumulated as lipid (Rodolfi *et al.* 2009).

Strains belonging to the genus *Nannochloropsis* have been also observed to be able to adapt their photosynthetic apparatus to different photon fluxes, suggesting that *Nannochloropsis* has a remarkable capacity of accumulating lipids in certain conditions (Simionato *et al.* 2011).

Therefore, *Nannochloropsis* is a promising microalgae for biotechnological purposes, and studying the light use efficiency of the cells will provide useful information for optimizing cultures for commercial purposes.

To this end, this thesis is divided into 4 data chapters; The first deals with the effect of light on photosynthesis and growth of batch cultures in *N. oculata*, the second focuses on the effect of light and temperature on photosynthesis and growth of *N. oculata* and the third one focuses on the mechanisms of light utilization for photosynthesis and growth under changes in pH and inoculum size of *N. oculata* cultures. The final data chapter examines the effects of high light exposure on photosynthetic performance, growth and macromolecular composition of *N. oculata* and *N. oceanica* cultures and the thesis concludes with a general summary and discussion of the results and how they cast light on constraints to productivity in mass culture

AIM

This thesis reports on investigations into the phenotypic plasticity of *Nannochloropsis*, with particular reference to light use efficiency. The light use efficiency of two species of the genus *Nannochloropsis*, was studied in response to a range of external conditions as well as to very high light exposure (potentially photo-inhibited conditions). This thesis aims to improve our understanding of the interactions between light and temperature, pH and inoculum size of the culture in order to be able

to develop optimal growth conditions for biomass and macromolecule composition of microalgal cultures.

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CHAPTER 2: 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. It has been observed that photosynthetic responses to light change with the stage of the culture, though little is known about how changes in the photosynthetic capacity with the time of batch culture affects the growth at the end/harvesting 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}. *N. oculata* showed a high ability to acclimate to changing light conditions; cells almost doubled their cellular chl *a* concentration under the low light conditions and increased their photosynthetic oxygen evolution capacity, which allows 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 followed by changes in the 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 the 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 on growth onwards. Incorporating an 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 optimization of the photosynthetic efficiency and growth of microalgal cultures.

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 out-door cultures; the efficiency of energy conversion of light into biomass has a significant effect on cost of the production. The highest efficiency of energy conversion into biomass found for algae is ~ 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.* 2013; 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 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). Therefore, a viable algal cultivation system requires better understanding of, and improvement to, the photosynthetic efficiency of the culture.

Microalgae have evolved a range of different strategies in order to efficiently convert sunlight into chemical energy for growth (Formighieri *et al.* 2012). When light is the limiting factor to the culture, light absorption and photochemical reactions may limit the overall process of photosynthesis (Formighieri *et al.* 2012). Therefore, microalgae adjust their light harvesting capacity to decreased light

conditions by either increasing the effective absorption cross-sectional area of the antennae serving individual photosynthetic reaction centres (increased antenna size) (Melis *et al.* 1998) or increasing the number of photosynthetic units (PSU) per cell (Quigg *et al.* 2006). On the other hand, under high light, if the absorbed light energy exceeds the capacity for energy dissipation through energy-requiring processes such as carbon assimilation via the Calvin - Benson cycle, cells can potentially become photoinhibited. In this regard, dissipation of excess absorbed light energy can be achieved by activation of non-photochemical quenching (NPQ) (Raven 2011).

The effect of light on photosynthesis has been observed in many species of microalgae, for instance *Phaeodactylum tricornutum* (Bohlin) (Quigg *et al.* 2006) and *Nannochloropsis gaditana* (L.M.Lubián) (Meneghesso *et al.* 2016), where the photosynthetic parameters were measured during the exponential phase of the culture. However, little is known about how the light intensity affects photosynthesis and therefore the growth toward the end of a batch culture period. Beardall and Morris (1976) studied the light acclimation strategy of *P. tricornutum* adapted to either low light (0.7 Klux, ~ 12 µmol photons m^{-2} s^{-1}) and high light (12 Klux, ~200 µmol photons m^{-2} s^{-1}) during the course of batch cultures, observing maximum photosynthetic rate on day 4 of the culture in cells grown under low light treatment, while the maximum photosynthetic rate in cells grown under high light was found on day 2 of the culture; therefore a comparison of the photosynthetic responses between low and high light treatment resulted in opposite findings depending on the day of observation. The ability of a species to maintain a high photosynthetic capacity across the entire period from exponential to stationary phase will have an important impact on their growth and biomass at the end of the culture period.

Many species of algae belonging to the classes Bacillariophyceae, Trebouxiophyceae and Eustigmatophyceae efficiently respond to changes of light availability for growth by photosynthetic acclimation, which make them promising for industrial commercialization. For instance, *P. tricornutum*, which is reported to be a high biomass producer (0.24 g L^{-1} d^{-1}) (Rodolfi *et al.* 2009) acclimates to high light by reducing its PS II functional cross-section (σPS II) from 128 $Å^2$ to 60 $Å^2$ and

can reverse this change nearly fully after only 30 min of darkness (Koblížek *et al.* 2001), changes also observed by Quigg *et al.* (2006) from photosynthetic unit size (PSU size) measurements. Some species belonging to the genus Chlorella (Trebouxiophyceae) also show high biomass productivity (~0.22 g L^{-1} d^{-1}) and have a high capacity for light acclimation; thus under low light, *Chlorella fusca* (Shihira & R. W. Krauss) was reported to be able to enhance its cellular chlorophyll content, but decrease its photosynthetic capacity, as a strategy to capture more light for growth (Senge & Senger 1990).

The genus *Nannochloropsis* (Eustigmatophyceae) is another promising algal candidate for industrial commercialization, showing 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 an important source of carotenoids (Nobre *et al.* 2013), eicosapentaenoic acid (EPA) (Renaud & Parry 1994) and triacylglycerol (TAG) (Pal *et al.* 2011). Photosynthetically, *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 (Sukenik *et al.* 2009). No differences in PSU size have been found in this genus when cells are exposed to different light intensities but instead they acclimate by undergoing changes in PSU number (Fisher *et al.* 1996); increasing light exposure from 30 to 650 µmol photons m^{-2} s^{-1} showed a decrease in PSU number from 36608 to 7730 PSU $cell^{-1}$ (Fisher *et al.* 1996).

The research described here was therefore aimed at monitoring the effect of light on photosynthesis at critical points during batch culture of *Nannochloropsis oculata* (D.J. Hibberd) culture and the consequences of this for growth.

To this end, we studied the effect of light intensity from 20 to 200 µmol photons m^{-2} s^{-1} on the photosynthetic apparatus and performance of photosynthesis as well as on growth from day 4 to 15 of *N. oculata* culture.

Materials and Methods

Strain and culture conditions

The unicellular green marine alga *Nannochloropsis oculata* (Eustigmatophyceae) (CS-179) was obtained from the CSIRO Australian National Algae Culture Collection, Hobart, Tasmania. For all treatments, three independent replicate 1 L batch cultures were grown in 2 L Erlenmeyer flasks using PHK medium (Rukminasari 2013), incubated under a 16:8 h light/dark cycle at 20^{o}C. To characterize the response of growth and photosynthesis, *N. oculata* was grown under four irradiance levels: 20, 50, 100 and 200 µmol photons m^{-2} s^{-1}.

Growth

Algal growth was monitored every two days by cell counting with a Neubauer hemocytometer under a light microscope and also by optical density $(OD)_{750}$ measurements determined spectrophotometrically with a Cary 50 Bio UV-visible spectrophotometer. The specific growth rate was determined by calculating the slope of log (cell number ml^{-1}) vs time in the exponential phase of the culture. The cultures were inoculated at an initial cell density of 2×10^5 cell ml^{-1} and mixed manually every day.

Chlorophyll Analysis

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) by centrifugation (10 min, 3500 rpm) at 4^{o}C and resuspended in 96% ethanol for 24 h at 4^{o}C. After extraction the suspension was centrifuged again and pigment concentration in the supernatant was determined spectrophotometrically, using a Cary-50 UV-V

Spectrophotometer, at 630, 647, 664 and 750 nm. Chlorophyll *a* concentration was then calculated using equation 1 (Jeffrey & Humphrey 1975).

$$\text{Chl } a = 11.85\ (A_{664} - A_{750}) - 1.54\ (A_{647} - A_{750}) - 0.08\ (A_{630} - A_{750}) \qquad \text{Equation 1}$$

Photosynthetic parameters

For all photosynthetic parameters, 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 g) and the pellet resuspended in 3 ml of fresh PHK medium containing 2 mM inorganic carbon. This suspension was then placed into an O_2 electrode chamber for measurement of oxygen exchange rates. 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. R_d measurement was taken after dark incubation of the cell suspension for 10 min. Maximum O_2 evolution rate of the culture was measured under a saturating light intensity previously determined from Rapid Light Curves performed in a Phyto-PAM phytoplankton analyzer system (Heinz Walz, Effeltrich, Germany).

Chlorophyll fluorescence of PSII was measured to determine maximum quantum yield (F_v/Fm), 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.

Flash fluorescence induction curves (FFI) were measured to determine the effective absorption cross sectional area of PSII α centers (σPSIIα) and antenna connectivity of PSIIα (J_{con}PSIIα), using a double-modulation fluorometer (Photon Systems Instrument, Brno, Czech Republic). Approximately $1x10^8$ cells were collected from the culture, resuspended in fresh PHK medium and incubated in the dark

for 5 min prior to measurement. For measurements, a single 50 µs turnover flash at a light intensity setting of 50% (1 x 10^6 µmol photons m^{-2} s^{-1}) was used and the detector sensitivity set at 5%. For the calculation of σPSIIα and J_{con}PSIIα, the area under the FFI curve was analysed according to Melis and Homann (1975) and Nedbal *et al.* (1999).

pH, temperature, salinity and inorganic carbon estimation

pH and temperature of the sample were measured using a Hach PH31 pH-meter, and salinity determined using a RHS-10ATC refractometer. A LI-840A CO_2/H_2O gas analyser (LI-COR) was used to measure the dissolved inorganic carbon (DIC) after conversion of DIC to CO_2 by acidification with I N HCl. DIC was then calculated using a standard curve based on a standard curve from 0 - 6 mM $NaHCO_3$. pCO_2 in the cultures was calculated from the calculated DIC, pH, salinity and temperature using an aquatic acid-base modelling environment (AquaEnv) package in R.

Statistical Analysis

Significant differences between treatments were tested by one way ANOVA (significance level $P<0.05$). Graphs were constructed and statistical analyses were carried out using the software Graphpad Prism 6.

Results

Effect of light intensity on N. oculata growth

The growth curves and growth rates obtained from cell counting and O.D measurements showed no differences between the two techniques. Growth curves based on cell numbers are given in Fig. 1A. In the range of light intensity between 20 and 100 µmol photons m^{-2} s^{-1}, the specific growth rate µ increased from 0.47 to 1.02 d^{-1} (data based on O.D measurements) (Fig. 1C). 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 *N. oculata* growth and that cells grown above this were in conditions of saturating light (Fig. 1B). 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 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} (insert graph in figure 1A).

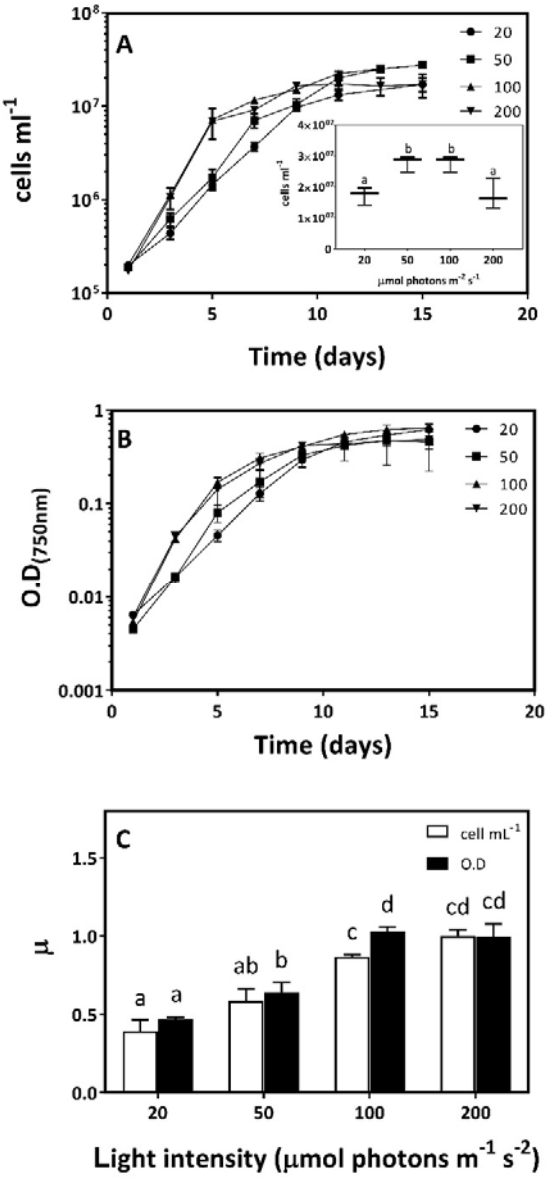

Figure 1. Growth responses of *N. oculata* grown under 20, 50, 100 and 200 µmol photons m^{-2} s^{-1}. A) Growth curve based on cell counts. The inserted graph shows the final cell density (day 15) achieved in each treatment. Different letters above each mean indicate statistically significant differences (Tukey test P>0.05). B) Growth curve based on $O.D_{750}$. C) Growth rate (d^{-1}) calculated at exponential phase of the culture (day 4). White bars correspond to calculations based on cell counts and black bars are $O.D_{750}$ based calculations. Data are means +/- SD, n = 3. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05).

Effect of light on chlorophyll *a* concentration and photosynthetic capacity

N. oculata grown under limiting light conditions (20 and 50 µmol photons m^{-2} s^{-1}) showed a >2 fold increase in chlorophyll *a* concentration per cell (Fig. 2A), 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 cell chlorophyll *a* concentration was observed under 20 µmol photons m^{-2} s^{-1} from day 8 of the culture (Fig. 2A). No effect of light intensity on the antenna size (σPSIIα) was observed on any of the measuring days (Fig. 2B), but an increase in connectivity of the antenna was observed in cells grown at 20 µmol photons m^{-2} s^{-1} across all 15 days of the experiment (Fig. 2C).

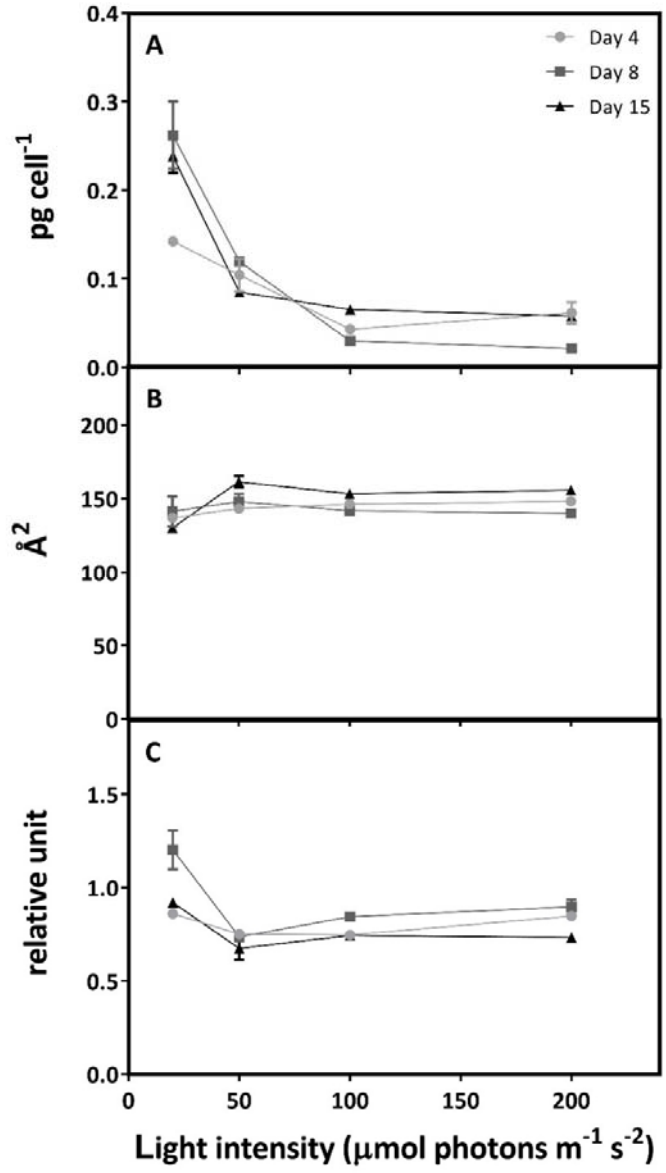

Figure 2. Composition of the photosynthetic apparatus of *N. oculata* measured at day 4, 8 and 15 of the cultures. A) Chlorophyll *a* concentration (pg cell^{-1}), B) Antenna size (σPSIIα), C) Antenna connectivity (J_{con} PSIIα). Data are means +/- SD, n = 3.

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. 3A), while a substantially decreased maximum photosynthetic capacity was observed in cells grown under 200 µmol photons m^{-2} s^{-1} (Fig. 3A), reflecting the organism's strategy of light acclimation (see discussion). 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. 3A). A comparison of P_{max} based on chl *a* between light treatments showed no effect of light on P_{max} per chl *a* either at day 4 (One Way Anova $p = 0.023$, $p > 0.05$ between pairs analysed by Tukey's multiple comparisons test), or on days 8 (One Way Anova $p = 0.073$) and 15 (One Way Anova $p = 0.84$) (Figure 4A).

On day 4 of the culture (Fig. 3B), 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 from day 8 of the cultures (Fig. 3B).). Figure 4B shows 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 $p = 0.004$).

The maximum quantum yield of PSII (F_v/F_m) of the cultures remained stable at all light treatments from day 3 to 8 of the cultures (Fig. 3C). However, a decreasing F_v/F_m was observed with increasing light intensities on day 15 of the cultures (Fig. 3C); thus at late stationary phase (day 15) of the cultures, increasing light intensity from 50 to 200 µmol photons m^{-2} s^{-1} severely impacted the photo-physiology of the cells.

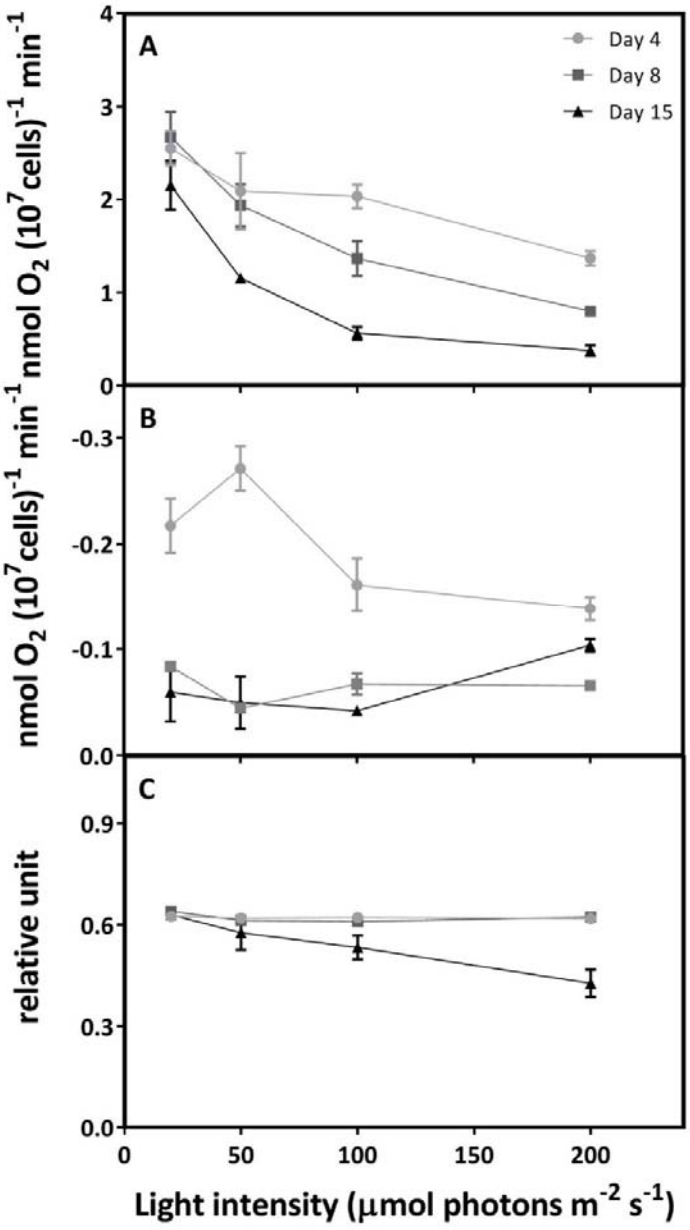

Figure 3. Performance of the photosynthesis of *N. oculata* measured at day 4, 8 and 15 of the cultures. A) Maximum rate of oxygen evolution per cell (P_{max}), B) Dark respiration per cell (R_d), and C) Maximum quantum efficiency of PSII (F_v/F_m). Data are means +/- SD, n = 3.

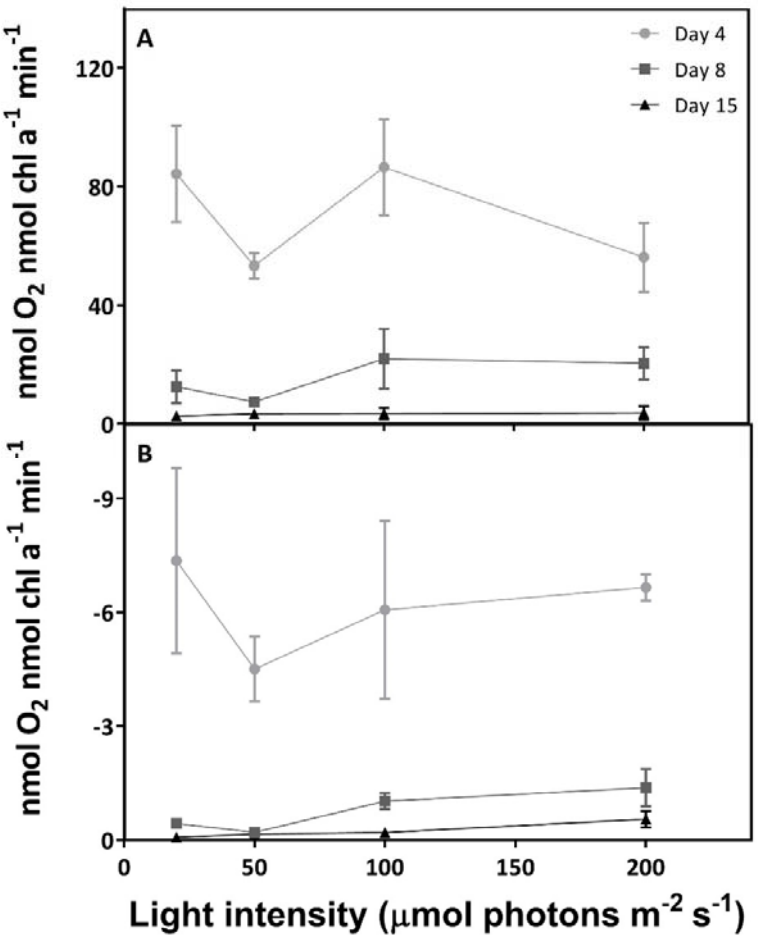

Figure 4. Performance of the photosynthesis of *N. oculata* measured at day 4, 8 and 15 of the cultures. A) Maximum rate of oxygen evolution per chl *a* (P_{max}), B) Dark respiration per chl *a* (R_d). Data are means +/- SD, n = 3.

pH and carbon estimation

A lower culture pH was observed in cells growing at 20 µmol photons m^{-2} s^{-1} than in all the other light treatments on day 4 of the cultures (Table 1). At later stages of culture, 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} (Table 1). At 20 µmol m^{-2} s^{-1}, DIC level remained stable across all days, while it decreased as cultures became older in all the other treatments, and by day 8 bicarbonate and CO_2 levels were extremely low in cultures growing at the 2 highest light levels (Table 1).

Table 1. pH and inorganic carbon composition of the medium at the days 4, 8 and 15 of *N. oculata* cultures grown under 20, 50, 100 and 200 µmol photons m^{-2} s^{-1}. Data are means +/- SD, n=3. Different letters above each value indicate statistically significant differences (Two Way ANOVA P<0.05).

	Day 4			
	20	**50**	**100**	**200**
pH	8.1 ± 0.09^{a}	8.4 ± 0.06^{a}	8.5 ± 0.06^{a}	8.5 ± 0.06^{a}
DIC (mM)	1.7 ± 0.07	1.7 ± 0.05	1.4 ± 0.05	1.5 ± 0.1
$[CO_2]$ (mM)	0.01 ± 0^{a}	0.004 ± 0^{b}	0.002 ± 0^{b}	0.003 ± 0^{b}
$[HCO_3^-]$ (mM)	1.59 ± 0.09^{a}	1.36 ± 0.04^{ab}	1.1 ± 0.13^{bc}	1.18 ± 0.06^{c}
$[CO_3^{2-}]$ (mM)	0.15 ± 0.02^{a}	0.28 ± 0.02^{b}	0.29 ± 0.02^{b}	0.3 ± 0.01^{b}
	Day 8			
	20	**50**	**100**	**200**
pH	8.6 ± 0.05^{ab}	9.5 ± 0.1^{cde}	9.8 ± 0.02^{de}	10 ± 0.05^{de}
DIC (mM)	1.3 ± 0.02^{a}	$0.5 \pm 0.2b^{b}$	0.2 ± 0.01^{b}	0.3 ± 0.01^{b}
$[CO_2]$ (mM)	0.001 ± 0^{a}	0.00005 ± 0^{b}	0.00003 ± 0^{b}	0.00001 ± 0^{b}
$[HCO_3^-]$ (mM)	0.9 ± 0.04^{a}	0.16 ± 0.05^{b}	0.029 ± 0.002^{b}	0.026 ± 0.004^{b}
$[CO_3^{2-}]$ (mM)	0.33 ± 0.09^{a}	0.33 ± 0.04^{a}	0.16 ± 0.13^{bc}	0.23 ± 0.06^{ac}
	Day 15			
	20	**50**	**100**	**200**
pH	8.9 ± 0.07^{abc}	9.3 ± 0.2^{bd}	9.7 ± 0.4^{d}	9.6 ± 0.4^{d}
DIC (mM)	1.5 ± 0.1^{a}	0.4 ± 0.05^{b}	0.2 ± 0.1^{b}	0.6 ± 0.4^{b}
$[CO_2]$ (mM)	0.0009 ± 0.3^{a}	0.00005 ± 0.1^{b}	0.00001 ± 0.01^{b}	0.00008 ± 0^{b}
$[HCO_3^-]$ (mM)	0.9 ± 0.09^{a}	0.15 ± 0.04^{b}	0.05 ± 0.13^{c}	0.21 ± 0.06^{b}
$[CO_3^{2-}]$ (mM)	0.59 ± 0.01^{a}	0.28 ± 0.01^{b}	0.1 ± 0.01^{b}	0.26 ± 0.1^{b}

Discussion

Growth

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 at 200 µmol photons m^{-2} s^{-1} represents a condition where at least the half of the available light energy is wasted. Sforza *et al.* (2012) observed similar effect of light on the growth rate of *Nannochloropsis salina* (D.J. Hibberd), where the highest growth rate obtained under around 170 µmol photons m^{-2} s^{-1} was 0.55 d^{-1}. Our results showed a highest growth rate of 1.02 d^{-1} under 100 µmol photons m^{-2} s^{-1}.

Photosynthetic responses

N. oculata has a high capacity for light acclimation. Under low light conditions, cells at least doubled their chlorophyll *a* concentration over the first 8 days of the culture. Higher chlorophyll concentration per cell under low light allow the cells to increase their capacity to absorb the photons available in order to maintain photosynthetic rates (Beer *et al.* 2014). As a photo-acclimation strategy, increasing cellular chlorophyll concentration is paralleled by either increasing the size of the antenna serving each reaction centre or increasing the number of photosynthetic units (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 increasing the antenna size of the photosynthetic apparatus. 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, rather a substantial increase of PSU number per cell was observed in cells grown under low light. Even

though the PSU number was not measured in the experiments reported here, the previous research on this genus suggests that *N. oculata* might employ the same photo-acclimation strategy.

Observations of increased chlorophyll *a* per cell in cells growing under light limitation (20 µmol photons m^{-2} s^{-1}) observed 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}) , where 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, especially to the deeper layers of the culture (Jain *et al.* 2015). Knowing that low light conditions lead to an increase the 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 the light availability by a self-shading effect, leading to increases in the cellular chlorophyll content toward the end of the culture growth period and the consequent stable P_{max} and F_v/F_m from day 4 to 15, , which is believed to reduce the impact of light limitation on the 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 may be contributing to the slowing down /cessation of growth as bicarbonate and especially CO_2 levels were very low in these cultures at day 8 and day 15. This is discussed further below.

It has been shown that changes either in antenna size or PSU numbers are paralleled by changes in the photosynthetic capacity of the cells (Richardson *et al.* 1983). Richardson *et al.* (1983) presented a model, showing that species that 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} 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* were observed between any of the light treatments studied, results that remained stable over the days of the culture. These results suggest that *N. oculata* follows the strategy of increased PSU number and P_{max} per cell under low light conditions instead of increasing the antenna size. The same tendency on the effect of light on P_{max} was observed at all three stages of the culture. DIC levels measured at day 4 of the cultures indicate that carbon limitation was unlikely to be affecting the photosynthetic rates in any of the light treatments, which supports our contention that increasing cellular chlorophyll concentration and P_{max} with decreasing light intensities is a clear photo-acclimation strategy driven by changing light conditions in *Nannochloropsis*.

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, ~ 12 µmol photons m^{-2} s^{-1}) and high light (12 Klux, ~200 µmol photons m^{-2} s^{-1}) on the photosynthetic capacity of *P. tricornutum* on days 1, 2, 3, 4 and 7 of batch cultures, observing that while at day 1 and 2 higher P_{max} was found in cells grown under high light, at day 4 and 7 higher P_{max} was observed in cells grown under low light. In our results, study of the photosynthetic characteristics from day 4 to day 15 shows 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 low light treatments at day 4 in order to adjust their light absorption capacity to the light availability, thereby maintaining a high photosynthetic efficiency which was reflected in higher growth rate during exponential phase of the culture compared with low light treatments. However, a slowdown in growth observed in linear and stationary phase of cultures grown under 100 and 200 µmol photons m^{-2} s^{-1} might be linked to a decrease in HCO_3^-, and especially CO_2, availability at later stages of the cultures.

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 are also in accord with the decrease in the level of CO_2 and HCO_3^- in the

medium. The the various inorganic carbon (C_i) forms in aquatic systems at pH levels from 9.3-10 comprise < 1 % CO_2, ~30-3 % HCO_3^- and ~60-90 % CO_3^{2-}, depending on the pH (Beer *et al.* 2014), which can be important for the photosynthetic and growth responses of *N. oculata*. *N. oculata* shows a preference for HCO_3^- use 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 catalyzes 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 $K_{0.5}$ value in DIC-dependent photosynthesis in *N. 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} at day might be carbon limited since at this time HCO_3^- concentrations in the medium were << the $K_{0.5}$ HCO_3^-. A clear decrease in bicarbonate availability in cells grown at days 8 and 15 of the 100 and 200 µmol photons m^{-2} s^{-1} treatments is linked with a dramatic decrease in the 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 with the fact that final cell density in these treatments did not increase compared to that 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.

Conclusions

N.oculata showed high capability of acclimation to changing light conditions, adjusting their photosynthetic apparatus and photosynthetic performance according to the light availability.

N. oculata decreased its cellular chlorophyll concentration with increasing light intensities, which was not followed by decreasing the antenna size of the photosynthetic apparatus. Therefore, it is believed 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 *N. oculata* culture. However, the lack of differences in the 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 *N. oculata* under changing light conditions in batch cultures, which is crucial to understand the light utilization process by microalgae and thus improve the use of light in microalgal cultures.

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CHAPTER 3: Combined effects of light and temperature on photosynthetic acclimation strategy and growth in *Nannochloropsis oculata*

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ABSTRACT

Light and temperature are the major factors effecting algal growth and productivity in mass culture. This research aimed to study the photosynthetic acclimation response and growth of *Nannochloropsis oculata* growing under combinations of low light (LL) or high light (HL) and temperatures of 20 and 25^{o}C. Furthermore we investigated the photosynthetic performance under the growth conditions of each treatment to see whether photosynthesis responses may provide a better understanding of the growth behaviour. Results indicate that the temperature levels studied affected the growth rate and maximum cell density when cells were grown under LL, but not under HL. *N. oculata* increases its photosynthetic capacity at LL under both temperature conditions, but a higher photosynthetic capacity was achieved at 25^{o}C. Under HL, decreased chlorophyll a and PSU numbers per cell were associated with a decrease in light harvesting efficiency and an increased energy dissipation mechanism (NPQ), likely a photoacclimation strategy to avoid photodamage. The NPQ values also show that under HL at both temperatures a high amount of energy is wasted. On the other hand, the effect of light and temperature on the performance of photosynthesis at the growth conditions did not explain the growth responses. In the future, understanding the acclimation strategy employed by *N. oculata* under the combined effects of light and temperature and knowing that factors other than

photosynthetic rates in situ explain growth behaviour will be crucial to improve growth of microalga in mass cultures.

Keywords: Photosynthesis, acclimation, growth, Nannochloropsis, PSU number

Introduction

Development of the biotechnology for microalgal production at an industrial scale production is being intensively studied aiming at increasing biomass productivity or specific accumulation of a high values product. Light and temperature are the main critical factors affecting outdoor algal culture productivity (Torzillo *et al.* 2003). The heterogeneous distribution of light in cultures has a negative impact on growth in both the surface (where light can be inhibitory) and deeper layers of the culture (where light can be limiting) (Lu & Vonshak 1999; Simionato *et al.* 2013; Vonshak *et al.* 2014), while large changes in temperature can affect both the structure of cell components and metabolic mechanisms (Carvalho *et al.* 2009; Mackey *et al.* 2013). Improving biomass production is a major focus for mass cultures, therefore it is necessary to continue to expand our knowledge on algal responses to changing conditions to better understand growth behaviour in the heterogeneous conditions of industrial scale cultures.

To this end, we studied the photoacclimation strategy of *N. oculata* under the combined effects of light and temperature and specifically investigated how photosynthesis is affected by the combined effects of these two main parameters. Furthermore we investigated whether the photosynthetic responses explain growth behaviour, in order to expand the knowledge from a cellular level to large scale microalgae culture.

Microalgae have evolved different strategies in order to efficiently convert light into chemical energy for growth (Formighieri *et al.* 2012). Under light-limiting conditions, growth rate increases linearly with increasing light (Simionato *et al.* 2013). Under such conditions cells adjust their light harvesting

capacity by either extending their effective absorption cross-section area of photosynthetic reaction centres PSII (increasing antenna size) (Melis *et al.* 1998) or increasing the number of photosynthetic units (PSU) per cell (Quigg *et al.* 2006). Under high light, growth rates increase non-linearly as a function of light (Simionato *et al.* 2013), corresponding to conditions where light reaction rates exceed the carbon dioxide fixation process (de Bianchi *et al.* 2010). To cope with this, microalgae activate an energy dissipation mechanism known as non-photochemical quenching (NPQ), to deal with the excess of absorbed energy (de Bianchi *et al.* 2010). However, when the light intensity absorbed by the cells is beyond the saturation limit of photosynthesis, light becomes inhibitory. The excess of light causes the photoprotective mechanisms to become impaired, resulting in a high number of reaction centres being damaged and the production of toxic levels of reactive oxygen species (ROS) (Asada 1999). All of these lead to a detrimental decrease in biomass production (Simionato *et al.* 2013).

When the effect of light on growth is studied in photosynthetic organisms, researchers need to consider the strong effect of the temperature of the culture. For instance Sandnes *et al.* (2005) showed that increasing light intensity from 34 to 80 µmol of photons m^{-2} s^{-1} has almost no effect on growth rate when cells of *Nannochloropsis oceanica* grew under low temperatures (14.5°C-17.3°C), while at higher temperature (25.6°C) growth rate increased rapidly from 1.2 d^{-1} at 34 µmol of photons m^{-2} s^{-1} to 1.6 d^{-1} at 80 µmol of photons m^{-2} s^{-1}. This interaction between light and temperature was also studied in outdoor cultures (Vonshak *et al.* 2001). Yet although previous research has examined the interaction between light and temperature on growth of a variety of microalgae, the relationship between photosynthetic acclimation of microalgae under both factors and their growth responses has not been fully addressed.

Members of the genus *Nannochloropsis* have received increasing interest for biodiesel production as well as for aquaculture nutrition due to their rapid growth rate (Rodolfi *et al.* 2009), ability to accumulate carotenoids (Nobre *et al.* 2013) and produce lipids such as eicosapentaenoic acid (EPA) (Renaud & Parry 1994) and triacylglycerol (TAG) (Pal *et al.* 2011). Therefore, in this work, *N. oculata*

was used to study the effect of low light (20 µmol of photons m^{-2} s^{-1}) (LL) and high light (200 µmol of photons m^{-2} s^{-1}) (HL) under 20°C and 25°C on the composition of photosynthetic apparatus and performance of photosynthesis as well as on growth.

Materials and method

Strain and culture conditions

Strain CS-179 of the unicellular marine green alga *Nannochloropsis oculata* (Eustigmatophyceae) was obtained from the CSIRO Australian National Algae Culture Collection, Hobart, Tasmania. For all experiments, cultures were grown in PHK medium (Rukminasari 2013) in controlled temperature (CT) rooms, using Erlenmeyer flasks bubbled with air at ~ 1 culture volume per minute. Batch cultures were incubated under a 16:8 h light/dark cycle, with an initial cell density of < 2×10^5 cell ml^{-1}, previously acclimated to their treatment condition for two weeks. Four experiments, each with triplicates 1L cultures at each condition were used; 20 µmol of photons m^{-2} s^{-1} was, on the basis of preliminary growth experiments, considered a low light treatment (LL) while 200 µmol of photons m^{-2} s^{-1} was considered a high light treatment (HL). Cultures at both light levels were illuminated with LED lights and grown at either 20°C or 25°C.

Growth parameters

Growth was followed by daily cell counts with a Neubauer hemocytometer under a microscope (Axio Scope.A1 Zeiss). The specific growth rate was calculated from the slope of log cell number vs time in the exponential phase of the cultures. Final biomass concentration per volume of cultures was also measured at the stationary phase of the cultures. This was done by filtering 10 ml of culture onto a previously dried and weighed glass microfiber filter (Whatman GF/C), rinsing with 10 ml of distilled

water to dissolve any remaining salts in the medium and drying for 24 hours at 100 ^{o}C. Once dried, the filter was weighed every 24 hours until a constant weight was achieved. The difference between the empty dried filter and the dried filter with biomass was used to calculate the biomass per volume of the culture at the sampling time.

Chlorophyll Analysis

For Chl *a* estimation, cells were harvested by centrifugation (10 min, 2600 g), resuspended in 96% ethanol and incubated for 24 h at 4^{o}C. The suspension was centrifuged again and pigment concentration in the supernatant was determined spectrophotometrically using a Cary-50 UV-V Spectrophotometer at 630, 647, 664 and 750 nm. Chlorophyll *a* concentration was then calculated using the equations of Jeffrey and Humphrey (1975).

Chl *a* (µg/ml) = $11.85(A_{664} - A_{750}) - 1.54(A_{647} - A_{750}) - 0.08(A_{630} - A_{750})$

Photosynthetic parameters

Composition of the photosynthetic apparatus and photosynthetic performance were analysed in the exponential phase of the cultures (day 3).

Chlorophyll fluorescence of PSII was measured to determine maximum quantum yield (F_v/F_m) and non-photochemical quenching (NPQ) using a pulse-amplitude modulated fluorometer (Phyto-PAM phytoplankton analyzer system, Heinz Walz, Effeltrich, Germany). Samples (3 ml) were taken from each culture and incubated in the dark for 15 min prior to measurements. For non-photochemical quenching (NPQ) measurements, samples were illuminated for 10 min with actinic light of 480 µmol

of photons m^{-2} s^{-1}. A saturating pulse was applied every 20 s until a stable maximum fluorescence yield under the light (Fm') was achieved. NPQ values were calculated using the Stern-Volmer equation:

$NPQ= (F_m/F_{m'}) – 1$

Flash fluorescence induction (FFI) curves were measured to determine the effective absorption cross-sectional area of PSII α-centers (σPSIIα) using a double-modulation fluorometer (Photon Systems Instrument, Brno, Czech Republic). Approximately $1×10^8$ cells were collected from the culture, resuspended in 3 ml of fresh PHK medium and incubated in the dark for 5 min prior to measurements. For measurement, a single 50 µs turn-over flash at a light intensity setting of 50% ($1x10^6$ µmol of photons m^{-2} s^{-1}) was used and the detector sensitivity set at 5%. For the σPSIIα calculation, the cross sectional area under the FFI curve was analysed according to Melis and Homann (1975) and Nedbal *et al.* (1999).

A Clark-type oxygen electrode (Hansatech, Norfolk, UK) was used to measure the dark respiration (Rd), photosynthesis vs irradiance (P vs I) curves, the number of photosynthetic units (PSU number) per cell and the PSU size in each treatment. All measurements were carried out at growth temperature conditions and between 20% and 50% of air equilibrium of O_2 concentration inside the O_2 electrode chamber. Approximately $1×10^8$ cells were harvested from the culture and resuspended in 2 ml of fresh PHK medium containing 2 mM of sodium bicarbonate and 40 mmol L^{-1} TRIZMA base pH 8.2 and placed into the O_2 electrode chamber. The R_d of the culture was measured after 5 minutes of dark incubation. After dark incubation, O_2 production rates were measured at increasing light intensities, each provided until rates reached a constant value, and these were used to build P *vs* I curves using the equations of Eilers and Peeters (1988a), calculated with the software Prism 6 (GraphPad Software, San Diego, CA, USA). From P *vs* I curves, maximum light-saturated photosynthesis (P_{max}), light harvesting efficiency (α) and light intensity required to achieve Pmax (I_k) were calculated as follow (Eilers & Peeters 1988b; Pierangelini *et al.* 2014):

$P= (I - Ic)/(a (I - Ic)2 + b (I - Ic) + c)$, where $P_{max} = 1/(b + 2 \sqrt{} ac)$, $\alpha = 1/c$ and $I_k = c/(b + 2 \sqrt{} ac)$

The oxygen evolution rate of the cells at the light intensity for growth was taken from the P *vs* I curve in order to calculate the oxygen production per cell under growth conditions and the net oxygen production per cell over 24 hours during the exponential phase of each treatment.

PSU number was measured using an Electro-Optics Xenon flash light (EG & G) as an external light source, flashing with a frequency of 35 Hertz and placed 10 cm away from the O_2 electrode chamber according to the method described in Quigg *et al.* (2006). In all experiments, oxygen evolution rate was measured after 3 minutes of incubation. PSU number and size were calculated according to the equation of Fisher *et al.* (1996) and Quigg *et al.* (2006): PSU number (PSU cell $^{-1}$) = (chl *a**N)/ (PSU size * 849X10^{1}); PSU size was calculated from the chlorophyll concentration of the sample and the oxygen evolution rates from one turnover saturating flash, expressed as mol chl *a* mol O_2^{-1} flash^{-1}.

Statistical analysis

Significant differences between treatments were tested by two way ANOVA (significance level $P<0.05$) and Tukey analysis as a posterior multiple comparison test (Significance level $P<0.05$) using the software Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

Growth parameters

Results showed a clear effect of light and temperature on *N. oculata* growth. High light stimulated growth rate and maximum cell density achieved in a temperature independent fashion (Figure 1, Table 1), but at low light, growth rate and maximum cell density were considerably decreased from 0.51 d^{-1} and 17.8 x 10^6 cell ml^{-1} at 25°C to 0.29 d^{-1} and 3.6 x 10^6 cell ml^{-1} at 20°C (Figure 1, Table 1). Final dry weight of cells in the culture was, however, unaffected by temperature at both LL and HL treatments,

though it was much higher in cultures grown at HL (Table 1). Biomass per cell revealed higher cell size in the LL-25°C treatment compared with the rest of the treatments (Table 1).

Table 1. Growth responses of *N. oculata* grown under LL (low light) and HL (high light) at 20°C and 25°C. Growth rate calculated during exponential phase, dry biomass per volume at stationary phase and biomass per cell number at stationary phase. n=3. Different letters above each value indicate statistically significant differences (Two Way ANOVA P>0.05).

	20°C		25°C	
	LL	HL	LL	HL
μ (d^{-1})	0.51 ± 0^{a}	0.92 ± 0.06^{b}	0.29 ± 0.03^{c}	0.83 ± 0.03^{b}
Dry Biomass (μg L^{-1})	88.33 ± 7.6^{a}	188.33 ± 36.8^{b}	60 ± 12.16^{a}	225.2 ± 49.8^{b}
Dry Biomass (mg (cell $10^{9})^{-1}$)	5.49 ± 1.4^{a}	6.55 ± 0.7^{a}	17 ± 2.6^{b}	10.56 ± 4.6^{a}

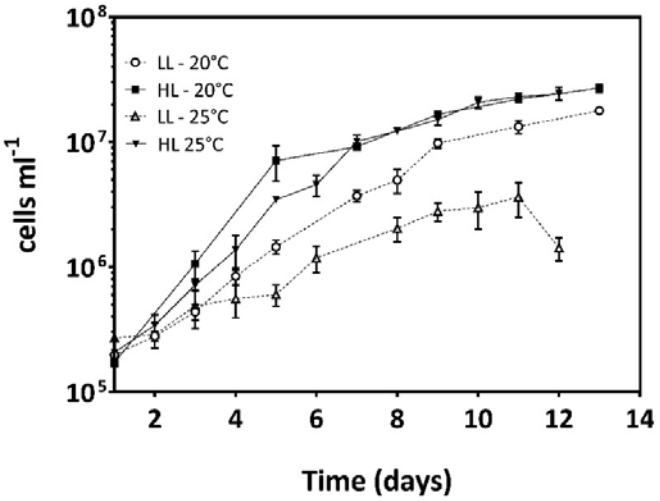

Figure 1. Growth curves of *N. oculata* grown under LL (low light) and HL (high light) at 20°C and 25°C. n=3.

Physiological analysis

Composition of the photosynthetic apparatus

Physiological changes of *N. oculata* cells were studied under different light and temperature conditions. Cells grown under LL increased their chl *a* per cell in both temperature treatments (Figure 2A). However, under 25°C, cells accumulated almost three times more chl *a* per cell than at 20°C (Figure 2A). In contrast, under HL cells decreased their chl *a* per cell but showed no statistically significant differences between 20 and 25°C treatments (Figure 2A). Furthermore cells increased their chl a at LL treatments by increasing their PSU number three-fold instead of increasing the size of the antenna (σPSIIα) (Figure 2C and E).

The size of PSU calculated from the equation of Fisher *et al.* (1996) showed no differences between treatments except for low light under 20°C, where cells show a decreased in PSU size (Figure 2F). The antenna size of photosystem II (σPSIIα) measured by FFI revealed no physiological differences between any treatments (Figure 2E).

Maximum quantum yield (F_v/F_m)

The photosynthetic efficiency of the cultures (F_v/F_m) remained stable between treatments at values ~0.6 (data not shown). This demonstrates that the photoacclimation strategy developed by *N. oculata* for all treatments is effective to maintain their quantum efficiency in all treatment conditions. In addition, NPQ analysis revealed that cells growing under HL at both 20 and 25°C increased their rates of energy dissipation (Figure 2D).

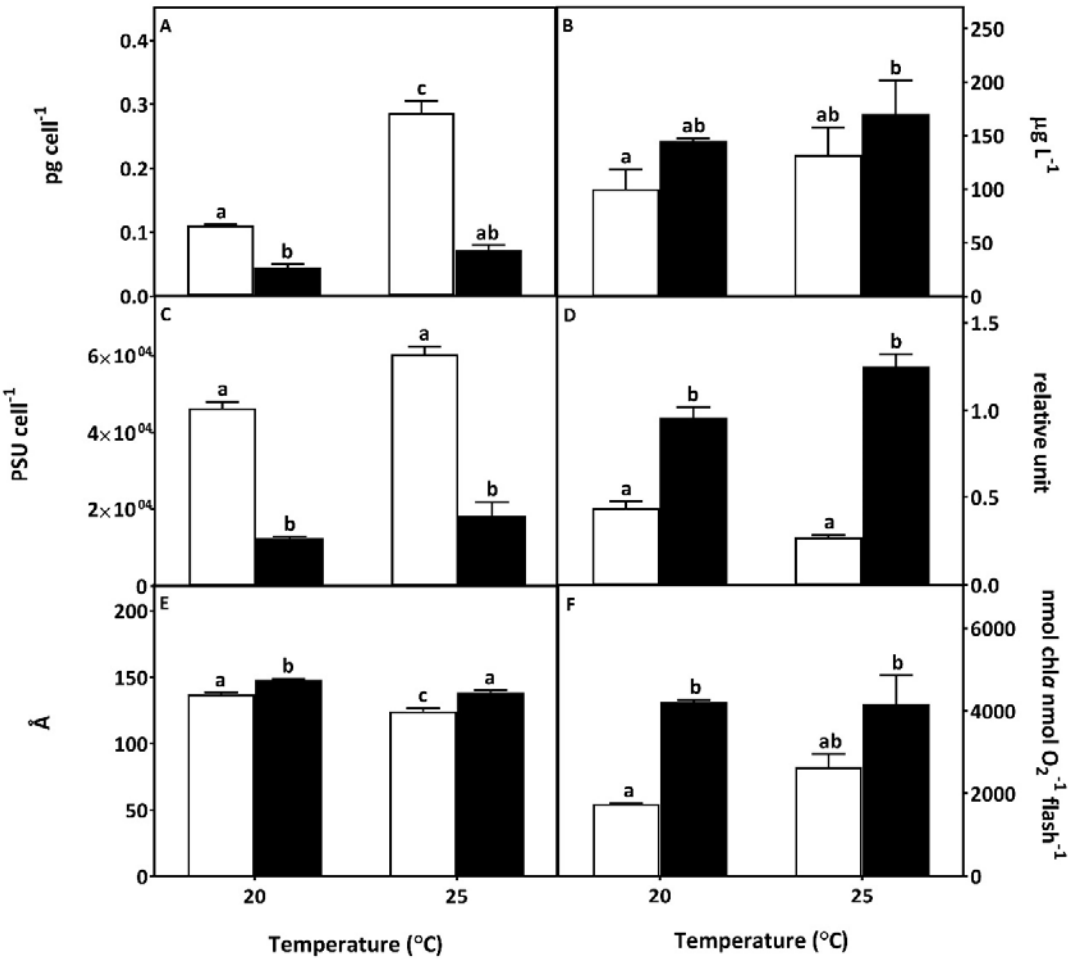

Figure 2. Photosynthetic apparatus composition of *N. oculata* measured during the exponential phase of growth (day 3), under 20°C and 25°C. White bars correspond to LL treatment and black bars to HL treatment. A) Chlorophyll *a* concentration (pg cell^{-1}), B) chlorophyll *a* concentration (mg L^{-1}), C) PSU number, D) NPQ , E) Antenna size, F) PSU size. n=3. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05).

P-I curve analysis

P vs I curves showed an increasing photosynthetic capacity (Pmax) in cells grown under LL at both 20 and 25°C, however greater capacity was achieved at 25°C compared with the LL-20°C treatment (Fig. 3), where cells almost doubled Pmax (t test: $P < 0.0001$) and their ability to harvest light (α) (t test: $P=0.0166$), though no statistically significant differences were observed in the light intensity required to achieve Pmax (Ik) (t test: $P = 0.19$). In the HL treatment, P vs I curves showed differences in Pmax between 20 and 25°C (t test: $P < 0.0001$), but no differences were observed either in α (t test: $P = 0.82$) or Ik (t test: $P = 0.062$) (Fig. 3).

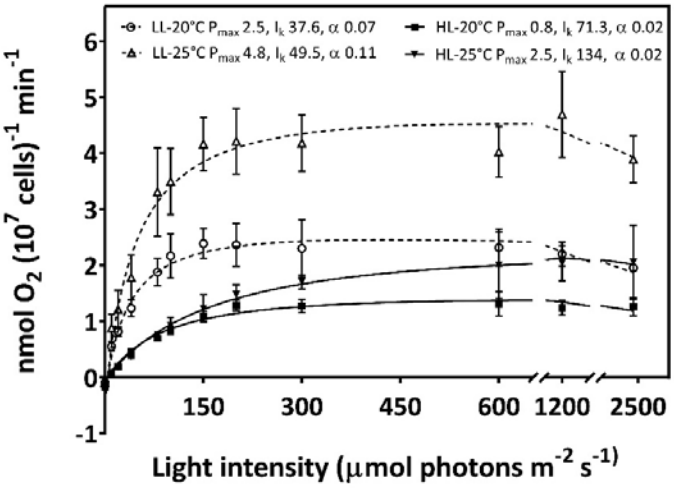

Figure 3. P *versus* I curve of *N. oculata* measured at exponential phase of the culture (day 3), grown under LL (low light) and HL (high light) at 20°C and 25°C. n=3.

Performance of photosynthesis under different growth conditions

Dark respiration (R_d) and oxygen production were measured under all four of the growth conditions. It was found that cells growing under LL had lower R_d at 20°C than at 25°C, while no differences were observed between 20 and 25°C when cells were grown under HL (Figure 4A). Conversely, when the oxygen evolution rate per cell was measured at the light intensity for growth in cells growing under

LL, no differences were found between temperature regimes, while cells grown under HL had a small difference in rates between 20 and 25°C (Figure 4B). The same trend was observed when the net oxygen production was calculated over 24 hours of the cultures (Figure 4C). This meant that oxygen consumption under a dark period of 8 hours had a minimal effect on the net oxygen production of the cultures over 24 hours.

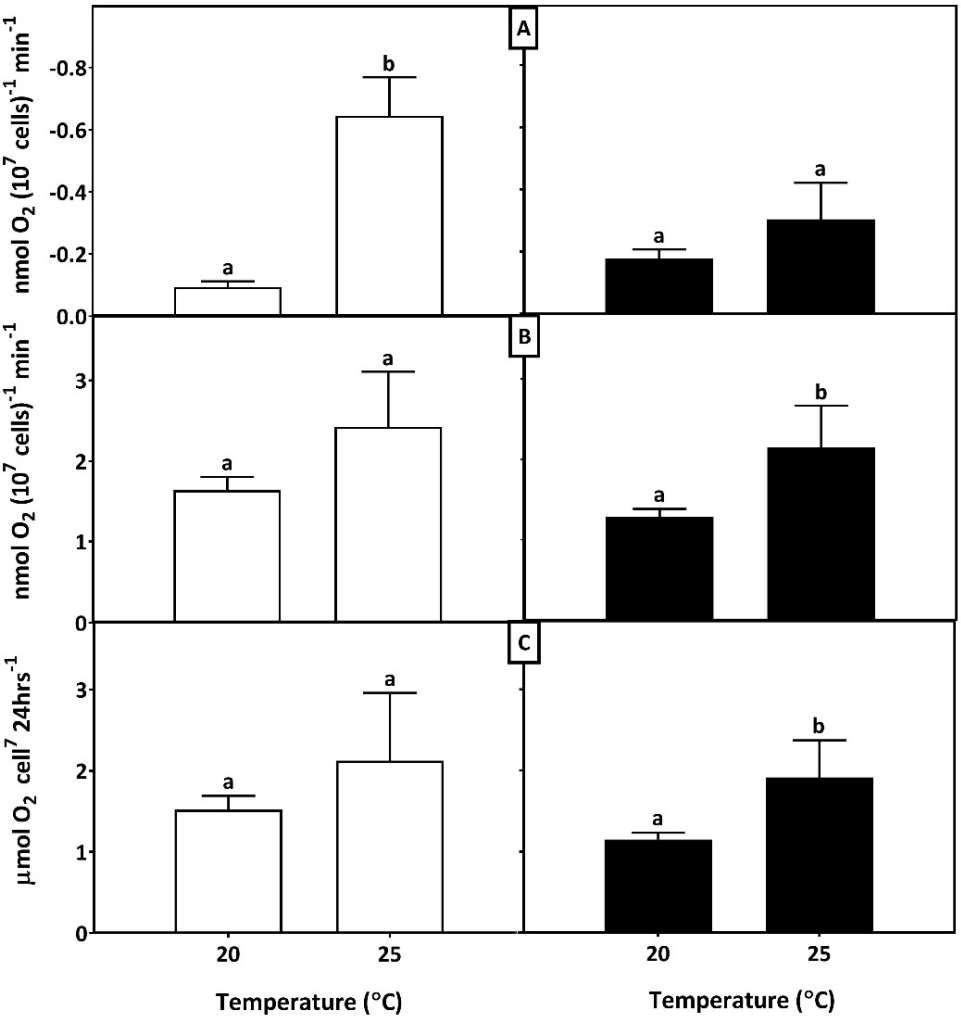

Figure 4. Photosynthetic performance of *N. oculata* measured under growth conditions during the exponential phase of the culture (day 3), grown under 20°C and 25°C. White bars correspond to LL treatment and black bars to HL treatment. A) Dark respiration, B) Oxygen production based on cell number, C) Net oxygen production over 24 h. n=3. Different letters above each bar indicate statistically significant differences (t test P>0.05).

Discussion

This research studied the photoacclimation strategy that *N. oculata* uses for growth under different light and temperature levels with the aim of applying this knowledge to the optimization of its potential for mass culture. Therefore, we studied the composition of the photosynthetic apparatus, performance of photosynthesis and growth under low and high light at different temperature levels.

Growth

In microalgae culture, when light is a limiting factor, growth is significantly affected as biomass concentration is increasing, and thus self-shading, increases (Vonshak *et al.* 1982; Vonshak *et al.* 2014). In this study, a significant effect of LL on growth rate, maximum cell density and biomass was observed, in agreement with results found by Sandnes *et al.* (2005) for *N. oceanica*. Other research also indicates that the effect of light on microalgae growth strongly depends on the temperature of the culture (Carvalho & Malcata 2003; Sandnes *et al.* 2005), in agreement with our results, where increasing temperature from 20°C to 25°C had a detrimental effect on growth rate and maximum cell density achieved at LL. However, at HL, our results reveal that temperature no longer had a negative effect on growth rate and maximum cell density, or on the biomass reached at stationary phase. Converti *et al.* (2009) reported an almost two-fold decrease in growth rate in *N. oculata* from 20°C to 25°C at 70 µmol photons m^{-2} s^{-1}. Therefore, temperature seems to start affecting the *N. oculata* growth rate at light intensities at least below 70 µmol photons m^{-2} s^{-1} (Converti *et al.* 2009).

Cells growing under LL at 25 °C presented the highest cell size of 17 mg $(cell^{9})^{-1}$, the effect of light and temperature on the size of microalgae cells have not been completely studied. Ojala (1993) observed higher cell volume in cultures grown under 21 °C compared with 16 °C treatments, under 30 µmol photons m^{-2} s^{-1}, which was correlated with a higher cell chlorophyll concentration under the 21 °C

condition. In our results, the highest chlorophyll a concentration per cell and photosynthetic capacity was observed under the LL-25 °C treatment, which seems to be beneficial for the size of the cells of this treatment.

At an industrial scale, biomass is one of the main parameters used to evaluate the success of a microalgae culture (Chisti 2007). Sandnes *et al.* (2005) determined the best treatments after calculating productivity in the photobioreactors based on daily biomass production. In our results, even though 25°C highly impacted the growth rate and maximum cell density achieved at LL, no significant differences in biomass were observed between these treatments at the stationary phase of the culture. No effect of temperature was also observed under HL treatments. From a mass culture point of view, our results indicate that the impact of light on the biomass during the exponential phase of the culture does not depend on the temperature. However, at LL, 25°C does appear to have an effect on cell number, even though biomass is not affected because cell size changes.

Composition of the photosynthetic apparatus

Adjusting the cellular chlorophyll concentration to environmental light conditions is a common photoacclimation strategy evolved by microalgae to optimise their light harvesting capacity (Sforza *et al.* 2012; González *et al.* 2013; He *et al.* 2015). Under light limitation, this strategy takes the form of either increasing the cross sectional area of photosystem II (increasing the antenna size serving each reaction centre) or increasing the number of photosynthetic units (PSU) (Melis *et al.* 1998; Quigg *et al.* 2006). In this study, we found that increasing chlorophyll *a* concentration in cells growing under LL under both temperature treatments is paralleled by at least a doubling of the PSU number per cell instead of increasing the size of the antenna as a photoacclimation strategy. Fisher *et al.* (1996) also observed an increased PSU number in *Nannochloropsis* sp. cells growing under LL (30 µmol photons m^{-2} s^{-1}) compared with a HL treatment of 650 µmol photons m^{-2} s^{-1}. Under these conditions, the effect

of light on chlorophyll content per cell was also observed to depend on the growth temperature of the culture; a higher temperature of 25°C is believed to increase the overall rate of metabolic process inside the cells, allowing them to accumulate almost three times more chlorophyll *a* per cell.

Meneghesso *et al.* (2016) studied the photoacclimation of photosytnthesis of *Nannochloropsis gaditana*, observing an increasing size of the antenna of the photosystem I and II under low light condition (10 µmol photons m^{-2} s^{-1}). These results show a different photoacclimation strategy employed by *N. gaditana* under light changes conditions compared with our results found in *N. oculata*, indicating that the photoacclimation strategy of *Nannochloropsis* species can be specie specific. However, it is important to indicate that *N. gaditana* has been recently relocated into the new species *Microchloropsis gaditana* based on DNA sequence analysis (Fawley *et al.* 2015).

A sharp decrease in chlorophyll and PSU number per cell was observed in cells growing under HL at both temperature treatments. This occurs in order to decrease the light absorption capacity in the photosynthetic apparatus as a photoacclimation strategy to avoid photodamage under HL exposure (He *et al.* 2015). This is also made clear by the non-photochemical quenching (NPQ) values observed, where cells at least doubled their rates of energy dissipation under the HL treatments. Low NPQ values are normally seen as advantageous in cultures with biotechnological application (Perin *et al.* 2015) as they reflect a higher effectiveness of light use. In our results, higher NPQ values in the HL treatments show that a substantial amount of energy is dissipated as heat, which has a negative effect on the light use efficiency, a critical point for microalgal mass culture (Simionato *et al.* 2013).

The combined effect of light and temperature on the composition of the photosynthetic apparatus is not yet described for many species of microalgae used in biotechnology. It is well known that decreasing light intensities increases the chlorophyll content in cells of many microalgae studied (Carvalho *et al.* 2009; Ferreira *et al.* 2016), including the genus *Nannochloropsis* (da Silva Ferreira & Sant'Anna 2016). Our results are in keeping with this, but further show that temperature did not affect the other parameters of the photosynthetic apparatus studied, a novel finding for *Nannochloropsis*.

Photosynthetic performance under growth conditions

Oxygen evolution rates allow us to appropriately evaluate photoacclimation strategies of microalgae (Vadiveloo *et al.* 2016). In this study, maximum photosynthetic capacity (P_{max}), light absorption efficiency (α) and light requirement for saturation of photosynthesis (I_k) were obtained from PI curves. Pulse amplitude modulated (PAM) was also used to study the performance of the photosynthesis through photosynthetic efficiency (F_v/F_m) measurements.

Is has been found that increasing light intensity impacts the photosynthetic electron transport rate in microalgae (Behrenfeld *et al.* 1998) as well as F_v/F_m, as was observed in *Chlorella* sp. where an increase from 40 to 200 µmol photons m^{-2} s^{-1} caused F_v/F_m values to drop dramatically from day 4 to day 12 of the culture (He *et al.* 2015). It has been also found that temperature can affect photosynthetic performance; for instance, a shift from 23 to 30°C clearly decreased rETR, α and I_K values in *Chlorella sorokiniana* and slightly decreased them in *Neochloris* sp. (Jazzar *et al.* 2016). Regarding the genus *Nannochloropsis*, the effect of variable light intensity on its photosynthetic features has been also studied (Kromkamp *et al.* 2009; Sforza *et al.* 2012). However, the combined effect of light and temperature on the performance of photosynthesis on *Nannochloropsis* has not yet been thoroughly investigated. In our results, it was found that *N. oculata* effectively acclimated to changing light conditions in order to optimise absorption of the light needed for photosynthesis, and this acclimation was also found to depend on the temperature of the culture. As a consequence of that, the photosynthetic efficiency (F_v/F_m) of the culture remained stable in all treatments.

In our results, the effect of light and temperature on the composition of the photosynthetic apparatus discussed above is in accord with the effect on the performance of photosynthesis observed in each treatment. The effect of increasing PSU number on the performance of photosynthesis in microalgae has been presented in a model by Richardson *et al.* (1983) built by the combined work of Prézelin and Sweeney (1979) and Prézelin (1981). When PSU numbers per cell increase as a response to light

limitation, all components of photosynthesis are enhanced as a consequence, therefore, the P_{max} based on cell number and α both increase and the I_k decreases under these conditions. Our results agree with this model, where cells growing under LL treatments increased their photosynthetic capacity at both temperature treatments. In addition to that, increased temperature was found to increase even more the photosynthetic capacity of the cells under light limitation.

From a mass culture point of view, a range of light between 20 and 200 µmol photons m^{-2} s^{-1} seems not to be negatively affecting the photosynthesis of the cells, because *N. oculata* can efficiently acclimate to both conditions.

Understanding photosynthesis as a predictor of growth

In photosynthetic organisms, light provides the energy that supports cellular metabolism and therefore, the higher the conversion of light into growth, the greater is the impact on mass microalgal culture (Simionato *et al.* 2013). Therefore, we were interested to determine whether the photosynthetic responses of the cells under each treatment explained their growth responses. Analysing the biomass, chl *a* per volume and oxygen evolution rate under all treatment conditions was required.

The relationship between photosynthesis and growth has been investigated in several microalgal species (Sforza *et al.* 2012; He *et al.* 2015). Abu-Ghosh *et al.* (2015), observed parallel changes between cell number, dry weight and the P *vs* I curve at day 3 of *Dunaliella salina* cultures. This indicates a direct relation between photosynthesis and growth responses. However, measuring the photosynthetic rate under growth conditions, instead of only observing the photosynthetic capacity of the culture, allows us to better decide if photosynthesis in situ explains growth behaviour. It is important when making these comparisons that light absorption and self-shading by cultures is the same across treatments (Huesemann *et al.* 2013). In our results, no differences were found either in biomass and chl *a* per unit

culture volume between 20 and 25°C under the LL treatments or under the HL treatments, which suggests that the same number of photons are absorbed between those temperature treatments and so no difference in shading effect is observed. Comparing photosynthesis and growth, we observed that the effect of temperature either between LL treatments or HL treatments on the net photosynthesis over 24 h in the cultures did not correspond to the same effect of temperature on the growth rate and maximum cell density achieved by those cultures. This supports the idea that photosynthetic responses in situ do not reflect the fact that growth rate and maximum cell density are affected by the temperature.

Conclusions

Light intensity had a significant effect on biomass, growth rate, and maximum cell density of *N. oculata*. Temperature had significant effects on growth rate during exponential phase and maximum cell density achieved, but only when cells were grown under light limitation (20 µmol photons m^{-2} s^{-1}). However, this effect was not observed in the biomass obtained in the stationary phase of the cultures.

N. oculata adapts a photosynthetic acclimation strategy dependent on the light intensity to which cells are exposed, increasing their PSU number per cell to increase the light absorption efficiency and so their photosynthetic capacity under light limitation. Temperature does not affect PSU number per cell under either LL or HL treatments. However, their photosynthetic capacity does change with temperature changes.

Oxygen evolution rates measured under growth conditions demonstrate that, under the light and temperature treatments studied, changes in net photosynthesis do not explain the effect of light or temperature on growth behaviour in *N. oculata*.

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CHAPTER 4: Effect of pH buffering *Nannochloropsis oculata* cultures and culture density on light utilization for photosynthesis and growth

This chapter will be submitted to *Journal of Applied Phycology*

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ABSTRACT

It is known that light is one of the most important factors affecting the growth of microalgal cultures, but light does not always have a straightforward effect as its utilization depends on other environmental factors such as temperature or nutrient availability. Little is known about how the effect of light on photosynthesis is influenced by pH buffering the microalgal culture and by the biomass density of the culture, both being factors that have important effects on the photosynthetic and growth responses of microalgae. A total of 16 experiments were carried out under a range of light intensity from 20 to 300 µmol photons m^{-2} s^{-1}, with cultures inoculated under combinations of low cell density (LD) or high cell density (HD) and in buffered (pH 7.4) or non-buffered (pH 6.9) culture medium. Results indicate that inoculation at low cell density and with buffering increased the growth rate of the cultures when cells were grown under higher light intensities, but not under low light. At day 3 of the culture (mid-exponential phase), the highest biomass of 0.9 g L^{-1} was obtained when cells were grown under pH 7.4 (buffered condition) and high light. The effects of light on cellular chlorophyll *a* concentration, photosynthetic unit (PSU) number and P_{max} were clearly observed at pH 7.4 in both LD and HD cultures, but not at pH 6.9. Results indicate that the photoacclimation strategy of this species

under different light conditions depended on the pH of the culture, which may explain why greater growth responses were found in buffered cultures. On the other hand, a comparison of growth and biomass between LD and HD cultures reveals that cultures inoculated at HD are more affected by self-shading. These results provide important information about how light utilization for photosynthesis and growth is influenced by pH buffering and the cell density of the culture, crucial information for optimization of microalgal mass cultures.

Introduction

For the last 70 years, microalgae have been a focus of attention for industrial scale applications (Tamiya 1957) due to their ability to accumulate a range of macromolecules of biotechnological interest (Singh *et al.* 2005; Borowitzka 2013). However, biotechnology still faces problems of maximising the productivity of the culture (Qiang & Richmond 1996; Hannon *et al.* 2010).

There are several factors known to affect microalgal growth, which have been well studied. Optimum temperature and nutrient concentration for algal cultures have been shown to increase the growth rate (Converti *et al.* 2009; Mackey *et al.* 2013). The effect of light on microalgal growth has also been studied in a wide range of microalgal species (Richardson *et al.* 1983; Richmond *et al.* 2003; Carvalho *et al.* 2011), and such studies have shown how important optimal light availability is to the cells in order to maximize the growth yield of the culture. For instance, in studies on *Nannochloropsis salina,* a four-fold increase in biomass of cultures was obtained by changing light intensity conditions (Huesemann *et al.* 2013).

Photosynthesis is the fundamental mechanism that converts solar energy into chemical energy, and is thus the major driver of microalgae growth. However, it is estimated that more than 90% of the photons absorbed by the antenna complexes of both PSII and PSI are dissipated as heat or fluorescence and therefore the photosynthetic efficiency of microalgae sees around 6% of the incident

light converted to chemical energy (Blankenship *et al.* 2011), and this has an important effect on growth. This emphasises that understanding algal photosynthesis is essential to understand and optimise the growth responses of microalgal cultures.

It is known that the impact of light on microalgae growth is not straightforward because the use of light depends on other factors such as the temperature of the culture (Sandnes *et al.* 2005). What has not been completely studied is how the use of light for photosynthesis and growth is affected by the cell density of the culture and buffering the culture, factors that are known to have important effects on photosynthetic rate and growth.

The objective of the research described here was to investigate the effects of buffering the media and culture density on the photosynthesis and growth of *N. oculata*, a species commonly utilised in algal mass culture for lipid production, and how these factors influence light utilization for photosynthesis and growth.

Effect of initial cell density on the photosynthesis and growth of the culture

The distribution of the light within microalgal cultures is clearly affected by the cell density of the culture, which in turn has an important impact on the growth of the culture (Qiang & Richmond 1996; Imaizumi *et al.* 2014). For instance, Huang *et al.* (2016) compared the biomass concentration and the chlorophyll content per dry weight obtained between four semi-continues cultures of *Chlorella vulgaris* growing at different cell densities in photobioreactors (PRBs) of 500 ml. Periodic dilution to maintain the biomass concentration at 1.5 g L^{-1} by harvesting 30% of the culture daily resulted in a total accumulative harvest of 3 g L^{-1} on day 7, which was 30% higher in the final biomass yield than in the undiluted control culture. At the same time, a decrease in chlorophyll per dry weight from around 20 mg g^{-1} to less than 10 mg g^{-1} was observed in the diluted culture, suggesting that the light conditions for growth in the PBRs improved when the cell density in the culture was lower. In this regard, the

initial cell density of the culture determines how dense the culture will be in subsequent days of growth and how available the light will be for cells growing in deeper layers of the culture. Therefore, comparing growth and photosynthetic responses between a dense culture and a diluted culture of *N. oculata* allows us to understand how light utilization by the cells is affected by the cell density of the culture.

Effect of buffering the culture on the photosynthesis and growth of the culture when CO_2 is added

As photosynthetic organisms, most microalgae use light as an energy source and CO_2 as the carbon source for photosynthesis respectively (Blankenship 2014).

When CO_2 is bubbled through microalgae cultures, the CO_2 forms carbonic acid, which would potentially cause the pH to drop, and conversely CO_2 use in photosynthesis runs counter to this, causing pH to rise, so buffering the culture has an important effect on the overall pH of the culture.

The pH of the culture impacts upon photosynthesis by direct effects on cellular processes and also by affecting the availability of the different inorganic carbon species in the medium. Thus at 20°C and 35‰ salinity at 6.5 pH the C_i proportion is ~80% HCO_3^-, less than 1% CO_3^{2-} and ~20% CO_2, while at 8.2 pH the C_i proportion changes to ~90% HCO_3^-, ~9% CO_3^{2-} and less than 1% CO_2 (Beer *et al.* 2014). Considering that *N. oculata* is normally grown at pH 8.2, studying the effect of light on the photosynthesis and growth responses under changes in pH are crucial to increasing our understanding of how to optimize microalgae mass culture.

In the research described here, photosynthetic performance and growth responses of *N. oculata* were studied under a range of light intensities from 20 to 300 µmol photons m^{-2} s^{-1} in low and high cell density cultures and under buffered and non-buffered conditions.

Materials and Methods

Strains and culture conditions

Strain CS-179 of *N. oculata* (Eustigmatophyceae) was obtained from the CSIRO Australian National Algae Culture Collection, Hobart, Tasmania. All experiments were carried out in a multi-cultivator (MC1000) from Photo Systems Instruments, Drásov, Czech Republic. Cultures were bubbled with 2% CO_2, and grown in PHK medium (Rukminasari 2013) under continuous light and 25°C. Sixteen experiments in triplicate were considered in the experimental design (Figure 1); eight cultures were buffered with 2g L^{-1} of TRIZMA and eight were non-buffered, while eight experiments were initiated with a low inoculum size (LD) of $1x10^6$ cells ml^{-1} (equivalent to around 0.01 µg of chl *a* ml^{-1}) and the other eight experiments were initiated with a high inoculum size (HD) of 10 x 10^6 cells ml^{-1} equivalent to 2 µg ml^{-1} of chl *a*. LD and HD experiments were studied under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1}. The experimental design is shown in Fig 1. All cultures were acclimated for 10 days under their corresponding light, pH, and size of their initial inoculum. After acclimation, cultures were initiated with their corresponding experimental conditions and samples were taken daily for growth and at exponential phase for physiological analysis.

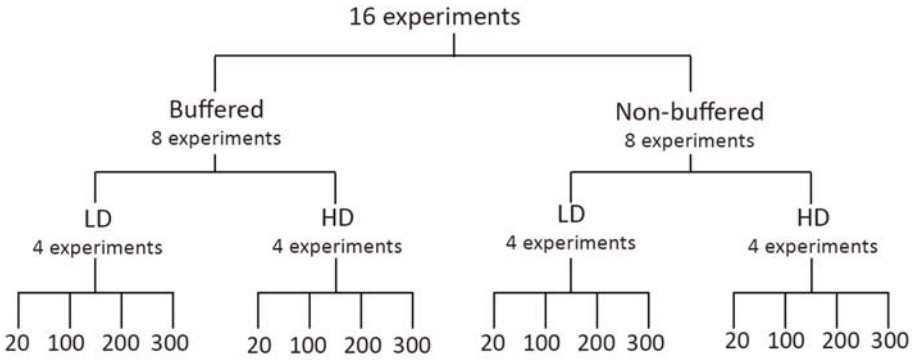

Figure 1. Experimental design: Cultures were grown in triplicate under buffered (2g L^{-1} of TRIZMA) and non-buffered conditions. Within this, cultures were run with either low inoculum size (LD) or high inoculum size (HD) treatments grown under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1}.

Growth

Growth curves from each experiment were constructed using cell counts. Cultures were sampled every day and counted with a Neubauer hemocytometer under a microscope (Axio Scope.A1 Zeiss). Sampling was stopped after 3 days, when cultures had passed mid-exponential phase. We were unable to grow the culture until stationary phase because of constraints of sampling volume for physiological measurements. The specific growth rate from each growth curve was calculated from changes in cell density during the exponential phase of the culture using the equation:

$$(\ln X_1 - \ln X_0)\,(t_1 - t_0)^{-1}$$

Where X_1 and X_0 are the cell concentration, measured at time t_1 and the initial time t_0 respectively.

Chlorophyll analysis

The timing of mid-exponential phase for physiological analysis was considered as day 2 for HD cultures and day 3 for LD cultures (see Figure 2). A sample of 1 ml of culture was harvested by centrifugation (10 min, 2600 g) at 4^{o}C and resuspended in 1 ml of DMSO. The suspension was heated at 70^{o}C for 3 minutes and centrifuged again. Chl *a* concentration in the supernatant was measured spectrophotometrically using a Cary-50 UV-V Spectrophotometer at 665 nm according to the equation (Seely *et al.* 1972)

$$\text{Chl}a\ (\mu g\ ml^{-1}) = OD_{665} * 13.7\ \mu g\ ml^{-1}.$$

Photosynthetic parameters

Composition of the photosynthetic apparatus and photosynthetic performance were analysed during the exponential phase of the culture (day 2 for HD cultures and day 3 for LD cultures).

A pulse-amplitude modulated phytoplankton analyzer (Phyto-PAM, Heinz Walz, Effeltrich, Germany) was used to measure the chlorophyll fluorescence of PSII in order to determine the maximum quantum yield (F_v/F_m) and non-photochemical quenching (NPQ). A sample of 3 ml was taken from each culture and incubated in the dark for 15 min prior to measurements. For NPQ measurement, an actinic light level of 480 µmol photons m^{-2} s^{-1} was provided to the sample and a saturating pulse applied every 20 sec for 300 sec, which was sufficient to allow the fluorescence yield (F_m') to plateau. NPQ was then calculated from the Stern-Volmer equation as follows:

$$NPQ = (F_m/F_m') - 1$$

Flash fluorescence induction (FFI) curves were measured, using a double-modulation fluorometer (Photon Systems Instrument, Brno, Czech Republic), in order to determine the effective absorption cross-sectional area of PSII α-centers (σPSIIα), PSII β-centers (σPSIIβ) and the antenna connectivity of PSIIα (J_{con}PSIIα). 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 measurements. For measurement, a single 50 µs turn-over flash at a light intensity setting of 50% ($1x10^6$ µmol photons m^{-2} s^{-1}) was used. For the calculation of all parameters, the area under the FFI curve was analysed according to Melis and Homann (1975) and Nedbal *et al.* (1999).

Dark respiration (R_d), maximum light-saturated photosynthesis (P_{max}) and photosynthetic unit (PSU) number were measured using a Clark-type oxygen electrode (Hansatech, Norfolk, UK). All measurements were carried out at 25°C and between 20% and 50% of air equilibrium of O_2 concentration inside the O_2 electrode chamber. Approximately 1×10^8 cells were harvested from the culture and resuspended in 2 ml of fresh PHK medium containing 2 mM of sodium bicarbonate and 40

mmol L^{-1} TRIZMA base pH 8.2 and finally placed into the O_2 electrode chamber for oxygen evolution measurements. R_d was first measured after 5 minutes of dark incubation. After that, the light-saturated O_2 production rate achieved by the culture was measured by providing the saturating level of light previously obtained from a rapid light curve measured with the PAM. PSU number was measured by determining the oxygen production rate using an Electro-Optics Xenon flash light (EG&G) as an external light source flashing with a frequency of 35 Hertz and placed 10 cm away from the O_2 electrode chamber. PSU number and size were calculated according to Fisher *et al.* (1996) and Quigg *et al.* (2006) using the equation: PSU number (PSU cell $^{-1}$) = (chl *a** (6.02 * 10^{23}))/ (PSU size * 849 X 10^{12}); PSU size was calculated from the chlorophyll concentration of the sample and the gross oxygen evolution rates of one turnover of the saturating flash, expressed as mol chl *a* mol O_2^{-1} $flash^{-1}$.

pH and carbon estimation

pH and temperature of the sample were measured using a Hach PH31 pH-meter. The salinity was also measured by using a RHS-10ATC refractometer. Finally, the dissolved inorganic carbon (DIC) in the culture was measured after conversion of DIC to CO_2 by acidification with 1 N HCl and analysis of the CO_2 (g) by the LI-840A CO_2/H_2O gas analyser (LI-COR). DIC was calculated a standard curve based on a standard curve from 0 - 6 mM $NaHCO_3$. pCO_2 in the cultures was calculated from the calculated DIC, pH, salinity and temperature using an aquatic acid-base modelling environment (AquaEnv) package in R.

Statistical analysis

Significant differences between treatments were tested by two-way ANOVA (significance level $P<0.05$) and Tukeys analysis as a posterior multiple comparison test (Significance level $P<0.05$) using the software Prism 6 (GraphPad Software, San Diego, CA, USA).

Results

In this study, the effects of pH and inoculum size on the photo physiology and growth of *N. oculata* were studied under increasing light intensities from 20 to 300 µmol photons m^{-2} s^{-1}. The characteristics of the photosynthetic apparatus, photosynthetic rates and growth parameters were measured for all treatments.

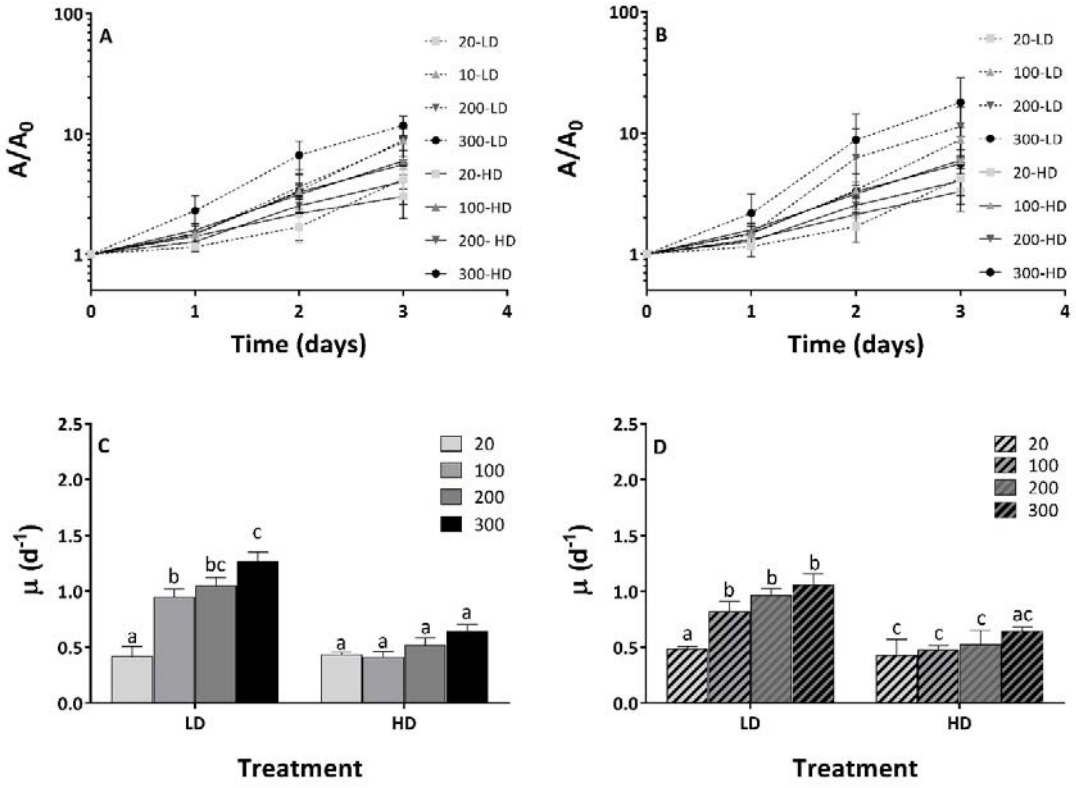

Figure 2. Growth responses of *N. oculata* grown under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). A) Growth curve of cultures buffered with TRIZMA, B) Growth curve of cultures without buffer, C) Growth rate of buffered cultures, D) Growth rate of non-buffered cultures. n=3. Different letters above each bar indicate statistically significant differences (Tukey P=0.05).

Growth

Comparison between LD and HD treatments

Clear differences in growth were observed between LD and HD treatments grown under both buffered and non-buffered conditions. Analysis of the growth rate in both buffered and non-buffered experiments showed that low cell density cultures had higher growth rates compared with high cell density treatments, except for the 20 µmol photons m^{-2} s^{-1} treatment (Figures 2A, B, C and D).

Comparison between buffered and non-buffered treatments

In LD treatments, higher growth rates were found in buffered cultures under 100, 200 and 300 µmol photons m^{-2} s^{-1} than in the corresponding unbuffered treatments. At 20 µmol photons m^{-2} s^{-1}, however, no effect on growth rate of buffering the culture was observed. In the HD treatments, no effect of buffering on the growth rate of any of the cultures was observed (Figure 2).

Increasing biomass during the exponential phase of the culture with increasing light intensities was observed in all treatments. An effect of buffering the culture on biomass accumulation was observed in both LD and HD treatments when cells were grown under 200 and 300 µmol photons m^{-2} s^{-1} (Figure 3A and B). However, under 20 and 100 µmol photons m^{-2} s^{-1} no differences between buffered and non-buffered cultures were observed. An interaction between light treatments and inoculum size treatments was observed when the biomass was analysed (p = 0.0003). Greater differences were observed between LD and HD treatments under buffered conditions, except for the 20 µmol photons m^{-2} s^{-1} treatment, where a larger difference was observed under the non-buffered condition (Figures 3A and B).

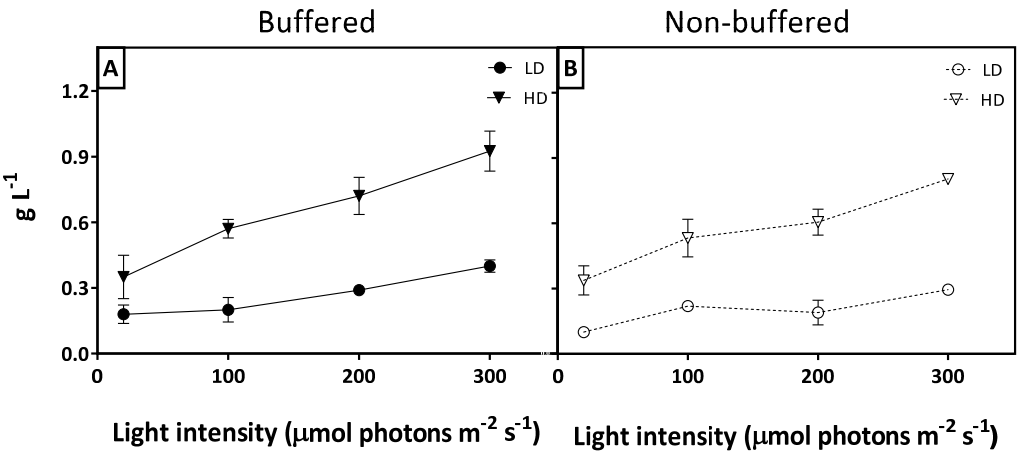

Figure 3. Dry biomass measured at Day 3 of exponential phase of *N. oculata* cultures grown under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). A) Cultures buffered with TRIZMA and B) Cultures treated without buffer.

Photosynthetic apparatus

Chl a

In LD-buffered treatments, cells grown under 20 µmol photons m^{-2} s^{-1} almost doubled their chlorophyll concentration compared with the rest of the LD-buffered treatments. From 100 µmol photons m^{-2} s^{-1} upwards though the cellular chlorophyll concentration remained stable with increasing light intensities. In the HD-buffered treatments, decreasing chl *a* per cell with increasing light intensity was observed (Figure 4A). In unbuffered cultures, the effect of increasing light intensities on cellular chlorophyll concentration was clearly less marked under both LD and HD conditions (Figure 4B). No differences were observed between 200 and 300 µmol photons m^{-2} s^{-1} under both LD (p>0.99) and HD (p>0.99) treatments.

PSU number

The effect of light on PSU number is clear in all buffered treatments; cells growing under 20 µmol photons m^{-2} s^{-1} showed the highest PSU number per cell under both LD and HD treatments, however higher values were observed in the LD than in the HD cultures (p=0.0005) (Figure 4C). From 100 µmol photons m^{-2} s^{-1} upwards, the PSU number slightly decreased with increasing light intensity in both LD and HD treatments (Figure 4C). Not buffering the cultures resulted in no observable effect of light on PSU number per cell in both LD and HD treatments (Figure 4D).

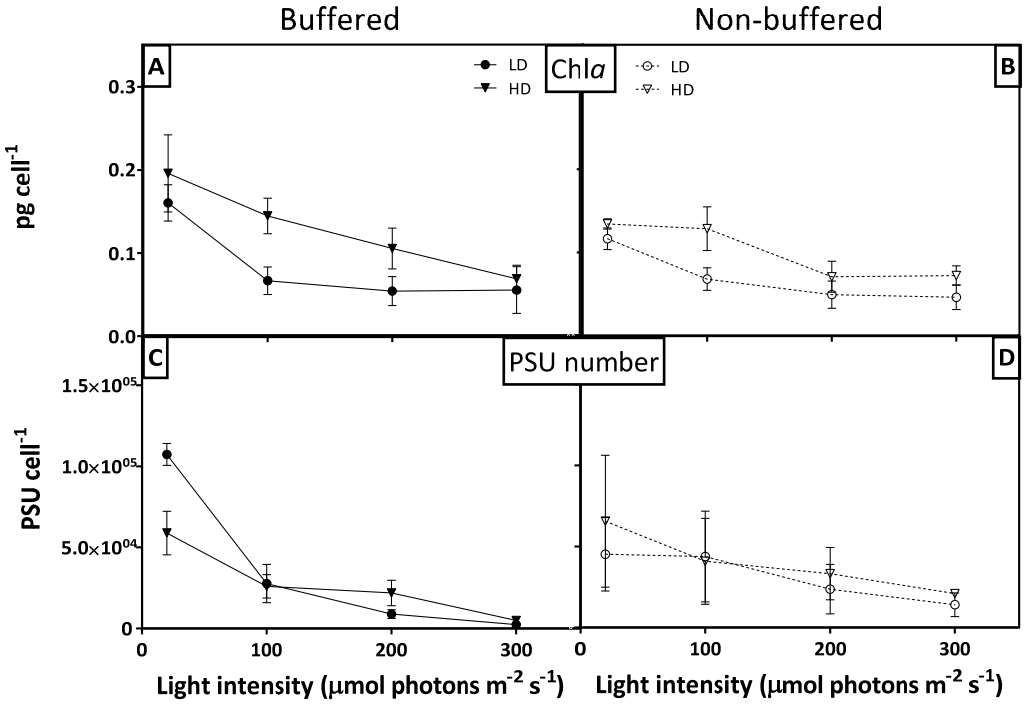

Figure 4. Composition of the photosynthetic apparatus of *N. oculata* grown under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). A and C) Cultures buffered with TRIZMA, B and D) Cultures treated without buffer. Chl *a* per cell (A and B) and PSU number per cell (C and D) were measured during exponential phase (Day 3).

Antenna features

The size of the antenna measured by FFI in all treatments revealed that σPSIIα was around three times higher than σPSIIβ in *N. oculata* (Figure 5A-D). Analysing the effect of light on σPSIIα in all buffered treatments, no statistically significant differences were observed (p = 0.4). In the non-buffered treatments, no differences in the σPSIIα size were observed in the HD treatment (p = 0.47), while a slight decrease was observed from 100 to 300 µmol photons m^{-2} s^{-1} in the LD treatments (Figure 5B) (p = 0.01). The J_{con}PSIIα values decreased with increasing light intensities in all buffered treatments, however a more marked effect was observed in the HD treatment (p = 0.0004) (Figure 5E). For the non-buffered treatment, no effect of light (p = 0.38) or cell density (p = 0.99) was observed in the J_{con}PSIIα of the cultures (Figure 5F).

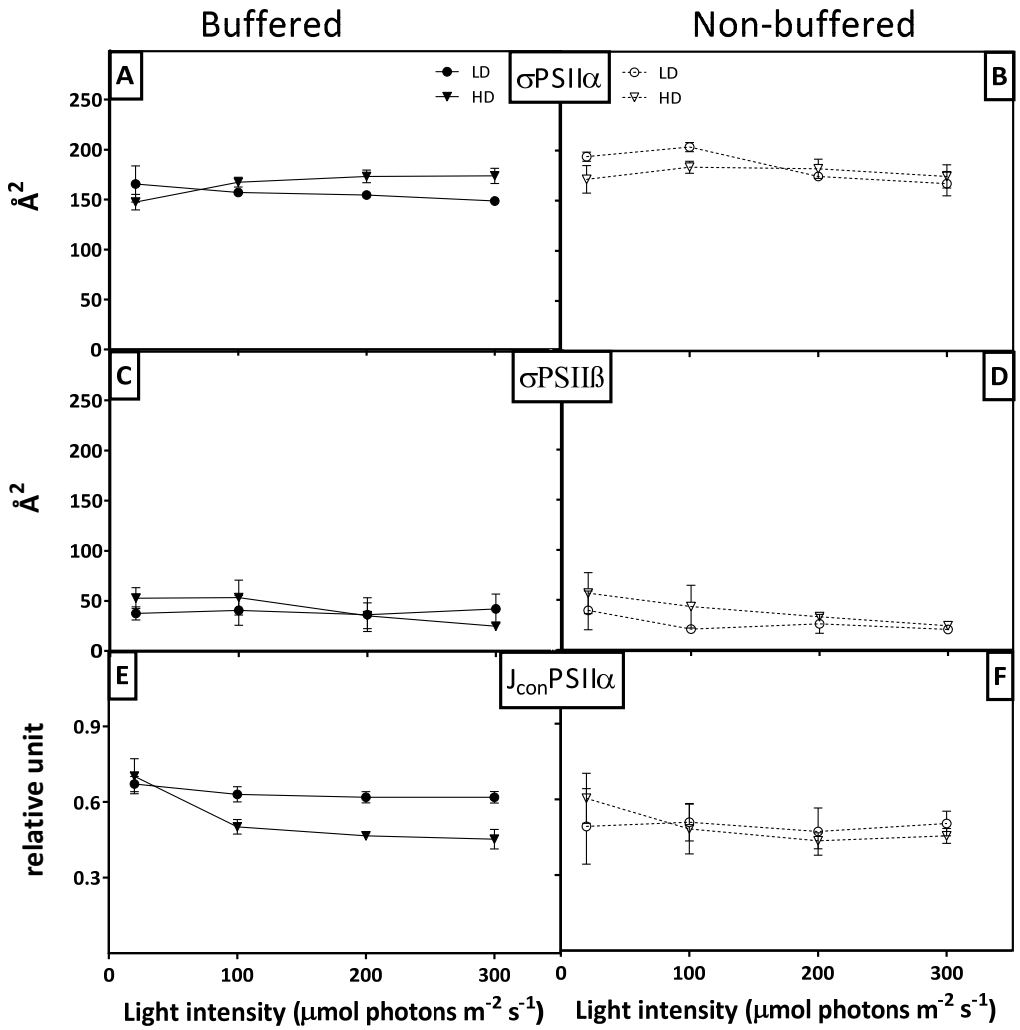

Figure 5. Composition of the photosynthetic apparatus of *N. oculata* grown under 20,100,200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). A, C and E) Cultures buffered with TRIZMA. B, D and F) Cultures treated without buffer. A and B) Cross sectional area of PSII *α*-centers (σPSIIα), C and D) Cross sectional area of PSII ß-centers (σPSIIß) and E and F) Antenna connectivity of the PSII α-centers (J_{con}PSIIα), measured at exponential phase of the culture (Day 3).

NPQ

In all treatments, NPQ was activated with increasing light intensities (Figure 6). In the buffered experiments, slight differences were observed between LD and HD cultures at 300 µmol photons m^{-2} s^{-1} (p = 0.01), while for the non-buffered treatment large differences were found between LD and HD cultures at 200 (p < 0.0001) and 300 µmol photons m^{-2} s^{-1} (p < 0.0001). Comparing between buffered and non-buffered conditions in the HD cultures, no statistically significant differences were observed at any light intensity. However, big differences appeared between LD-buffered and LD-non-buffered culture under 200 (p = 0.0002) and 300 µmol photons m^{-2} s^{-1} (p < 0.0001) (Figure 6).

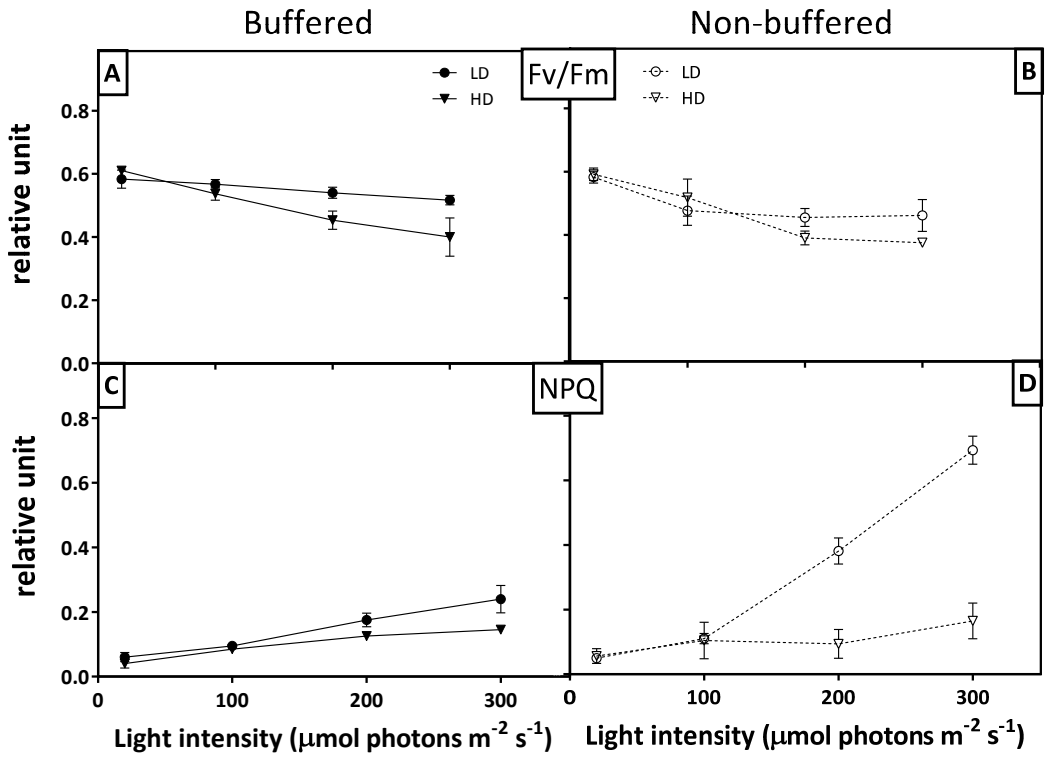

Figure 6. Photosynthetic responses of *N. oculata* grown under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). A and C) Cultures buffered with TRIZMA, B and D) Cultures treated without buffer. Photosynthetic efficiency (Fv/Fm) (A and B) and Non-photochemical quenching (NPQ) (C and D) were measured at exponential day of the culture (Day 3).

Photosynthetic Performance

Fv/Fm

The highest Fv/Fm of 0.6 was observed in all treatments at 20 µmol photons m^{-2} s^{-1} (Figure 6), but this decreased with increasing light intensity in all treatments. However, a smaller effect of increasing light intensity on Fv/Fm was found under both LD-buffered and LD-non-buffered treatments compared with the HD treatments (Figure 6).

Oxygen exchange

The dark respiration (R_d) and oxygen evolution rates were measured during exponential phase for all treatments (Figure 7). The analysis of R_d revealed no effect of light (p = 0.1) or inoculum size (p = 0.34) on R_d in buffered cultures as well as in non-buffered cultures (p = 0.5 and p = 0.94 respectively). The P_{max} results showed the highest values at 20 µmol photons m^{-2} s^{-1} under both LD and HD buffered treatments indicating that under these conditions cells increased their photosynthetic capacity. In both LD and HD buffered treatments, slightly deceasing P_{max} values were observed from 100 to 300 µmol photons m^{-2} s^{-1}. Unbuffered cultures showed no significant differences in P_{max} between any of the treatments studied (p = 0.08).

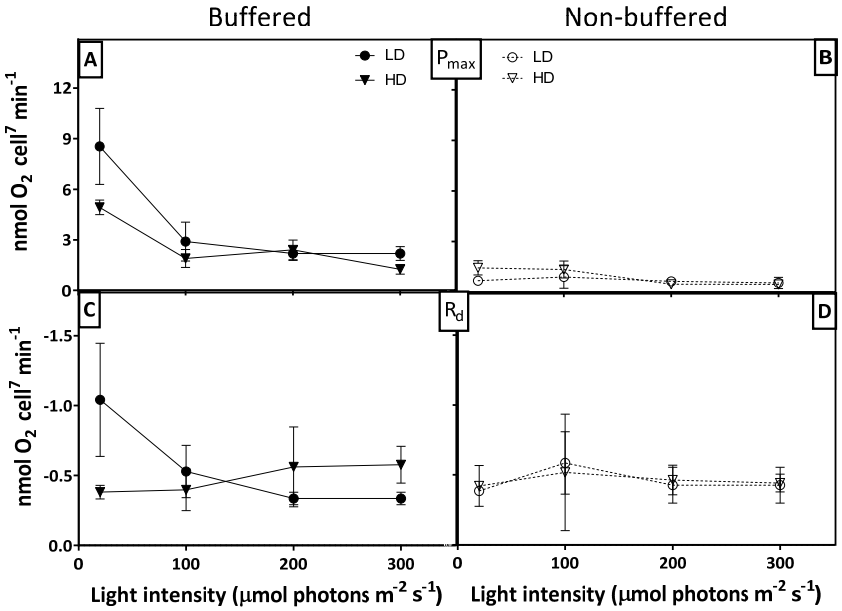

Figure 7. Performance of the photosynthesis of *N.oculata* grown under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). A and C) Cultures buffered with TRIZMA, B and D) Cultures treated without buffer. Maximum photosynthetic rate (P_{max}) based on cell number (A and B) and dark respiration of the culture (R_d) (C and D) were measured at exponential phase of the culture (Day 3).

Chemical analysis of the medium

The composition of the carbonate system in the medium in all treatments is summarised in Table 1. No effect of light intensity on the pH of the medium was found in any of the treatments (p = 0.83), while not buffering the cultures saw the pH drop from around 7.4 to 6.9 in all light and cell density conditions (p < 0.0001). The level of CO_2 remained stable in all light treatments (p = 0.9) as well as between buffered and non-buffered cultures (p = 0.17). Instead, a clear effect of not buffering the culture was observed in the bicarbonate concentration in the medium (p < 0.0001), while no effect of light on the bicarbonate concentration was found in any of the treatments (p = 0.72). Nutrient analysis (data not shown) showed that nitrate reminded stable in all treatments and only in the HD-buffered treatment did phosphate levels eventually reach potentially limiting concentrations.

Table 1. The carbonate system and pH of the medium at day 3 of *N. oculata* cultures grown with 2% CO_2 in the gas stream under 20, 100, 200 and 300 µmol photons m^{-2} s^{-1} at low inoculum size (LD) and high inoculum size (HD). Upper values correspond to cultures buffered with TRIZMA and lower values correspond to non-buffered cultures.

	Buffered							
	LD				**HD**			
	20	**100**	**200**	**300**	**20**	**100**	**200**	**300**
pH	7.5 ± 0.1[a]	7.4 ± 0.1[a]	7.4 ± 0.1[a]	7.4 ± 0.1[a]	7.4 ± 0[a]	7.4 ± 0[a]	7.4 ± 0.1[a]	7.5 ± 0.1[a]
DIC (mmol L^{-1})	10.7 ± 3.3[a]	12.0 ± 4[a]	12.2 ± 4.3[a]	12.5 ± 4.2[a]	12.8 ± 4.6[a]	13.9 ± 5.2[a]	14.4 ± 5.1[a]	15.4 ± 6[a]
[CO_2] (mmol L^{-1})	0.2 ± 0[a]	0.4 ± 0[a]	0.4 ± 0.1[a]	0.3 ± 0.1[a]	0.5 ± 0.1[a]	0.4 ± 0.1[a]	0.4 ± 0.2[a]	0.4 ± 0.2[a]
[HCO_3^-] (mmol L^{-1})	10.1 ± 3.1[a]	11.4 ± 3.8[a]	12.5 ± 3.2[a]	12.5 ± 3.2[a]	11.9 ± 4.6[a]	12.9 ± 5.4[a]	13.4 ± 5.3[a]	14.6 ± 5.7[a]
[CO_3^{2-}] (mmol L^{-1})	0.3 ± 0.2[a]	0.2 ± 0.1[a]	0.3 ± 0[a]	0.3 ± 0[a]	0.3 ± 0.1[a]	0.3 ± 0.2[a]	0.3 ± 0.1[a]	0.4 ± 0.1[a]
	Non-Buffered							
	LD				**HD**			
	20	**100**	**200**	**300**	**20**	**100**	**200**	**300**
pH	6.9 ± 0.1[a]	6.9 ± 0[a]	6.9 ± 0[a]	6.9 ± 0[a]	7.0 ± 0.1[ab]	7.0 ± 0[a]	7.0 ± 0[a]	7 .0 ± 0.1[b]
DIC (mmol L^{-1})	4.0 ± 0.8[a]	5.2 ± 0.8[a]	4.8 ± 0.2[a]	4.2 ± 0.5[a]	4.4 ± 0.9[a]	6.4 ± 1.1[a]	5.8 ± 1.3[a]	6.5 ± 1.3[a]
[CO_2] (mmol L^{-1})	0.4 ± 0[a]	0.4 ± 0[a]	0.4 ± 0.1[a]	0.4 ± 0[a]	0.4 ± 0.1[a]	0.4 ± 0[a]	0.5 ± 0[a]	0.4 ± 0.2[a]
[HCO_3^-] (mmol L^{-1})	3.6 ± 0.8[ab]	4.3 ± 0.2[a]	3.8 ± 0.6[a]	4.3 ± 0.3[a]	4.9 ± 0.4[a]	4.3 ± 1.2[a]	6.4 ± 0.2[b]	6.0 ± 1.1[a]
[CO_3^{2-}] (mmol L^{-1})	0.02 ± 0.01[a]	0.03 ± 0[a]	0.03 ± 0[a]	0.03 ± 0.01[a]	0.04 ± 0[a]	0.03 ± 0.01[a]	0.05 ± 0[a]	0.06 ± 0[a]

Discussion

The effects of inoculum size and buffering of cultures on the photosynthesis and growth of *N. oculata*, were studied in cells growing under increasing light intensities from 20 to 300 µmol photons m^{-2} s^{-1} under 2% CO_2.

Growth

The effect of CO_2 on growth has been reported for several species of microalgae (Kumar *et al.* 2014). In *N. oculata*, a comparison between 4 levels of CO_2 between 2 and 15% showed the highest biomass of 0.4 g L^{-1} measured on the day 3 of cultures grown under 2% CO_2, 26± 1°C and 300 µmol photons m^{-2} s^{-1}, where the initial cell density was 1 x 10^5 cell mL^{-1} (Chiu *et al.* 2009). In our results, 2% CO_2 in *N. oculata* cultures resulted in a similar biomass on the day 3 of the LD culture grown under 300 µmol photons m^{-2} s^{-1} when the culture was buffered. Under those same conditions, a lower biomass (0.3 g L^{-1}) was observed in the non-buffered culture. These results indicate a negative effect on the biomass of a pH drop from 7.4 in buffered cultures to 6.9 in non-buffered cultures.

Maintaining pH stability in microalgal cultures is a crucial challenge due to the effect of pH on photosynthesis and microagal growth. In large volume flat photobioreactors, Wang *et al.* (2016) observed an increasing biomass from around 0.7 g L^{-1} at 20 µmol photons m^{-2} s^{-1} to around 2.2 g L^{-1} at 250 µmol photons m^{-2} s^{-1} in *N. oceanica* under pH controlled conditions, indicating the importance of controlling the pH when CO_2 is supplied to the culture. In our results, comparing between LD treatments as well as between HD treatments, a decreased biomass was observed at 200 and 300 µmol photons m^{-2} s^{-1} when the pH was not controlled by buffering the culture, showing the negative effect of a pH decrease from 7.5 to 6.9 on the growth when cells are exposed to high light intensities. Thus, to counter the negative effects of higher CO_2 levels on culture pH, and hence on growth rate and yield, buffering is recommended.

Clear differences in growth rate were observed between LD and HD cultures under both buffered and non-buffered cultures, where the inoculum size seems to be the main influence. Improving light distribution in mass cultures by decreasing the self-shading effect between cells has been recently studied (Jain *et al.* 2015). In smaller cultures (500 ml), Huang *et al.* (2016) observed the effect of three different level of culture dilution on the growth of Chlorella vulgaris cultures, finding a higher growth rate of around 0.55 d^{-1} under a 40% diluted culture compared with around 0.47 d^{-1} under a 20% diluted culture. A higher chlorophyll content per dry cell weight was also observed in the 20% diluted culture compared with the 40% diluted culture, indicating improved light supply in the more diluted culture. In our results, lower chlorophyll per cell was observed in LD-buffered treatments under 100 and 200 µmol photons m^{-2} s^{-1}, suggesting that a better light distribution in LD cultures improved cell division in these treatments, compared with HD treatments.

Photosynthetic apparatus

Changes in the photosynthetic apparatus provide information that explain the effects of pH and inoculum size on growth behaviour discussed above. It has been observed that increasing chlorophyll concentration in *Nannochloropsis* cells exposed to decreasing light intensity is a photoacclimation strategy to increase the light absorption efficiency of the cells growing under light limitation (Sukenik *et al.* 1989; Fisher *et al.* 1996; Sforza *et al.* 2012; He *et al.* 2015). In accord with previous research, our results also showed a gradual increase in chlorophyll content per cell with decreasing light intensities in the culture under all treatments studied. It has been also found that the photoacclimation strategy employed by this genus involves increasing the cellular chlorophyll concentration as a consequence of an increased PSU number per cell under light limitation, rather than an increase in their antenna size (Fisher *et al.* 1996), and this was also observed in both LD and HD buffered cultures in our experiments. However, an inhibitory effect of decreasing pH in the non-buffered culture was also observed on the

photoacclimation strategy that *N. oculata* utilizes under changing light conditions; under low light treatments, unbuffered cells showed a lower chlorophyll concentration at both LD and HD compared with the buffered treatments and a lack of a trend in the PSU number across all light treatments. The effect of pH on photosynthetic pigments and photosynthesis has been observed in some species such as *Chlamydomonas acidophila* (Gerloff-Elias *et al.* 2005) and *Schizothrix calcicola* (West & Louda 2011). Gerloff-Elias et al (2005) observed significant inhibitory effects of pH on photosynthetic parameters when *C. acidophila* cells were grown under suboptimal pH conditions, which consequently affected the growth of the culture.

As previously mentioned, changes in the pH of the culture can affect cellular processes and also the availability of the different inorganic carbon species in the medium (Beer *et al.* 2014). In our results, higher levels of HCO_3^- under pH 7.4 than under 6.5 are in accord with what might be expected (Beer *et al.* 2014). These last results are crucial to understand the effect of pH on the photosynthetic process of *N. oculata* because previous research has shown that this species has a preference for HCO_3^- (rather than CO_2) use between pH 8.2 and 7.5 (Huertas & Lubián 1998). Huertas *et al.* (2000) also observed a CO_2 concentrating mechanism based on a bicarbonate uptake system in *Nannochloropsis* sp., which increases the intracellular CO_2 level. In our results therefore, a higher availability of HCO_3^- in the buffered medium would favour HCO_3^- availability for transport to increase the CO_2 availability for fixation in the Calvin cycle compared with non-buffered cultures. These observations point to the fact that a lower pH in the unbuffered treatments could not just have an inhibitory effect on the cellular chlorophyll *a* concentration and PSU numbers, but might also affect inorganic carbon acquisition and the photosynthetic performance of those cultures and consequently adversely affect biomass production.

Knowing that higher cellular chlorophyll concentrations are observed when microalgal cells are exposed to decreasing light intensities (Richardson *et al.* 1983), our observation that under buffered conditions the increased chlorophyll concentration per cell in HD treatments grown under 100 and

200 µmol photons m^{-2} s^{-1} compared with LD treatments at the same light intensities suggests a self-shading effect is taking place in the HD treatments, reducing light availability and thus reducing the growth rate of these cultures.

Changes in NPQ were also observed under the applied treatments. This mechanism of thermal dissipation of excess absorbed energy has been less studied in algal groups compared with higher plants, and this is certainly the case with the genus *Nannochloropsis* (Cao *et al.* 2013). Therefore, the mechanism and response of NPQ under changing conditions in *Nannochloropsis* requires further investigation. Cao *et al.* (2013) observed increasing activation of NPQ from 100 to 1594 µmol photons m^{-2} s^{-1} actinic light exposure in *Nannochloropsis* sp. Our results also showed increasing NPQ values with increasing light intensities for growth from 20 to 300 µmol photons m^{-2} s^{-1} in all treatment conditions, with minimum values observed under light limitation. The LD non-buffered treatments presented higher NPQ responses with increasing light intensities than did the LD buffered cultures, where differences in external pH seems to be the main influence. The higher availability of HCO_3^- under buffered treatments and higher affinity for this inorganic carbon by *N. oculata* explained above also might explain why NPQ is lower in the LD-buffered cultures as carbon fixation and CO_2 concentrating mechanism activity might act as a sufficient energy sink, reducing the need to induce NPQ. In HD cultures however, a different response to buffering is shown as no differences in NPQ values were observed between these treatments. Even though higher levels of bicarbonate were found in HD-buffered cultures, the self-shading effect seems to be affecting the light availability of both buffered and non-buffered cultures. A reduced light availability in HD cultures might reduce the CO_2 demand of those cultures, as reported by Ho *et al.* (2012) for *Scenedesmus obliquus*, which creates a balance between CO2 fixation and light energy supply and therefore a reduction in the energy dissipation activity.

Photosynthetic performance

A decrease in maximum quantum yield of PSII (F_v/F_m) was observed with increasing light intensity in all treatments, which is in accord with previous research in *Chlorella*, *Monoraphidium dybowskii*, *Phaeodactylum tricornutum*, and *Dunaliella tertiolecta* (Quigg *et al.* 2006; He *et al.* 2015). He *et al.* (2015) observed a decrease in F_v/F_m in *Chlorella* sp. from around 0.75 under 40 µmol photons m^{-2} s^{-1} to 0.63 under 400 µmol photons m^{-2} s^{-1} at day 3 of the culture, suggesting that the decline in F_v/F_m might be attributed to changes in light availability. Another aspect in the study of the photosynthetic performance is the maximum rate of photosynthesis (P_{max}). In our study, a clear effect of light on P_{max} was observed from 20 to 100 µmol photons m^{-2} s^{-1} under buffered conditions, while no effect of light on P_{max} was observed under non-buffered treatments, where a drastic drop of P_{max} was observed in all light treatments. It has been shown that changes in external pH affect the internal pH in several species of microalga (Gehl & Colman 1985; Dason & Colman 2004; Suffrian *et al.* 2011), and has shown to have an impact on the photosynthetic capacity of the algae (Gehl & Colman 1985; Blankenship 2014). Dason and Colman (2004) observed in two dinoflagellates that the internal pH decreased in parallel with the external pH between a pH of 7 and 8, which had a significant negative effect on the P_{max} of the culture. Even though, in our research, the internal pH was not measured it is believed that the decreased external pH in non-buffered cultures would have affected the internal pH of the cells and therefore a wide range of metabolic process, including the P_{max} values and hence the achieved biomass in all light treatments was likely affected.

Dark respiration

Dark respiration (R_d) as a process of energy production in the dark has been found to be related to the growth rate of microalgal species such as *Phaeodactylum tricornutum*, *Isochrysis galbana* and *Nannochloris atomus* (J.Geider & Osborne 1989). In such species is been found increasing dark

respiration with increasing growth rates (J.Geider & Osborne 1989). However, in the experiments reported here no relationship between dark respiration and growth rate was found in any of the light and buffered treatments. On the other hand, the ratio between R_d and P_{max} is been studied, which has shown a range between 0.11-0.59 in Dinophyceae, 0.05-0.22 in Chlorophyceae and 0.0004-0.17 in Cyanophyceae (J.Geider & Osborne 1989). Fisher *et al.* (1996) also studied the effect of low light (30 µmol photons m^{-2} s^{-1}) and high light (650 µmol photons m^{-2} s^{-1}) on R_d and P_{max} of *Nannochloropsis*, observing a ration between R_d and P_{max} of 0.13 in low light treatment and 0.55 in high light treatment. In our results, the effect of light on R_d and P_{max} was observed only in the buffered treatments, observing a ratio from low to high light of 0.11, 0.17, 0.15 and 0.15 in the LD treatments and 0.07, 0.15, 0.23, 0.46 in HD treatments (data not shown). The ratio between R_d and P_{max} obtained in our results are in accord with the previous results shown by Fisher et al 1996. This suggests that the influence of light on the interaction between R_d and P_{max} could influence the growth responses of *Nannochloropsis oculata*, when the cultures are buffered.

Conclusions

A lower pH of 6.9 in unbuffered medium had a significant effect on the biomass accumulation in *N. oculata* cultures when cells were exposed to high light. The effect of lower pH also affected the photoacclimation strategy that *N. oculata* is able to employ under altered conditions, though in unbuffered conditions this was independent of the cell density of the culture.

However, differences in the density of the culture had important effects on the growth rate of the cultures in a pH- independent fashion, where a self-shading effect seems to be affecting the availability of light and therefore the growth rate of the culture in all light treatments.

Both factors, pH and culture density, were shown to interact with the effect of light on the photosynthetic apparatus, photosynthetic performance and growth of *N. oculata* cultures, where the

buffered and low cell density conditions showed the clearest effect of light on both photosynthesis and growth. Such data have implications for strategies to optimise productivity in mass cultures of *N. oculata* and other species.

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CHAPTER 5: The effect of photoinhibition on growth, photosynthesis and macromolecular composition in *Nannochloropsis oculata* and *Nannochloropsis oceanica* previously acclimated to low and high light conditions

This chapter will be submitted to *Algal Research*

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ABSTRACT

Microalgal cultures are daily exposed to a heterogeneous light distribution as well as to potentially excess light conditions, which have a high impact on the photosynthetic responses, growth and macromolecular composition of the cells. How photosynthesis is affected by photoinhibition in cells previously acclimated to different light conditions and how this consequently affects the growth and macromolecular composition of the cells has not been completely defined. Here we studied the effect of 24 hours exposure to 1250 µmol photons m^{-2} s^{-1} on photosynthesis, growth and macromolecular composition of *N. oculata* and *N. oceanica* cultures, previously acclimated to a range of light intensities from 50 to 300 µmol photons m^{-2} s^{-1}. The photosynthetic apparatus and the photosynthetic performance of cells acclimated to low light conditions (50 µmol photons m^{-2} s^{-1}) were highly affected by photoinhibition in both species studied,

however a larger effect was observed in *N. oculata*. The impact of photoinhibition on photosynthesis decreased as cells were acclimated to higher light conditions in both species, indicating that the effect of photoinhibition can be mitigated by manipulating the pre-acclimation conditions of the cells. After photoinhibition, a drop in F_v/F_m of 55 % on average in *N. oculata* and of 40 % in *N. oceanica* was followed by a clear increase in lipid content in cells pre-acclimated to 50, 100 and 200 µmol photons m^{-2} s^{-1}, which suggests that the physiological state of cells as reflected in F_v/F_m values can be used as an indicator of the level of lipids in both *N. oculata* and *N. oceanica*. Macromolecular analysis showed that photoinhibition mainly affected the lipid content of the cells but not the level of protein and carbohydrates. These results show that the response of cells to photoinhibitory light levels is highly related to the level of light under which cells are pre-acclimated, showing a possible path to reduce the impact of photoinhibition on microalgal cultures.

Introduction

Microalgae have received increasing attention from the industrial sector over the past 60 years, due to their promising use in the production of biomass rich in lipids, proteins or carbohydrates (Tamiya 1957; Spolaore et al. 2006). As photosynthetic organisms, the growth of microalgal cells is normally affected by the heterogeneous light distribution experienced in mass cultures (Vonshak et al. 2014). In fact, the light intensity in mass microalgal cultures can reach levels where excess light dramatically affects the physiological responses, growth and macromolecular accumulation of cells. Understanding how cells respond to such photoinhibition is a fundamental question that has to be addressed in order to improve the production rate and quality of microalgal biomass.

In microalgal cultures, cells grow under exposure to low, optimum and high light conditions, depending on their position in the water column or growth vessel (Simionato et al. 2013). Light limitation in the deepest layers of the culture dramatically reduces the growth rate, which increases only when the light availability increases in cells growing closer to the surface (Sforza et al. 2012). An optimum light condition for growth is considered as a light intensity level where cells receive the maximum number of photons that they are able to absorb and convert into growth. Therefore, the outermost layer of the culture can be considered as a high light condition for the cells as growth rate does not increase with any further increasing light intensities (Sforza et al. 2012) and the excess of energy is either dissipated as a heat or fluorescence by the photosynthetic apparatus (Cao et al. 2013). Beyond the high light conditions, the light intensity in the outer layer of mass microalgal cultures can reach levels where the light exposure is beyond the saturation limit of photosynthesis, causing photodamage and a significant reduction of the growth rate (Vonshak et al. 2014).

Under photoinhibitory conditions, the absorption of light by chlorophyll exceeds the capacity of the photosynthetic apparatus to convert light energy into chemical energy, causing a saturation of the photochemical reactions (Li et al. 2009). Excess energy can over-excite the chlorophyll molecule, increasing the amount of triplet excited state chlorophyll (^{3}Chl*) which leads to the formation of reactive oxygen species such as singlet oxygen ($^1O_2$) and a consequent degradation of pigments, lipids and proteins (Takahashi & Badger 2011), including the proteins of the light harvesting complex of photosystem II (Vass 2012). Effects of photoinhibition on light harvesting may also translate into changes in metabolism, with a consequent alteration to the macromolecular composition of the cells. Thus, studying the effect of excess light on macromolecular composition is also crucial to predict changes in the final product of microalgal biomass production.

Microalgal genera such as *Nannochloropsis* show a high capability for acclimation to changing light conditions. This could allow a reduction in the effect of photoinhibition on the culture if cells also possess

an ability to acclimate to excess of light. It has been found in *Nannochloropsis* that cells growing under light limitation adopt a photoacclimation strategy that involves an increase in their chlorophyll concentration, associated with an increase in photosynthetic unit (PSU) numbers and photosynthetic capacity so as to increase their light absorption efficiency (Fisher et al. 1996). Cells growing under high light conditions instead decrease their light absorption efficiency by decreasing their PSU numbers and photosynthetic capacity (Fisher et al. 1996). However, Gallegos et al. (1983), found that microalgal cells are more susceptible to photoinhibition when they are acclimated to low light conditions. Several research groups have studied the effect of low and high light on the growth of microalgal cultures (Yeh et al. 2010; Martínez et al. 2012; Vonshak et al. 2014), however little is known about how photoinhibition affects microalgal cultures and whether changes in the photosynthetic apparatus and capacity caused by pre-acclimation to different light intensities can cause different responses under photoinhibitory conditions. If changes in the photosynthetic acclimation strategy also cause changes in their physiological and growth responses to photoinhibition, understanding these processes better may enable us to predict and reduce the effect of photoinhibition on microalgal cultures. To this end, the present research aimed to study the effect of photoinhibition on growth, photosynthesis and macromolecular composition of *N. oculata* and *N. oceanica* cultures previously acclimated to a range of light intensities from low light to high light.

Material and Methods

Strains and culture conditions

Strain CS-179 of *N. oculata* (Eustigmatophyceae) was obtained from the CSIRO Australian National Algae Culture Collection, Hobart, Tasmania and strain CCALA 804 of *N. oceanica* from the CCALA Culture Collection of Autotrophic Organisms, Czech Republic. All experiments were carried out in a multi-cultivator (MC1000) from Photo Systems Instruments, Drásov, Czech Republic. Cultures were bubbled with 2% CO_2, and grown in PHK medium (Rukminasari 2013) under continuous light and 25°C. *N. oculata* and *N. oceanica* cultures were pre-acclimated for 7 days under 50, 100, 200 or 300 µmol photons m^{-2} s^{-1}. After pre-acclimation, cultures were inoculated in duplicate at an initial density of 2 µg ml^{-1} of chl *a* under their corresponding light intensity treatment for 3 days. On the third day of growth, cultures were diluted to 5 µg ml^{-1} of chl *a*, resuspended into new PHK medium and exposed to an excess light of 1200 µmol photons m^{-2} s^{-1} for 24 hours.

Growth curve

The growth curve of each culture was measured on a cell count basis. Samples of 500 µl were taken daily from each culture and counted with a Neubauer hemocytometer under a microscope (Axio Scope.A1 Zeiss). The specific growth rate during the exponential phase of each growth curve was calculated using the equation:

$$(\ln X_1 - \ln X_0)\,(t_1 - t_0)^{-1}$$

Where X_1 and X_0 are the cell concentration, measured at time t_1 and the initial time t_0 respectively.

Chlorophyll analysis

Chl *a* measurements were taken on day 2 of the culture (i.e. before the photoinhibition treatment) and after 24 hours of light saturation exposition (i.e. after the photoinhibition treatment). A sample of 1 ml was taken from each culture for chl *a* extraction. Cells were harvested by centrifugation (5 min, 2600 g) at 4^{o}C and resuspended in 1 ml of DMSO. The suspension was heated for 3 minutes at 70° C and centrifuged at 2600 g for 5 minutes. Chlorophyll *a* concentration in the supernatant was measured at 665 nm using a Cary-50 UV-V Spectrophotometer and calculated as Chl *a* (µg ml^{-1}) = OD_{665} * 13.7 µg ml^{-1} (Seely et al. 1972).

Photosynthetic parameters

The composition of the photosynthetic apparatus and photosynthetic performance were measured before photoinhibition (BPH) treatment and after photoinhibition (APH) treatment.

Chlorophyll fluorescence of PSII was measured to determine the maximum quantum yield (F_v/F_m) of the cultures by using a pulse-amplitude modulated phytoplankton analyzer (Phyto-PAM, Heinz Walz, Effeltrich, Germany). A sample of 3 ml was taken from each culture and incubated in the dark for 15 minutes prior to measurements. After dark acclimation, cells were exposed to a saturating flash (2600 µmol photons m^{-2} s^{-1}) of red light to measure the maximum fluorescence (F_m) and calculate F_v/F_m.

The effective absorption cross-sectional area of PSII α-centers (σPSIIα) and the antenna connectivity of PSIIα (J_{con}PSIIα) were determined by flash fluorescence induction (FFI) curve measurements 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 measurements. A single 50 µs turn-over flash at a light intensity setting of 50% ($1x10^6$ µmol photons m^{-2} s^{-1}) was used. After measurements, the area under the FFI curve was analysed according to (Melis & Homann 1975; Nedbal et al. 1999).

Maximum light-saturated photosynthesis rate (P_{max}) and photosynthetic unit (PSU) number were measured using a Clark-type oxygen electrode (Hansatech, Norfolk, UK). Approximately $1×10^8$ cells were harvested from the culture and resuspended in 2 ml of fresh PHK medium, containing 2 mM of sodium bicarbonate and 40 mmol L^{-1} TRIZMA base pH 8.2 and placed into the O_2 electrode chamber for oxygen evolution measurements. Measurements were taken at 25°C and between 20% and 50% of air equilibrium of O_2 concentration. The maximum photosynthetic rate (P_{max}) was measured by using the saturating level of light previously obtained from a rapid light curve measured with the PhytoPAM. PSU number was measured by determining the oxygen production rate in cells exposed to an Electro-Optics Xenon flash light (EG&G) as an external light source, flashing with a frequency of 35 Hertz and placed 10 cm away from the O_2 electrode chamber. PSU number and size were calculated according to Fisher et al. (1996) and Quigg et al. (2006) using the equation: PSU number (PSU $cell^{-1}$) = (chl *a** ($6.02 * 10^{23}$))/ (PSU size * 849 X 10^{12}); PSU size, expressed as mol chl *a* mol O_2^{-1} $flash^{-1}$, was calculated from the chlorophyll concentration of the sample and the oxygen evolution from one turnover of the saturating flash.

Spectroscopic methods

A sample of 10 ml of culture was taken from each treatment and centrifuged at 2600 g for 10 minutes. The algal pellets were washed twice with MilliQ water, resuspended in 20 µl MiliQ water and stored at 5 °C for a maximum of 5 days before analysis. For the final preparation of the samples, an average of 7 dots per sample were placed in a Polysine™ microscope glass slide and dried in a desiccator for 24 hours.

A FTIR spectrometer (Bruker Equinox 55; Bruker Optics Inc., Etligen, Germany) coupled to an ATR accessory (Golden Gate; Thermo Fisher Scientific, Inc, Waltham, Ma, USA), controlled by the software OPUS (Bruker), was used to analyse the samples, using a liquid nitrogen cooled MCT detector.

Before measurement, the diamond window was cleaned with Isopropanol and the background spectra without sample was acquired. After that, the absorbance spectrum was obtained in the mid-infrared spectral region (4000-800 cm^{-1}) at 8 cm^{-1} spectral resolution with 50 scans co-added. ATR correction was conducted in all the data from the measured samples by using an extended ATR correction in OPUS software. The correction was done using one internal reflection, a 45° angle of the incident IR beam, and mean refraction index of 1.5 (Smith 2011).

The software Unscrambler 10.3 (Camo, Oslo, Norway) was used to analyse the data. The Savitsky-Golay algorithm was used to calculate the second derivative of the raw data with 9 points smoothing, which is used to avoid overlapping of the bands or obscured bands in the raw spectra. Line plots of the second derivative were used to visualise differences between treatments. Secondly, the data were normalized with the Extended Multiplicative Signal Correction (EMSC) to avoid possible differences in sample thickness (Heraud et al. 2006). Band assignments to specific functional groups were made according to (Zeroual et al. 1994; Jackson & Mantsch 1995; Zeroual et al. 1995; Zhbankov et al. 1997; Giordano et al. 2001) (Table 1).

Principal Component Analysis (PCA) was finally used to differentiate between groups of spectra by using the software Unscrambler 10.3 (Camo, Oslo, Norway). For this analysis, only the spectral regions of biological bands (3057-2796 cm^{-1} and 1801-798 cm^{-1}) were used. Scores plots and loading plots were used to find differences between treatments and the spectral region that contributed most to the variance of the data.

Finally, the area under the bands was calculated to determine the total lipid, protein and carbohydrates content of each sample by using the Beer-Lambert Law, which describes a direct relationship between the absorbance and the concentration of the component (Wagner et al. 2010).

Statistical analysis

The statistical significance of differences between treatments was tested by two-way ANOVA (significance level $P<0.05$) and Tukey analysis as a posterior multiple comparison test (significance level $P<0.05$) using the software Prism 6 (GraphPad Software, San Diego, CA, USA).

Table 1. Assignment of the major bands for FTIR spectroscopy analysed in this study.

Wavenumber values (cm^{-1})	Assignment	Functional group or molecules	References
2920	ν C-H	Primarily from lipids	(Giordano *et al.* 2001)
2850	ν C-H		
1735	ν C = O	Ester functional groups from lipids and fatty acids	(Zeroual *et al.* 1995)
1650	ν C = O	Amide I associated with proteins	(Jackson & Mantsch 1995)
1540	ν C-N δ N-H	Amide II associated with proteins	
1153, 1080, 1040, 1020	ν C-O-C of polysaccharides ν C-OH side groups	Carbohydrates	(Zeroual *et al.* 1994; Zhbankov *et al.* 1997)

Results

In this research, the effects of excess light treatment on the photosynthetic apparatus, performance of photosynthesis, growth and macromolecular composition were studied in *N. oculata* and *N. oceanica* cultures previously acclimated to levels of light intensity from low light to high light.

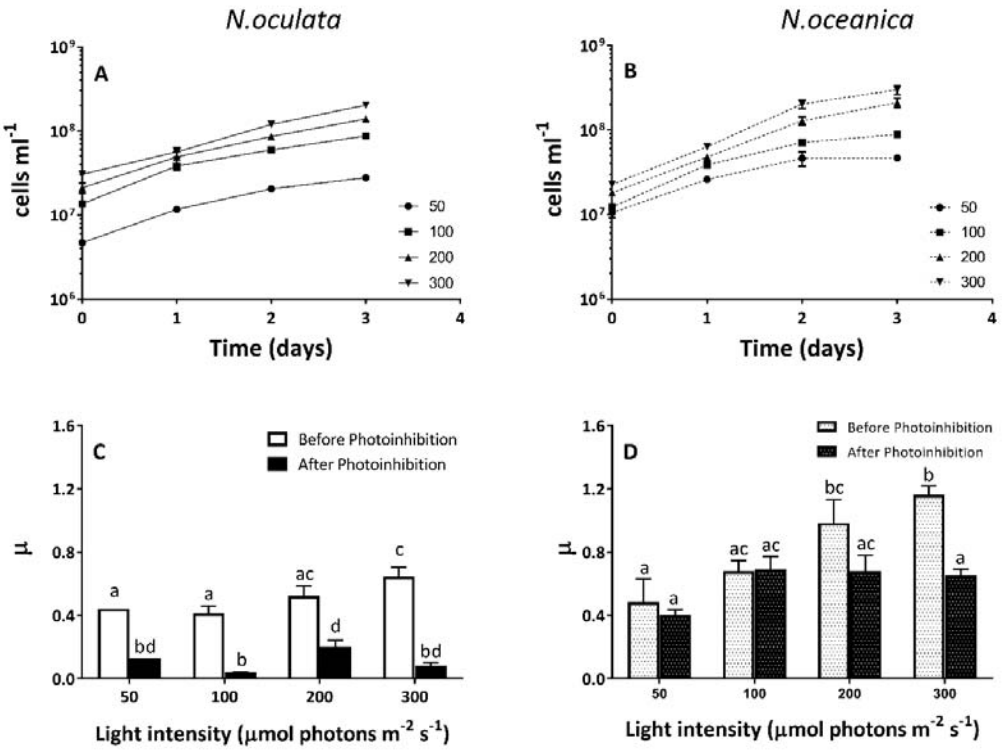

Figure 1. Growth responses of *N. oculata* and *N. oceanica* grown under 50, 100, 200, 300 µmol photons m^{-2} s^{-1} and after excess light treatment (1200 µmol photons m^{-2} s^{-1}). A and B) Growth curve of cultures of *N. oculata* and *N. oceanica* respectively. C and D) Growth rate before and after excess light exposure of *N. oculata* and *N. oceanica* respectively; white bars correspond to growth rate of cells pre-acclimated to their corresponding light treatment and black bars correspond to growth rate of cells exposed to photoinhibition. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05). In A) and B) initial cell densities are different between treatments as the inocula were based on chlorophyll content /ml and cellular chlorophyll differed between cells acclimated to different light intensities.

Growth

Increasing growth rate with increasing light intensities was observed in both species (Figure 1). However, under 200 and 300 µmol photons m^{-2} s^{-1}, higher growth rates were observed in *N. oceanica* than in *N. oculata* (Figure 1C and D). A dramatic effect of photoinhibition treatment on the growth rate of *N. oculata* was observed in all light treatments, while in *N. oceanica* no effect of high light exposure on the growth rate was observed in cells growing under 50, 100 and 200 µmol photons m^{-2} s^{-1} (Figure 1C and D). Under 300 µmol photons m^{-2} s^{-1}, the highest percentage drop of 95.3 % and 43.78 % in the growth rate of *N. oculata* and *N. oceanica* respectively was observed after photoinhibition.

Photosynthetic apparatus

Chl a *and PSU number*

A clear effect of light intensity for growth on chl *a* and PSU numbers per cell was observed in both *N. oculata* and *N. oceanica* (Figure 2); cells decreased their chl *a* and PSU number with increasing light intensities as a photoacclimation strategy under changing light conditions (Figure 2A, B, C and D). A clear effect of photoinhibition treatment on chl *a* and PSU numbers per cell was also observed in *N. oculata* (Figure 2A and C), but this effect decreased as cells were acclimated to higher light intensities until no effect of photoinhibition on cellular chl *a* concentration was observed in cells pre-acclimated to 300 µmol photons m^{-2} s^{-1} (Figure 2A).

A less marked effect of photoinhibition on the photosynthetic apparatus was observed in *N. oceanica* (Figure 2B and D); cells acclimated to 50 µmol photons m^{-2} s^{-1} decreased their PSU number per cell after photoinhibition; however, no effect of photoinhibition on chl *a* concentration was observed

under that treatment. Cells pre-acclimated to intensities above 100 µmol photons m^{-2} s^{-1} showed no effect of photoinhibition on either chl *a* concentration or PSU number (Figure 2B and D).

Photosynthetic Performance

In *N. oculata*, cells growing under 50 µmol photons m^{-2} s^{-1} showed the highest maximum photosynthetic rate (P_{max}), which deceased with increasing light intensities (Figure 2E). A clear effect of photoinhibition was observed in both species of *Nannochloropsis* as cells were pre-acclimated from 50 to 200 µmol photons m^{-2} s^{-1}, while no effect of photoinhibition treatment on P_{max} was observed in cells pre-acclimated to 300 µmol photons m^{-2} s^{-1} (Figure 2E and F).

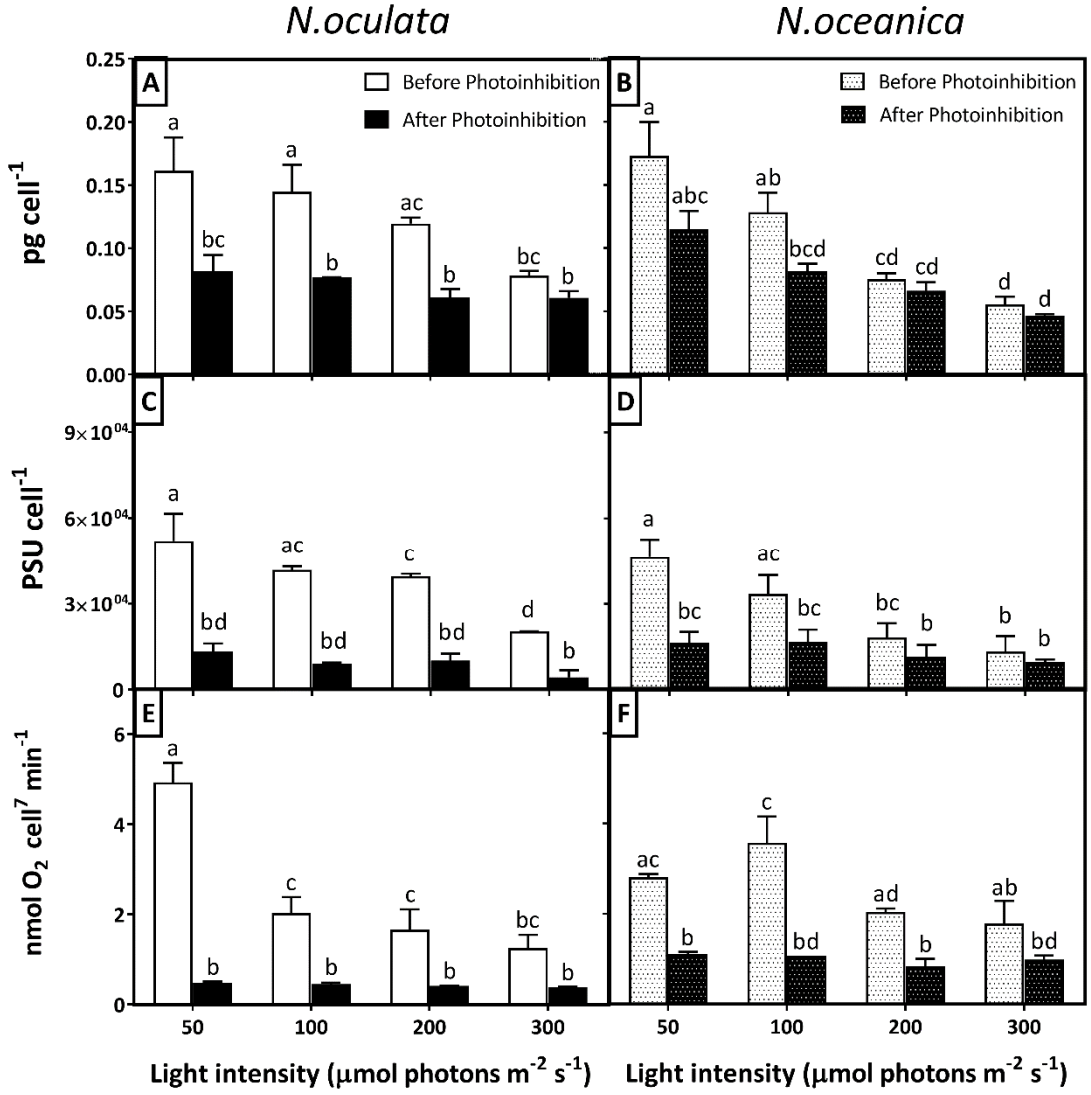

Figure 2. Composition of the photosynthetic apparatus and photosynthetic performance of of *N. oculata* and *N. oceanica* grown under 50, 100, 200, 300 µmol photons m^{-2} s^{-1} and after high light treatment (1200 µmol photons m^{-2} s^{-1}). A and B) Chl *a* concentration (pg cell-1) of *N. oculata* and *N. oceanica* respectively. C and D) PSU number (PSU $cell^{-1}$) of *N. oculata* and *N. oceanica* respectively. E and F) Maximum photosynthetic rate (P_{max}) (nmol O_2 $cell^{7}$ min^{-1}) of *N. oculata* and *N. oceanica* respectively. White bars correspond to cells pre-acclimated to their corresponding light treatment and black bars correspond cells exposed to photoinhibition. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05).

The effect of high light exposure on the photosynthetic efficiency (F_v/F_m) was also measured before and after the photoinhibition treatment in both species (Figure 3). Decreasing F_v/F_m with increasing light intensities for growth was observed in both species, while a dramatic effect of photoinhibition on F_v/F_m was observed in all treatments (Figure 3A and B), showing an average of 50% and 40% drop in the photosynthetic efficiency in *N. oculata* and *N. oceanica* respectively (Figure 3C and D).

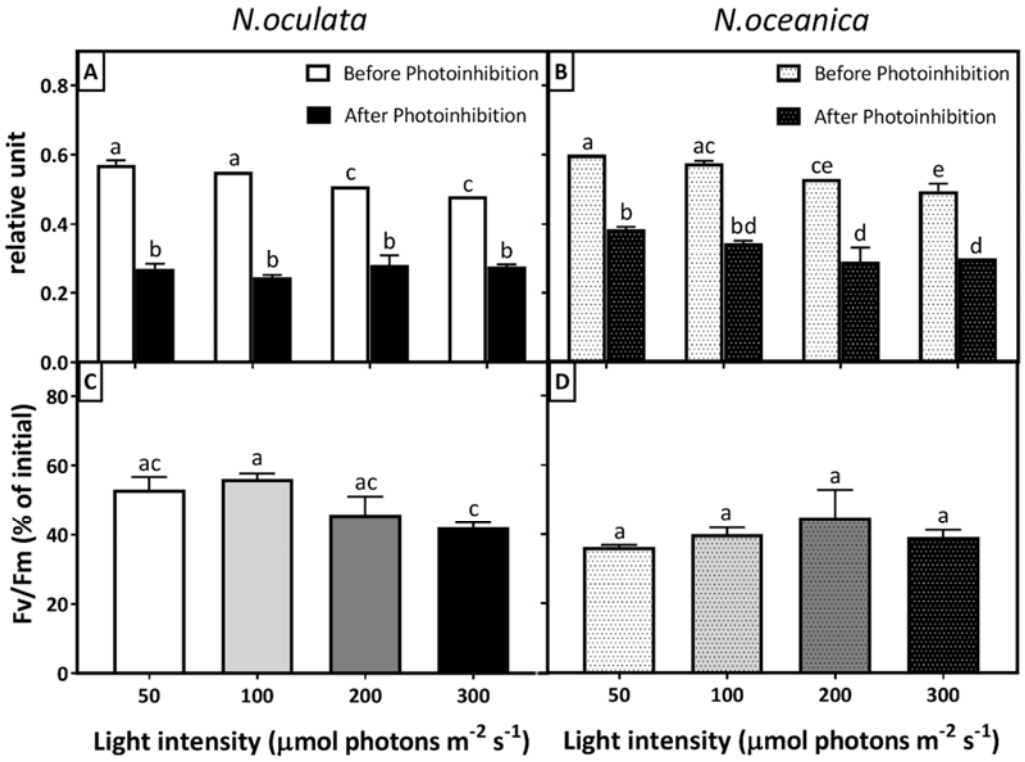

Figure 3. Maximum photosynthetic quantum yield (F_v/F_m) of *N. oculata* and *N. oceanica* grown under 50, 100, 200, 300 µmol photons m^{-2} s^{-1} and after high light treatment (1200 µmol photons m^{-2} s^{-1}). A and B) F_v/F_m of *N. oculata* and *N. oceanica* respectively. White bars correspond to cells pre-acclimated to their corresponding light treatment and black bars correspond cells exposed to photoinhibition. C and D) The decrease in F_v/F_m after high light exposure in cells pre-acclimated to their corresponding light intensity treatment. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05 (A and B) and One Way ANOVA P>0.05 (B and C)).

Antenna features

Results obtained from measurements of σPSIIα and J_{con}PSIIα revealed no effect either of increasing light intensity in the pre-acclimation stage or photoinhibition on the antenna size of *N. oculata* (Figure 4A and B), while statistically significant differences were observed between treatments for some parameters in *N. oceanica* (Figure 4C and D); however, the differences are small and not considered physiologically significant. An exception was the antenna connectivity which was higher in cells growing under 50 µmol photons m^{-2} s^{-1} in both species (Figure 4C and D), and decreased after photoinhibition. In *N. oculata* there was also a negative effect of excess light treatment on the antenna connectivity of cells growing under 100 µmol photons m^{-2} s^{-1} (Figure 4C). For all other treatments, no effect of high light treatment on the antenna connectivity was observed in either species (Figure 4C and D).

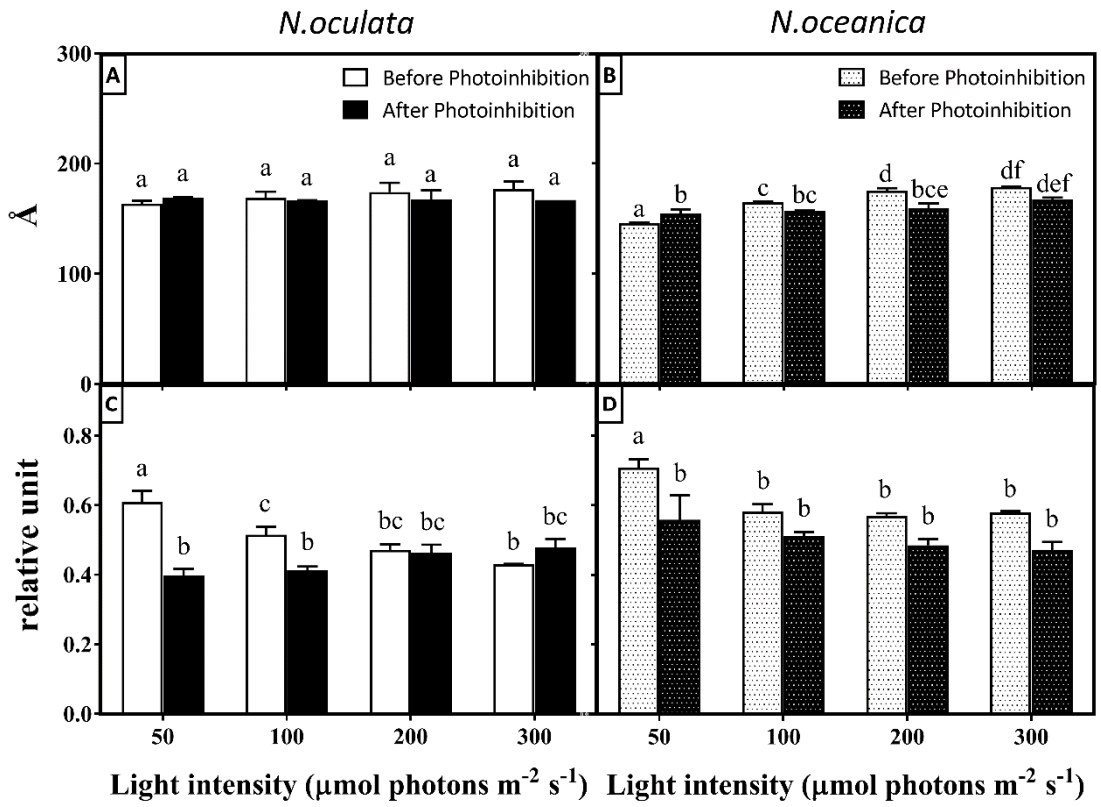

Figure 4. Antenna features of *N. oculata* and *N. oceanica* grown under 50,100,200, 300 µmol photons m^{-2} s^{-1} and after high light treatment (1200 µmol photons m^{-2} s^{-1}). A and B) Cross sectional area of PSII ß-centers (σPSIIß) of *N. oculata* and *N. oceanica* respectively. C and D) Antenna connectivity of the PSII α-centers (J_{con}PSIIα) of *N. oculata* and *N. oceanica* respectively. White bars correspond to cells pre-acclimated to their corresponding light treatment and black bars correspond cells exposed to photoinhibition. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05).

Macromolecular composition

The macromolecular composition of *N. oculata* and *N. oceanica* was analysed using Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy. Clear differences in the bands assigned to functional groups from lipids, proteins and carbohydrates were observed between cells before and after excess light treatment. Figure 5 shows the second derivative spectra of both species. In all treatments, results showed higher absorbance for bands in the 2960 and 1735 cm^{-1} range, attributed to lipids, than in the spectral region from 1153 to 1020 cm^{-1}, associated with carbohydrates (Figure 5). The analysis of the lipids bands revealed increasing absorbance for bands in the 2960 and 1735 cm^{-1} range with increasing light intensities in both *N. oculata* and *N. oceanica* cells (Figure 5A, B, C and D). Moreover, after photoinhibition these bands showed the highest absorbance, independent of the light intensity to which the cells were previously acclimated.

The PCA analysis explained 93% (85% for PC-1 and 8% for PC-2) and 91% (77% for PC-1 and 14% for PC-2) of the total variance in the data of *N. oculata* and *N. oceanica*, respectively (Figure 6). Before photoinhibition, all samples clustered with positive scores for PC-1, whereas after photoinhibition samples clustered with negative scores for PC-1 (Figure 6A and C). The data indicate a clear separation between 'before-' and 'after-photoinhibition' groups in both species, indicating clear changes in the chemical composition of the cells after they had been exposed to excess light treatment. In *N. oculata*, a separation of two groups between 50-100 µmol photons m^{-2} s^{-1} and 200-300 µmol photons m^{-2} s^{-1} was observed before photoinhibition, while after photoinhibition no separation of groups was observed between cells pre-acclimated to different the light intensities (Figure 6A). In *N. oceanica* a progressive separation between treatments was observed from 50 to 300 µmol photons m^{-2} s^{-1}, while clear differentiation between cells pre-acclimated to different light intensities was observed after excess light exposure (Figure 6C).

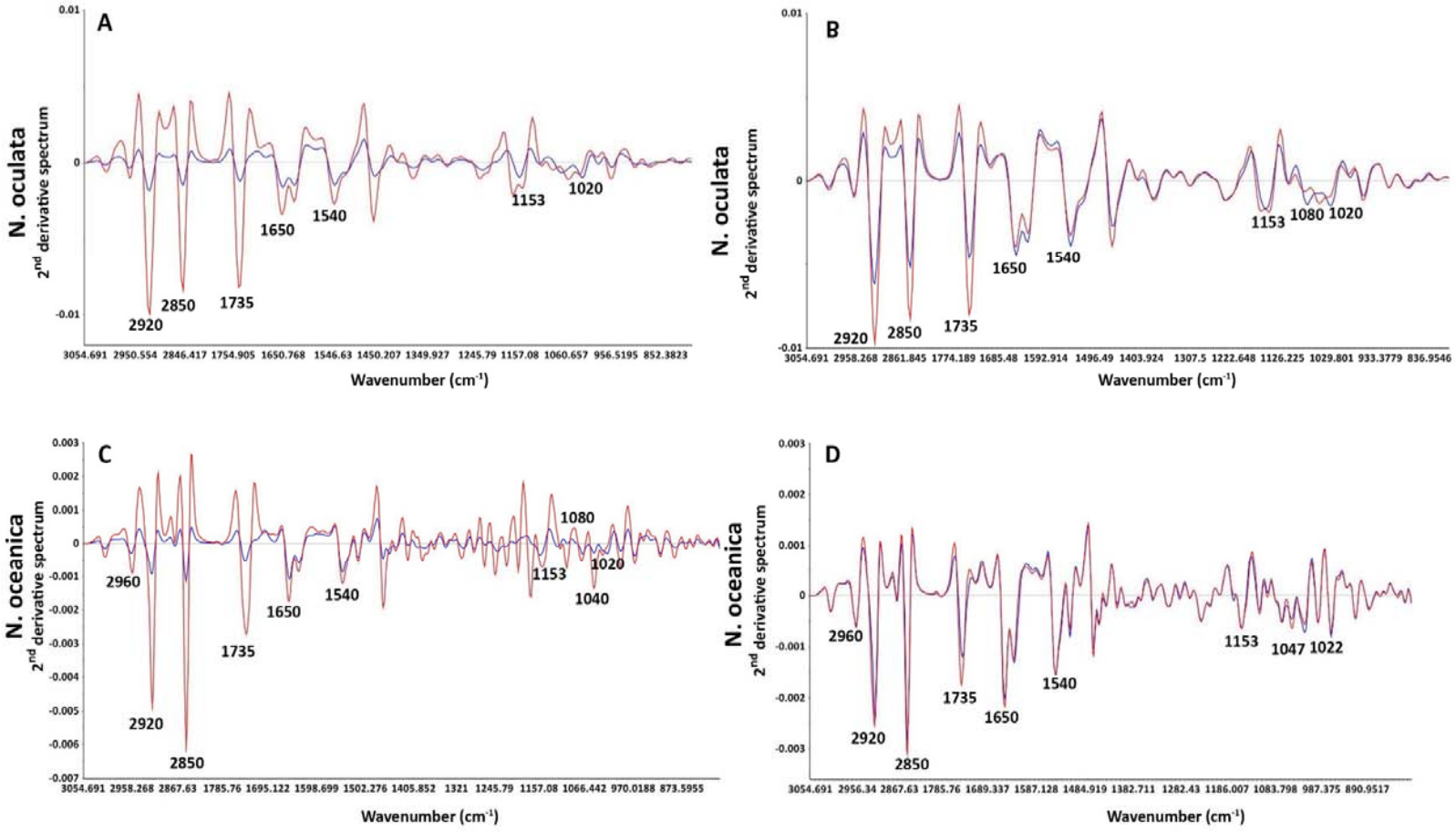

Figure 5. Second derivative ATM-FTIR spectra for *N. oculata* (A and B) and *N. oceanica* (C and D) in BPH (▬) and APH (▬) conditions. A and C correspond to cells pre- acclimated to 50 µmol photons m^{-2} s^{-1} and B and D correspond to cells pre-acclimated to 300 µmol photons m^{-2} s^{-1}.

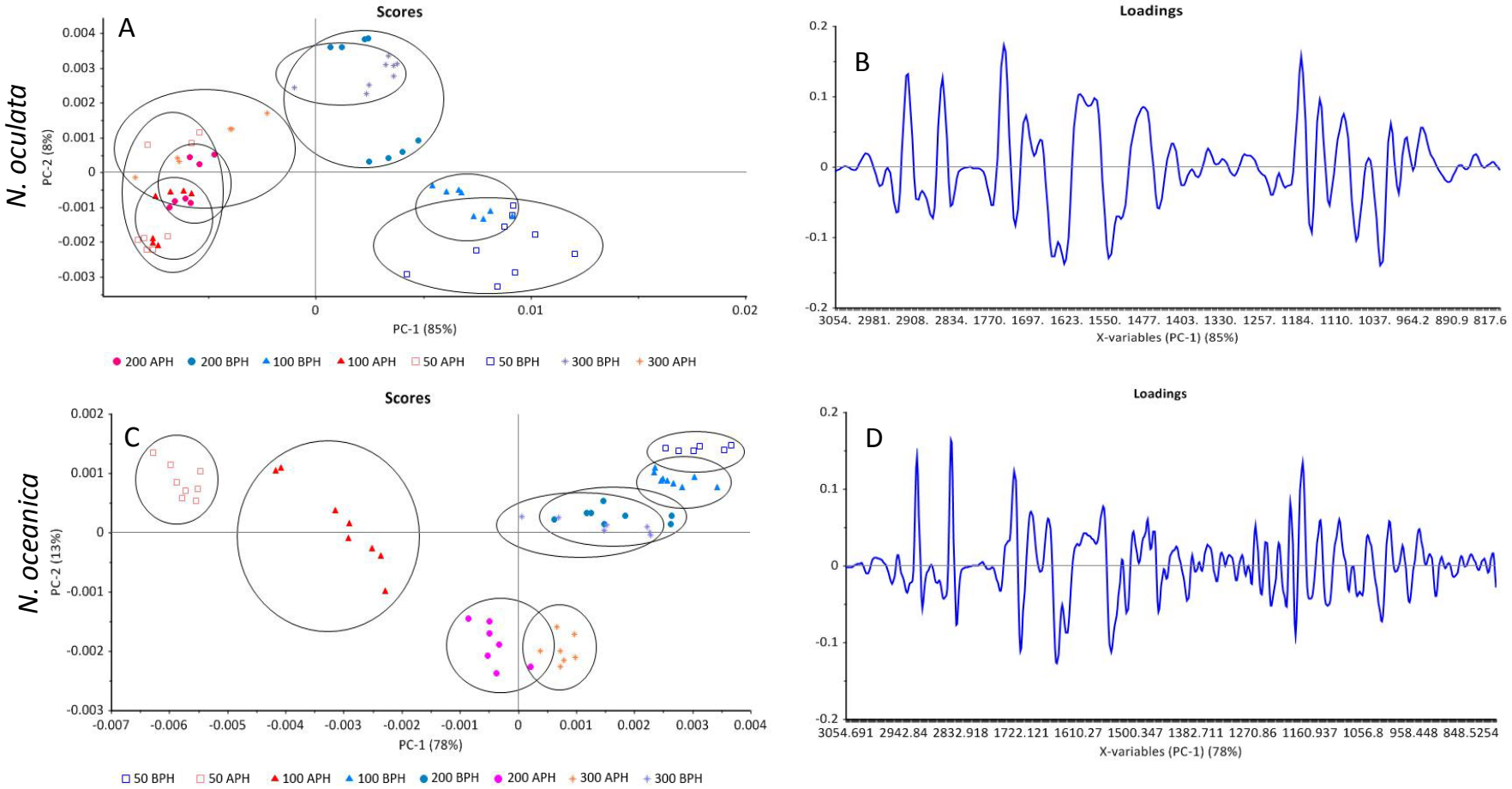

Figure 6. PCA analysis. A and C) PCA scores plots of *N. oculata* and *N. oceanica* respectively before photoinhibition (BPH) treatment and after photoinhibition (APH) treatment. B and D) PCA loadings plots of *N. oculata* and *N. oceanica* respectively before photoinhibition (BPH) treatment and after photoinhibition (APH) treatment.

The peak area of all studied bands was calculated from the second derivative data (Figure 7). In *N. oculata*, before exposing the cells to excess light the lipid content, as well as the protein content, increased with increasing light intensities (Figure 7A and C). However, no effect of light on the carbohydrate content was observed (Figure 7E). In *N. oceanica* in contrast, no effect of acclimation light intensity was observed on either lipid, protein or carbohydrates contents in cells prior to exposure to excess light (Figure 7B, D and F). However, after photoinhibition treatment, the lipid content increased at least three times in the cells of both species pre-acclimated to 50 µmol photons m^{-2} s^{-1}, differences that gradually decreased as cells were pre-acclimated to higher light intensities, until no effect of photoinhibition on the lipid content could be observed in cells pre-acclimated to 300 µmol photons m^{-2} s^{-1} (Figure 7A and B). No effect of excess light exposure was observed either on the protein or carbohydrate content in both species (Figure 7C, D, E and F). A higher protein content was also observed in *N. oculata* compared with *N. oceanica* when cells were growing under 200 and 300 µmol photons m^{-2} s^{-1} (Figure 7C and D).

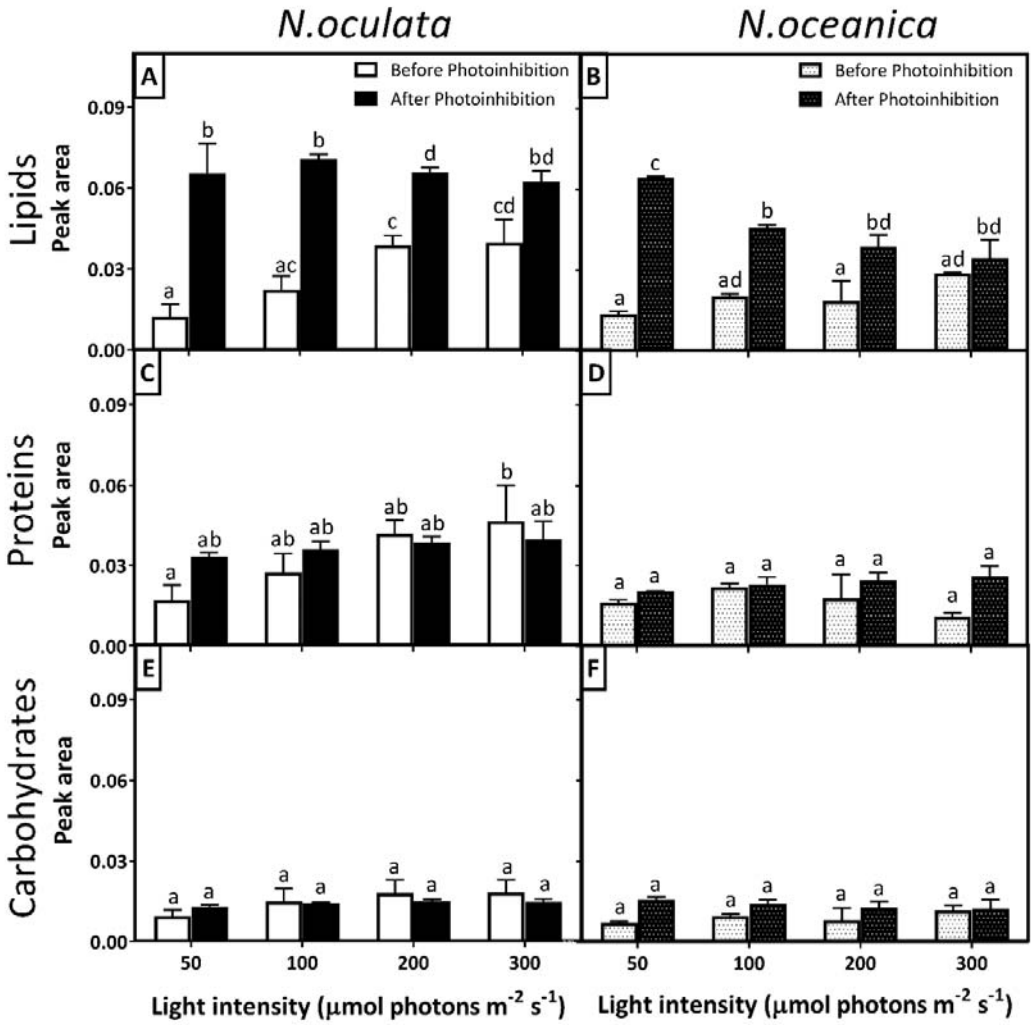

Figure 7. Macromolecular composition based on infrared spectroscopy analysis of *N.oculata* and *N. oceanica* grown under 50,100,200, 300 µmol photons m^{-2} s^{-1} and photoinhibition (1200 µmol photons m^{-2} s^{-1}). A and B) Lipids content of *N. oculata* and *N. oceanica* respectively. C and D) Protein content of *N. oculata* and *N. oceanica* respectively. E and F) Carbohydrate content of *N. oculata* and *N. oceanica* respectively. White bars correspond to cells pre-acclimated to their corresponding light treatment and black bars correspond cells exposed to photoinhibition. Different letters above each column indicate statistically significant differences (Two Way ANOVA P>0.05).

Discussion

The effects of light intensity on microalgal growth, photosynthesis and macromolecular composition have been studied separately extensively in the past. In our study, I have investigated the concurrent effects of increasing light intensity and exposing cells to excess light on *N. oculata* and *N. oceanica* growth, photosynthesis and macromolecular composition. Here we discuss how physiological processes reflect growth and macromolecular composition responses and from that expand our understanding of how cells respond to photoinhibition.

Growth

Light, as the source of energy for photosynthesis, has a major impact on the growth of microalgal cultures (Falkowski & Owens 1980; Carvalho et al. 2011; Vilém et al. 2016). Species like *Dunaliella tertiolecta*, *Phaeodactylum tricornutum*, and *Arthrospira platensis* have shown higher growth rate in cultures where cells grow under optimum light conditions compared with low light conditions (Beardall & Morris 1976; Falkowski & Owens 1980; Vonshak et al. 2014). Similarly, in our results, increasing growth rate with increasing light intensities was observed in both *N. oculata* and *N. oceanica* cultures. However, *N. oceanica* showed higher growth rate than *N. oculata* in cells grown under 100, 200 and 300 µmol photons m^{-2} s^{-1}, but the effect of excess light on growth rate was lower than in *N. oculata* in all light treatments studied. The effect of photoinhibition on the growth rate of *N. oceanica* was in accord with a study in *Dunaliella tertiolecta*, which showed that cells pre-acclimated to 50 µmol photons m^{-2} s^{-1} presented no differences in growth rate between the control (50 µmol photons m^{-2} s^{-1}) and cultures exposed to 1000, 1500 and 2000 µmol photons m^{-2} s^{-1} (Seepratoomrosh et al. 2016). A comparison between 9 species of *Nannochloropsis* showed that growth rate can vary from 0.11 to 0.21 (day^{-1}) depending on species (Ma et al. 2014), supporting the differences in growth response between *N. oculata* and *N. oceanica* found in our results.

Photosynthesis

The effect of light on the cellular chlorophyll concentration of microalgae has been much studied in previous research (Yentsch & Lee 1966; Beardall & Morris 1976; Carvalho et al. 2011). For example, Simionato et al. (2011) observed an increased from 0.067 to 0.178 µg chl*a*/10^6 cell in *Nannochloropsis gaditana* cells grown from 1200 to 15 µmol photons m^{-2} s^{-1}. Similarly, in our results *N. oculata* and *N. oceanica* showed increasing cellular chlorophyll concentration with decreasing light intensities for growth. Moreover, changes in cellular chlorophyll concentration in both species was clearly followed by changes in the PSU number per cell, but not in the antenna size. These data indicate that, in both *N. oculata* and *N. oceanica*, instead of increasing the antenna size, cells increase their PSU number with decreasing light intensities in order to enhance their light absorption capacity under light limitation conditions. Similar results were observed by Fisher et al. (1996) in *Nannochloropsis* sp.

Under photoinhibitory conditions, it has been observed that the excess of light causes a decrease in thylakoid lumen pH and induces the production of reactive oxygen species, mainly as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$*), and ultimately causes perturbation of chlorophyll production (Li et al. 2009), which consequently negatively affects photosynthetic performance. The effect of photoinhibition on the cellular chlorophyll concentration of *N. oculata* and *N. oceanica* was clearly observed in cells pre-acclimated to 50 µmol photons m^{-2} s^{-1}, however this effect decreased in cells acclimated to higher light intensities. This suggests that in both species cells acclimated to low light condition are more susceptible to photoinhibition and therefore, the effect of photoinhibition on photosynthesis is mitigated when cells are acclimated to higher light intensities, decreasing possible photodamage.

The effect of excess light on the performance of photosynthesis in both *Nannochloropsis* species was also studied. It was observed that both *Nannochloropsis* species were able to acclimate to the changes in their light environment by increasing P_{max} and maintaining, or even increasing, F_v/F_m with decreasing

light intensities, as a consequence of increasing their cellular chlorophyll *a* concentration and PSU number. Under excess light, however, a detrimental effect of light on both P_{max} and F_v/F_m was observed. Similarly, Seepratoomrosh et al. (2016) also found a decrease in the F_v/F_m of cells of *Dunaliella tertiolecta* from around 0.6, in cells grown under 50 µmol photons m^{-2} s^{-1}, to around 0.3 in cells exposed to 2000 µmol photons m^{-2} s^{-1}.

In my study, photosynthetic performance was more affected by photoinhibitory conditions in cells pre-acclimated to low light conditions, especially in *N. oculata*, which suggests that possible photodamage in the photosynthetic apparatus occurred. In contrast, cells acclimated to higher light intensities would be less prone to damage by high light exposure and recover after removal of the light stress. Further investigations are required to clarify this however. Photoacclimation to excess light can also occur after cells are exposed to an excess light for a prolonged period of time. Seepratoomrosh et al. (2016) studied the effect of excess light on photoinhibition of *Dunaliella tertiolecta*, a comparison between 1000, 1500 and 2000 µmol photons m^{-2} s^{-1} showed a decline of F_v/F_m from around 0.62 to around 0.42, 0.39 and 0.15 respectively after 5 hours of light exposure. After 6 hours however, the F_v/F_m started to increase in all excess light treatments until reaching almost 0.6 again after 2 days, indicating that cells of *D. tertiolecta* are able to photoacclimate after a prolonged period of excess light exposure. In our experiments, cells were exposed to 24 hours of excess light, and showed decreasing differences in the photosynthetic apparatus and performance of photosynthesis between before and after photoinhibition as cells were pre- acclimated to higher light intensities. Therefore, further investigations are required to determine whether the photosynthetic apparatus and consequently performance of photosynthesis in *N. oculata* and *N. oceanica* are able to recover over time periods longer than 24 h.

Macromolecular composition

The effect of photoinhibition on the macromolecular composition of *N. oculata* and *N. oceanica* was studied by Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy and showed clear differences between treatments and species of *Nannochloropsis*. Previous research has found no differences between results using ATR-FTIR spectroscopy and conventional, wet-chemistry based, macromolecular determination (Dao et al. 2017). Moreover, ATR-FTIR spectroscopy is a faster, non-destructive and inexpensive technique for the study of changes in cellular composition.

The genus *Nannochloropsis* is of biotechnological interest due to the capacity of species of this genus to produce high biomass and accumulate lipid (Sukenik et al. 1989; Bondioli et al. 2012), which has been related to the photosynthetic efficiency of the cells (He et al. 2015). Ma et al. (2014) studied 9 strains of *Nannochloropsis*, finding a lipid content of 52.1 % in *N. oculata* and 52.9 % in *N. oceanica*. Those results were found in cells bubbled with 2 % CO_2 and grown under 100 µmol photons m^{-2} s^{-1} at 25 ˚C, where the F_v/F_m in the culture was around 0.67 in both species at day 4 of the culture. The relation between F_v/F_m and lipid content has been previously discussed; exposing microalgal cultures to high light conditions causes cells to prioritise the synthesis of lipid (Gwak et al. 2014) above other macromolecules, as was observed in our results. He et al. (2015) observed, in *Chlorella* sp., a drop in F_v/F_m from around 0.75 to 0.63 in cells grown at 40 and 400 µmol photons m^{-2} s^{-1} respectively, while the lipid content increased from around 250 to 350 mg/g under 40 and 400 µmol photons m^{-2} s^{-1} respectively. Similar results were observed in *Monoraphidium dybowskii* (He et al. 2015). In our results, cells grown under 100 µmol photons m^{-2} s^{-1} showed F_v/F_m values around 0.6 in both *N. oculata* and *N. oceanica*, but the lipid content was found not to increase in cells grown under 200 and 300 µmol photons m^{-2} s^{-1}. However, after photoinhibition an average drop of 55 % in F_v/F_m in *N. oculata* and 40 % in *N. oceanica* was followed by a clear increase in lipid content in cells grown under 50, 100 and 200 µmol photons m^{-2} s^{-1}. A comparison between 50 and 300 µmol photons m^{-2} s^{-1} treatment showed, in both *Nannochloropsis* species, that cells grown under low light have higher F_v/F_m and lower

lipid content, while cells grown under high light showed statistically lower F_v/F_m and higher lipid content. The correlation between the photosynthetic efficiency and the lipid accumulation in cells of both *N. oculata* and *N. oceanica* suggests that changes in the F_v/F_m, indicating stress, will also be an indicator of increases in the lipid content.

Interestingly, cells acclimated to high light intensities showed the lowest effect of high light exposure on the organization of the photosynthetic apparatus and photosynthetic performance; at the same time these cells showed no effect of high light treatment on their lipids, proteins and carbohydrates levels. For industrial-scale production of microalgae, these results confirm that the measurement of the quantum yield of PSII could be used as an indicator of stress and thus the lipid content in the cells.

Secondly, after excess light exposure of *N. oculata*, the dramatic increase of lipid content in cells pre-acclimated to 50, 100 and 200 µmol photons m^{-2} s^{-1}, reaching the same level as cells pre-acclimated to 300 µmol photons m^{-2} s^{-1}, and a clearly decreased growth rate in cells subjected to all pre-acclimation treatments, suggest that 24 h of excess light conditions led cells to use the energy from photosynthesis to increase the accumulation of lipids rather than support cell division and growth. The same tendency was observed in *N. oceanica* in some treatments. For industrial scale production, this may be a promising treatment to increase lipid content before harvesting and therefore, increasing the biomass quality.

Conclusions

In the research reported here, the effect of photoinhibition on photosynthesis, growth and macromolecular composition was studied in *N. oculata* and *N. oceanica*. The photosynthetic response of these two species to exposure to excess light was found to depend on the level of light to which the cells were pre-acclimated, although this effect was more marked in *N. oculata*. It is proposed that

changes in the F_v/F_m may be an indicator of changes in the lipid accumulation of the cells as decreasing values of F_v/F_m were clearly paralleled by increasing lipid accumulation in all treatments studied. Despite big changes in F_v/F_m, PSU number and maximum rates of oxygen evolution, changes in other aspects of the organisation of the photosynthetic apparatus such as antenna size and connectivity were minimal. A clear increase in lipid content, after excess light exposure, of cells of *N. oculata* pre-acclimated to 50, 100, and 200 µmol photons m^{-2} s^{-1}, reaching as high as the lipid content of cells pre-acclimated to 300 µmol photons m^{-2} s^{-1}, suggests that 24 h of high light stress is a promising alternative to increase the biomass quality before harvesting.

The results found in this research are a clear contribution toward understanding the effect of excess light on culture of *Nannochloropsis* strains and how their responses will depend on the position of the cells in the water column of the culture, and hence light exposure. In future research, studying the photo-acclimation capability of *Nannochloropsis* to photoinhibition will be important to predict for how long cells can cope with excess-light conditions and their ability to recover, crucial information to help predict decreases in the microalgal biomass production and to avoid losses in mass cultures.

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CONCLUSIONS

In this work, I investigated the effect of light on the phenotypic plasticity of *Nannochloropsis*, a promising genus for biotechnological purposes. My research was particularly focused on the importance of light for optimization of microalgal cultures and the influence of other external conditions, namely temperature, pH, inoculum size and excess light exposure, on light utilization.

Light is a major factor affecting microalgal growth and photosynthesis and, therefore, it represents one of the key factors to optimize microalgal cultures (Torzillo et al. 2003; Vonshak et al. 2014). At the industrial scale in open ponds or in dense cultures in photobioreactors, microalgal cells are exposed to low, optimum or high light, depending on their position in the water column or the photobioreactor surface. In *Nannochloropsis*, light limitation (50 µmol photons m^{-1} s^{-2}) can decrease the growth rate from 0.55 d^{-1} in optimum light conditions (150 µmol photons m^{-1} s^{-2}) to 0.3 d^{-1} 1 (Sforza et al. 2012). On the other hand, under high light growth does not increase beyond that at the optimum and the excess of light energy is dissipated as heat or fluorescence to avoid photoinhibition (de Bianchi et al. 2010; Cao et al. 2013). Under photoinhibitory conditions, the growth rate can be highly affected, which has a major impact on biomass production as mass microalgal cultures can be daily exposed to excess light (Raven 2011). Microalgae have a strong photosynthetic acclimation capacity under changing light conditions, and can adjust their photosynthetic apparatus and performance to the level of light. Therefore, how cells respond to changing light conditions at the level of photosynthesis is crucial to understanding how the biomass production of microalgal cultures can be optimized.

The research in this thesis demonstrates that *Nannochloropsis oculata* is able to acclimate and alter its photosynthetic response when grown under a range from low to high light conditions. For instance, cells at least doubled their cellular chlorophyll concentration and PSU number under light limitation, resulting in almost a doubling of their maximal photosynthetic capacity (P_{max}). These acclimation

responses were crucial to the cells to increase their light harvesting ability and therefore maintain their photosynthetic efficiency under light limitation conditions during the progress of batch culture.

Our results also demonstrate that other external conditions have an important effect on the utilization of light for photosynthesis and growth of *N. oculata* cultures. The effect of light limitation on growth rate was found to be closely related to the growth temperature conditions, while at high light treatment, temperature did not have any effect. Some photosynthetic parameters, such as the cellular chlorophyll concentration, were also affected by temperature only under light limitation, but the maximum photosynthetic capacity and light harvesting efficiency were influenced by the temperature under both low and high light treatments, which suggest that the effect of light on the photosynthetic capacity of the cells was highly dependent on the temperature of the culture.

The study of pH and inoculum size revealed that both factors interact with the effect of light on photosynthesis and biomass measured in the exponential phase of *N. oculata* cultures; under high light, controlling the pH by buffering the cultures showed a 25% and 13% increase in biomass in cultures initiated with a low inoculum and a high inoculum respectively, compared with the non-buffered cultures. The effect of light on the photosynthetic capacity, photosynthetic efficiency and NPQ was also found to depend on the pH of the culture, as well as on the inoculum size.

In addition, the effect of excess light on photosynthesis, growth and macromolecular composition was also studied in cells of *Nannochlorpsis oculata* and *Nannochloropsis oceanica* pre-acclimated to a range of light from low to high light conditions. Results revealed a clear relationship between the decrease of photosynthetic efficiency and increase of lipid content after exposing the cultures to excess light, suggesting that F_v/F_m values could be used as an indicator of the level of stress-induced lipid formation in both *N. oculata* and *N. oceanica*. Furthermore, a dramatic increase in lipid content was observed, especially in *N. oculata*, after 24 h of excess-light exposure, which was independent of the light intensity to which cells were pre-acclimated. It was revealed that in all light treatments cells divert energy and carbon flow to increase the accumulation of lipids rather than enhancing growth

after exposure to excess light, suggesting that this could be a promising treatment to increase lipid content in mass cultures.

The optimization of microalgal cultures still requires additional research to better understand the main critical points that affect biomass production or macromolecular composition. The research reported here has contributed with a better understanding about the use of light by the microalgal genera *Nannochloropsis* as being one of the main factors affecting photosynthesis, growth and the macromolecular composition, indicating how light, temperature, pH and inoculum size influence the biomass of *N. oculata* cultures, as well as how treatment under excess light might represent an alternative to commonly used techniques, such as nitrogen limitation, to increase the lipid content in the biomass of *N. oculata* and *N. oceanica*.

In future research, studying details of the photoacclimation capability of *Nannochloropsis* to cope with photoinhibition will be crucial to predict for how long cells can be exposed to excess light without affecting its biomass and lipid content and their ability to recover. This would be important information to prevent dramatic decreases of biomass production.

It is difficult to extrapolate from small scale laboratory cultures to the larger scale. Thus, from an industrial point of view, testing the combination of environmental conditions studied in this thesis and improving our physiological understanding of *Nannochloropsis* at a larger scale of microalgal culture will be crucial to prove the success of the optimization strategies for cultures. After optimizing the biomass production, testing the excess-light treatment in mass culture will be crucial to reveal if it is possible to increase the biomass quantitatively and qualitatively. A success in the application of the knowledge acquired in this thesis on the optimization of microalgal mass culture would be a clear contribution to the utilization of *Nannochloropsis* in industrial-scale production and also to the importance of interdisciplinary work between the range of experts involved in an Industrial scale design of microalgal culture facilities, such as between physiologist and engineers.

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