Journal of Plant Physiology 180 (2015) 18–26

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Physiology

# The impact of fluctuating light on the dinoflagellate *Prorocentrum micans* depends on $NO_3^-$ and $CO_2$ availability

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ARTICLE INFO

*Article history:*
Received 1 April 2014
Received in revised form
18 November 2014
Accepted 10 January 2015
Available online 24 March 2015

*Keywords:*
$CO_2$
Fluorescence
Growth
Light fluctuations
Nitrate

ABSTRACT

Increasing atmospheric $pCO_2$ and its dissolution into oceans leads to ocean acidification and warming, which reduces the thickness of upper mixing layer (UML) and upward nutrient supply from deeper layers. These events may alter the nutritional conditions and the light regime to which primary producers are exposed in the UML. In order to better understand the physiology behind the responses to the concomitant climate changes factors, we examined the impact of light fluctuation on the dinoflagellate *Prorocentrum micans* grown at low (1 μmol $L^{-1}$) or high (800 μmol $L^{-1}$) [$NO_3^-$] and at high (1000 μatm) or low (390 μatm, ambient) $pCO_2$. The light regimes to which the algal cells were subjected were (1) constant light at a photon flux density (PFD) of either 100 (C100) or 500 (C500) μmol $m^{-2}$ $s^{-1}$ or (2) fluctuating light between 100 or 500 μmol photons $m^{-2}$ $s^{-1}$ with a frequency of either 15 (F15) or 60 (F60) min. Under continuous light, the initial portion of the light phase required the concomitant presence of high $CO_2$ and $NO_3^-$ concentrations for maximum growth. After exposure to light for 3 h, high $CO_2$ exerted a negative effect on growth and effective quantum yield of photosystem II ($F_v'/F_m'$). Fluctuating light ameliorated growth in the first period of illumination. In the second 3 h of treatment, higher frequency (F15) of fluctuations afforded high growth rates, whereas the F60 treatment had detrimental consequences, especially when $NO_3^-$ concentration was lower. $F_v'/F_m'$ responded differently from growth to fluctuating light: the fluorescence yield was always lower than at continuous light at 100 μmol $m^{-2}$ $s^{-1}$, and always higher at 500 μmol $m^{-2}$ $s^{-1}$. Our data show that the impact of atmospheric $pCO_2$ increase on primary production of dinoflagellate depends on the availability of nitrate and the irradiance (intensity and the frequency of irradiance fluctuations) to which the cells are exposed. The impact of global change on oceanic primary producers would therefore be different in waters with different chemical and physical (mixing) properties.



## Introduction

Phytoplankton cells are exposed to changes in irradiance, with very different periodicity depending on the temporal scale considered and the environmental conditions. The light field can be altered with a periodicity of fractions of a second in the case of the influence of surface waves on the light field; with frequencies of minutes in coastal waters, and of hours or days in deeper oceanic waters, due to turbulent and thermal mixing. Seasonal changes can occur over months (Litchman, 2000; MacIntyre et al., 2000). The impacts of light fluctuations on phytoplankton physiology varies, leading to short term physiological changes (e.g., regulation of energy transfer from the antennae) and potentially major repercussions at the ecosystem level through their influence on primary production (Litchman, 2000 and references therein).

*Abbreviations:* LNLC, cells grown under low $NO_3^-$ low $CO_2$ nutrient condition; LNHC, cells grown under low $NO_3^-$ high $CO_2$ nutrient condition; HNLC, cells grown under high $NO_3^-$ low $CO_2$ nutrient condition; HNHC, cells grown under high $NO_3^-$ high $CO_2$ nutrient condition; C100, cells cultured under constant light of 100 μmol $m^{-2}$ $s^{-1}$; C500, cells cultured under constant light of 500 μmol $m^{-2}$ $s^{-1}$; F15, cells cultured under fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min; F60, cells cultured under fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min; UML, upper mixed layer; PAR, photosynthetically active radiation; PFD, photon flux density; CCM, carbon concentrating mechanism; NPQ, non-photochemical quenching.

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http://dx.doi.org/10.1016/j.jplph.2015.01.020
0176-1617/

On a global scale, the warming of surface waters associated with the increase of atmospheric $pCO_2$ enhances stratification, resulting in a shoaling of the upper mixed layer (UML) (IGBP, 2013). This is expected to cause a higher residence time of phytoplankton at depths where the irradiance is high and therefore causes changes in the frequency of light fluctuation. Concomitantly, water stratification decreases nutrient supply via upwelling from deep waters. The ongoing increase of atmospheric $pCO_2$ also leads to a differential increase of $CO_2$ concentration in the oceans at different latitudes according to Henry's Law (Henry, 1803). Additionally, the ocean acidification associated with increased $CO_2$ dissolution results in a change in the relative abundance of inorganic carbon species, with higher $CO_2$ and bicarbonate and lower carbonates concentrations. The combined effects of these events on phytoplankton are of general concern in predicting impacts of future ocean changes on marine productivity and related ecosystem-scale processes.

The ability of photosynthetic organisms to cope with high irradiance is associated with specific responses for the prevention (e.g., synthesis of protective pigments and antioxidants, regulation of energy transfer in the antennae and from the antennae to the electron transfer chain, and avoidance mechanisms) and repair of damage to crucial functional molecules (Garcia-Pichel, 1994; Barros et al., 2003; Jahns and Holzwarth, 2012). The tolerance to high irradiance, however, is also decisively dependent on the availability of sinks for the electrons that facilitate the dissipation of excess energy and minimize the persistence of excited states (Raven, 2011).

In photosynthetic organisms, the main sink for electrons is $CO_2$ fixation, followed by nitrate reduction to ammonium (Giordano and Raven, 2014). In most cases (but not all), phytoplankton cells are not C limited due to the fact that they are equipped with $CO_2$ concentrating mechanisms that assist in the acquisition of inorganic carbon (Giordano et al., 2005a). Carbon concentrating mechanism (CCM) is energetically costly and their operations can contribute to the dissipation of excess light energy (Raven et al., 2014). Therefore, the fact that, at the $CO_2$ concentration expected for the end of this century, the CCMs of many organisms will be down-regulated and is likely to have consequences on the cell responses to the exposure to excess energy (Beardall and Giordano, 2002; Giordano et al., 2005a; Gao et al., 2012a,b).

In open oceans, phytoplankton is usually N limited, while in coastal waters N can be abundant and limitation can be shifted to other nutrients (e.g., P) or other environmental factors (Beardall et al., 2001 and references therein). Since most new N production in the ocean's surface is associated with upwelling of deep waters, increased stratification may lead, especially in mid and high latitudes, to a reduction in the standing nitrate concentration (Doney, 2006). N is required by cells in constrained stoichiometric ratios with C (Giordano et al., 2005b; Norici et al., 2011; Jebsen et al., 2012; Giordano, 2013), due to the fact that adequate C:N ratios are necessary to make amino acids (C:N = 53:17; Granum et al., 2002; Norici et al., 2011), protein (C:N = 53.4:16.3; Geider and La Roche, 2002), and nucleic acids (C:N = 32.7:14.5; Sterner and Elser, 2002). N limitation may thus hamper protein synthesis, growth rate, and contextually, C allocation and acquisition (Palmucci et al., 2011; Giordano, 2013).

The amount of rubisco, the main photosynthetic carboxylase, is closely related to N availability (Losh et al., 2013) and photosynthetic carbon fixation and strongly depends on rubisco amount (Spreitzer and Salvucci, 2002; Reinfelder, 2011). The impact of N limitation on photosynthesis may also be exerted through the CCMs, via the indirect effect it has on the synthesis of CCM-related proteins (e.g., carbonic anhydrases, pyrenoid components, inorganic carbon transporters) (Beardall and Giordano, 2002; Raven et al., 2011, 2012). It should, however, be noted that a number of algal species do not down-regulate C fixation to match a reduced availability of N (Raven et al., 2005; Domenighini and Giordano, 2009; Palmucci et al., 2011; Li et al., 2012; Palmucci and Giordano, 2012). These organisms may thus maintain a larger sink for electrons associated with C fixation that may be functionally relevant in the case of exposure to supra-optimal irradiance.

Light fluctuations happen frequently in marine environments due to mixing caused by wind or tides or changes of seawater gravity or due to cloud movements (Litchman, 2000 and references therein). Although phytoplankton cells are exposed to fluctuating irradiance in situ, their photo-physiological performance in the lab is usually examined under monotonic light regimes, either with continuous light or with alternation of light and dark phases (Litchman, 2000 and references therein). Under fluctuating light, the diatoms *Skeletonema costatum* and *Stephanodiscus neoastraea* increased the number of photosynthetic units (chlorophyll *a* to produce $O_2$) but decreased their size. This was interpreted as a response aimed at collecting additional energy to support photo-acclimation, which is presumably more demanding of energy in fluctuating light than when the photon supply is constant (Kromkamp and Limbeek, 1993; Fietz and Nicklisch, 2002). In the coccolithophore *Gephyrocapsa oceanica*, photosynthetic carbon fixation rates were lower under fluctuating light than under constant light, even if the PAR radiation dose the cells received was the same in the two light regimes (Jin et al., 2013). The picoeukaryote *Pelagomonas calceolate*, instead, showed the highest maximal electron transport rate under fluctuating light (Dimier et al., 2009). From this information, a generalization is difficult, due to the different parameters measured and the different nutritional regimes used as a background for the experiments.

The nutritional background has rarely been considered in the few studies conducted so far. Consequently, very little is known on the combined effects on phytoplankton of fluctuating light, nitrate availability, and $CO_2$ concentration. This study intends to contribute to filling this gap in the current knowledge. We decided to address this matter in one of the less studied groups of phytoplankton, the dinoflagellates. We selected *Prorocentrum micans* Ehrenberg, as our experimental organism. *P. micans* is common and a recurrent bloom constituent of coastal phytoplankton (Vernet et al., 1989). Although seldom studied, dinoflagellates are an important component of many aquatic ecosystems and are major contributors to the global biological carbon pump (Raven and Johnston, 1991). Previously, it was shown that growth, photosynthetic, and dark respiration rates increased significantly upon exposure of the dinoflagellate *Protoceratium reticulatum* to $pCO_2$ of 1000 and 5000 μatm, in a medium buffered at pH 8.0 (Montechiaro and Giordano, 2010). However, both growth and photosynthetic rates of the dinoflagellates *Amphidinium carterae* and *Heterocapsa oceanica* were fully inhibited when these algae were cultured at a $pCO_2$, causing the pH to decline to 7 (Dason and Colman, 2004). *Scrippsiella trochoidea* and *Alexandrium tamarense* were instead not affected by elevated $CO_2$ (Eberlein et al., 2014). Building on the scant existing literature on dinoflagellate responses to elevated $CO_2$ (Montechiaro and Giordano, 2010; Eberlein et al., 2014), N assimilation (Harrison, 1976; Dixon and Holligan, 1989; Lomas and Glibert, 1999; Clark et al., 2002) and photo-biological performance (Prézelin and Matlick, 1983; Lindström, 1984; Hartz et al., 2011), we hypothesize that the size of the electron sinks driven by $CO_2$ and $NO_3^-$ acquisition and assimilation affects the responses of *P. micans* to light fluctuation and photon flux density.

## Materials and methods

### Culture condition

The marine dinoflagellate *Prorocentrum micans* (CCMBP1883), originally isolated from the South China Sea, was obtained from

the Center for Collections of Marine Bacteria and Phytoplankton (Xiamen, China). In preparation for the experiments, the algae were grown in 1000 mL Erlenmeyer flasks containing 800 mL of media (described below), at 20 °C, under a 12 h light (photosynthetically active radiation [PAR] = 100 μmol $m^{-2}$ $s^{-1}$ with cool white fluorescent tubes) – 12 h dark (12L:12D) cycle. The growth medium was prepared according to the f/2 recipe of Guillard and Ryther (1962). In the high N treatment (HN), the $NO_3^-$ concentration (883 μmol $L^{-1}$) of the original f/2 recipes was used; for the low N treatment (LN), the amount of $NO_3^-$ was decreased to 1 μmol $L^{-1}$. Both the HN and LN cultures were continuously aerated (350 mL $min^{-1}$) with gas phases containing either 390 μatm (ambient $CO_2$, LC) or 1000 μatm (high $CO_2$, HC) $CO_2$, in a $CO_2$ plant chamber (HP1000G-D, Wuhan Ruihua Instrument and Equipment Co. Ltd., Wuhan, China), with $pCO_2$ variations <4%. Cells were acclimated to each of these four conditions (HNLC, HNHC, LNLC, LNHC) for not less than 20 generations, prior to their utilization for the experiments. During the acclimation phase, all cultures were diluted every 24 h with a dilution rate of 37.5% $d^{-1}$ (and thus an approximate growth rate of 0.47 $d^{-1}$), maintaining the cultures at low cell concentration (500–800 cell $mL^{-1}$), to minimize changes in nutrient concentration and seawater carbonate chemistry (Supplementary Table S1). A Benchtop pH 510 pH meter (OAKTON, USA) was used to measure the pH in the cultures. The pH meter was calibrated daily, before and after dilution, with standard buffers (Hanna). Other parameters of the seawater carbonate system (Supplementary Table S1) were calculated using the CO2SYS software (Lewis and Wallace, 1998) taking into account salinity (35 psu), total dissolved inorganic carbon (DIC), pH, nutrient concentrations, and temperature (20 °C). Total DIC was measured with a DIC analyzer (AS-C3, Apollo SciTech, USA). The equilibrium constants $K_1$ and $K_2$ for carbonic acid dissociation and $K_B$ for boric acid were determined according to Roy et al. (1993) and Dickson (1990), respectively.

The HNLC treatment at C100 light regime was used as the control group, since these conditions closely resemble those of the mother cultures from where all cells used for this work were obtained (100 μmol $m^{-2}$ $s^{-1}$, 12L:12D cycle, f/2 medium).

### *Experimental design*

The cells grown under the above conditions were collected at the beginning of the light period and transferred to 100 mL glass tubes. The tubes were subjected to four different light treatments with cool white fluorescent tubes, for a period of 6 h: (1) continuous exposure to PAR at a PFD of 100 μmol $m^{-2}$ $s^{-1}$ (C100); (2) continuous exposure to 500 μmol $m^{-2}$ $s^{-1}$ (C500); (3) fluctuating light between a PFD of 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min (F15); (4) as in (3) but with a frequency of 60 min (F60). We chose 500 μmol $m^{-2}$ $s^{-1}$ as the high light level, because the cruise data we obtained for South China Sea (not published) indicate that the average daily PAR at the surface was about 500 μmol $m^{-2}$ $s^{-1}$, when the surface seawater temperature was about 20 °C as in our culture condition. Light fluctuation was enforced by manually adding or removing neutral filters between the light source (cool fluorescent lamp) and the tubes. For the two treatments at continuous light and the F60 regime, measurements of the effective quantum yield of PSII ($F'_v/F'_m$) were performed every 15 min for the first 2 h, then every 30 min. The measurements for the F15 treatment were conducted every 15 min for the entire duration of the experiment. After 6 h, the cells incubated at a constant PFD of 500 μmol $m^{-2}$ $s^{-1}$ were exposed to 100 μmol $m^{-2}$ $s^{-1}$ and those at 100 μmol $m^{-2}$ $s^{-1}$ were exposed to 500 μmol $m^{-2}$ $s^{-1}$. Measurements were then carried out for further 3 h, every 15 min for the first two data points, after 30 min for the third point and then every hour. For the C100 and C500 treatments, all measurements were carried out on 6 independent cultures. In the case of the F15 and F60 treatments, measurements were effected on 3 independent cultures.

Additional experiments conducted on the cells acclimated to growth at 100 μmol $m^{-2}$ $s^{-1}$ and at all $CO_2$ and $NO_3^-$ growth regimes were used for this study: cells acclimated to growth at each of the four combinations of $pCO_2$ and $NO_3^-$ concentration (HNLC, HNHC, LNLC, LNHC) were collected every hour for the first 14 h and then at 24 h, in a 12:12 light:dark period. The chlorophyll *a*, carotenoid and MAAs contents were measured at time 0, 3, and 6 h (time zero being the time lights were switched on) in order to investigate acclimation responses.

### *Cell number and growth rates*

Cells numbers were determined with a Z2 Coulter Counter (Beckman Coulter Inc., Brea, CA, USA). The cell counts were used to construct growth curves and the growth rates were calculated from the slope of the logarithmic function describing these growth curves.

### *Chlorophyll content*

The chlorophyll content was determined after extraction in 100% methanol for 12 h, at 4 °C, in the dark. The intensity of the absorption bands at 340 nm, 480 nm, 652 nm, 665 nm, and 750 nm were measured with a DU800 UV-VIS spectrophotometer (Beckman). The amount of chlorophyll *a* was calculated using the equation of Porra (2002), the carotenoid cell content was calculated using the equation of Parsons and Strickland (1963), and the MAAs abundance was calculated using the equation of Sinha (2003).

### *Variable fluorescence measurements*

The values for the effective photochemical efficiency of PSII ($F'_v/F'_m$) were derived from variable fluorescence measurements detected with a Xe-PAM. The measurements were carried out with actinic light of 100 μmol $m^{-2}$ $s^{-1}$ and a saturating white light pulse of 5000 μmol $m^{-2}$ $s^{-1}$ in 0.8 s (Walz, Effeltrich, Germany). The samples were collected from the culture bottles with a sterile syringe, which for the measurements of cells exposed to 100 μmol $m^{-2}$ $s^{-1}$ PFD was wrapped in the same neutral filters that were around the bottles. The measurements were conducted immediately after sampling. The fluorescence parameters were estimated according to Genty et al. (1990). The effective quantum yield ($F'_v/F'_m$) was calculated as:

$$F'_v/F'_m = (F'_m - F_t)/F'_m$$

where $F'_m$ is the maximal fluorescence in the presence of actinic light (same PFD used for growth), and $F_t$ is the fluorescence at growth irradiance. The non-photochemical quenching (NPQ) was estimated according to the following equation:

$$NPQ = (F_m - F'_m)/F'_m$$

### *Statistics*

The results were expressed as the mean of all measurements and their standard deviations. Interactive effects of light, $NO_3^-$ and $CO_2$ on growth and $F'_v/F'_m$ were statistically analyzed using a 3-way ANOVA test to detect two- and three-way interactions among the variables. The post hoc Tukey's test was used to determine the difference between the means. The limit of significance was set at

**Table 1**
Significance test results of photosystem II quantum yield ($F'_v/F'_m$) and growth rates for effects among $NO_3^-$, $CO_2$ and light during the experiment periods based on the 3-way ANOVA (Tukey's post hoc tests). The limit of significance was set at 95%. The bold numbers indicate significant difference.

| Source of variation | df | Post hoc test results Growth rate $F'_v/F'_m$ | |
|---|---|---|---|
| $NO_3^-$ (low vs. high) | 1 | **low < high** | **low < high** |
| $CO_2$ (low vs. high) | 1 | 0.643 | **low > high** |
| Light (the four light regimes) | 3 | ***p* < 0.001** | ***p* < 0.001** |
| Light * $NO_3^-$ | 1 | ***p* < 0.001** | ***p* < 0.001** |
| Light * $CO_2$ | 1 | ***p* = 0.002** | ***p* < 0.001** |
| $NO_3^-$ * $CO_2$ | 1 | *p* = 0.641 | ***p* < 0.001** |
| Light * $NO_3^-$ * $CO_2$ | 3 | *p* = 0.486 | ***p* < 0.001** |

**Table 2**
Significance test results of growth rates for $NO_3^-$ and $CO_2$ under C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min) light regimes during different periods (0–3, 3–6 and 0–6 h), based on the 2-way ANOVA (Tukey's post hoc tests). The limit of significance was set at 95%. The bold numbers indicate significant difference.

| Light | 0–3 h | | 3–6 h | | 0–6 h | |
|---|---|---|---|---|---|---|
| | $NO_3^-$ | $CO_2$ | $NO_3^-$ | $CO_2$ | $NO_3^-$ | $CO_2$ |
| C100 | **0.009** | **0.001** | 0.139 | **0.013** | 0.178 | 0.744 |
| C500 | **0.003** | **0.001** | **0.006** | **0.009** | **<0.001** | **0.009** |
| F15 | 0.426 | 0.056 | 0.437 | **0.005** | 0.133 | 0.757 |
| F60 | 0.164 | 0.363 | **0.005** | 0.762 | **0.036** | 0.14 |

95%. All tests were conducted with the SPSS software 16.0 (IBM, USA) (Tables 1–4).

## Results

### *Impact of nutritional conditions at constant light levels*

*C100 treatment* – When *P. micans* was cultured at a PFD of 100 μmol $m^{-2}$ $s^{-1}$ (C100), its growth rates averaged over the first 6 h were not appreciably affected by the nutritional conditions (Fig. 1A). Interestingly, if this period is divided into two three hour portions, a more complex picture of the interactions between nutritional and light treatments emerges: cells grown at concomitantly high concentrations of $NO_3^-$ and $CO_2$ (HNHC) showed by far the highest maximum growth rate in the first three hours and maintained similar rates for the subsequent period (with a slight decline) (Fig. 1B and C). In all other treatments, the growth rate was appreciably lower in the first three hours of light than in the ensuing three hours period. The cultures maintained at high [$NO_3^-$] and low [$CO_2$] (HNLC) showed the largest increase of growth rate in the period between hours 3 and 6. During this period, they grew faster than all other cultures. At HN, HC slightly increased the effective quantum yield ($F'_v/F'_m$; *p* = 8.98E−6; Fig. 2C). However, at LN, HC reduced $F'_v/F'_m$ and did so increasingly with the passing of time (Fig. 2D).

*C500 treatment* – When grown at a PFD of 500 μmol $m^{-2}$ $s^{-1}$ (C500), the HNHC cultures were the only ones able to grow, and at a very high rate (0.76 $d^{-1}$), in the initial light phase (Fig. 1B). In all other conditions, cells tended to die, in the first 3 h, but the cultures then recovered in the ensuing 3 h (Fig. 1B and C). Over the whole duration of the experiment, the concomitant availability of elevated concentrations of $NO_3^-$ and $CO_2$ (HNHC) stimulated growth rate as compared to the LNLC, and the growth rate of HNHC cells was fairly constant (Fig. 1). A comparison between the results for C100 and C500 shows that, even when the PFD was 100 μmol $m^{-2}$ $s^{-1}$, light was not limiting growth and 500 μmol $m^{-2}$ $s^{-1}$ was a supra-optimal light intensity (Fig. 1).

In HNLC, the $F'_v/F'_m$ value was initially significantly lower than in HNHC (Fig. 2A and C). The detrimental effect of low [$CO_2$] combined with high [$NO_3^-$], however, faded away with time: in the 3–6 h period, LC cells showed similar or higher growth rates than the HC cells (Fig. 1C). Also the effective quantum yield of HNLC cells recovered (within about an hour), to reach values similar to those of HNHC cells. In the C500 cultures grown in the presence of high $NO_3^-$ concentration, differently from the C100 treatment, there was no significant difference between LC and HC cells with respect to their $F'_v/F'_m$ (*p* = 0.908), after the $F'_v/F'_m$ values reached a steady state (Fig. 2A and C).

In LN, however, the $F'_v/F'_m$ values of LNHC were appreciably lower than in LNLC, with a slight downward trend (Fig. 2B and D). In general, HC decreased $F'_v/F'_m$ and increasingly so as the treatment continued (Figs. 2D and 3). Comparing the HN and LN cell, the LN ones had $F'_v/F'_m$ values invariably much lower than in HN (Figs. 2 and 4), although the growth rate in LN, after the first three hours of illumination, was significantly lower than that in HN only at low $CO_2$.

HC cells had higher NPQ then cells cultured in LC under both the C100 and the C500 treatments; this was especially obvious in LN cells (Fig. S1).

### *Impact of light fluctuations*

When we applied a light fluctuation between 100 and 500 μmol $m^{-2}$ $s^{-1}$, the outcomes of the treatments differed from C100 and C500 and depended on the frequency of the fluctuations, especially for the 3–6 h period (Fig. 1C).

**Table 3**
Significance test results of growth rates for different light regimes: C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min) during different periods (0–3, 3–6 and 0–6 h), based on the 3-way ANOVA (Tukey's post hoc tests). The limit of significance was set at 95%. The bold numbers indicate significant difference.

| | 0–3 h | 3–6 h | 0–6 h |
|---|---|---|---|
| C100 vs. C500 | **<0.001** | 0.973 | **0.006** |
| C100 vs. F15 | 0.47 | 0.768 | 0.169 |
| C100 vs. F60 | **0.002** | **<0.001** | 0.271 |
| C500 vs. F15 | **<0.001** | 0.941 | **<0.001** |
| C500 vs. F60 | **<0.001** | **<0.001** | **<0.001** |
| F15 vs. F60 | **0.047** | **<0.001** | 0.987 |

**Table 4**
Significance test results of photosystem II quantum yield ($F'_v/F'_m$) for different light regimes: C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), low light (F15L) and high light (F15H) period of F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and low light (F60L) and high light (F60H) period of F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min), based on the 3-way ANOVA (Tukey's post hoc tests). The limit of significance was set at 95%. The bold numbers indicate significant difference.

| Factors | Post hoc test results |
|---|---|
| C100 vs. C500 | ***p* < 0.001** |
| C100 vs. F15L | ***p* < 0.001** |
| C100 vs. F15H | ***p* < 0.001** |
| C100 vs. F60L | ***p* < 0.001** |
| C100 vs. F60H | ***p* < 0.001** |
| C500 vs. F15L | ***p* < 0.001** |
| C500 vs. F15L | ***p* < 0.001** |
| C500 vs. F60L | ***p* < 0.001** |
| C500 vs. F60L | ***p* < 0.001** |
| F15L vs. F60L | ***p* < 0.001** |
| F15L vs. F60H | ***p* < 0.001** |
| F15H vs. F60H | ***p* = 0.785** |

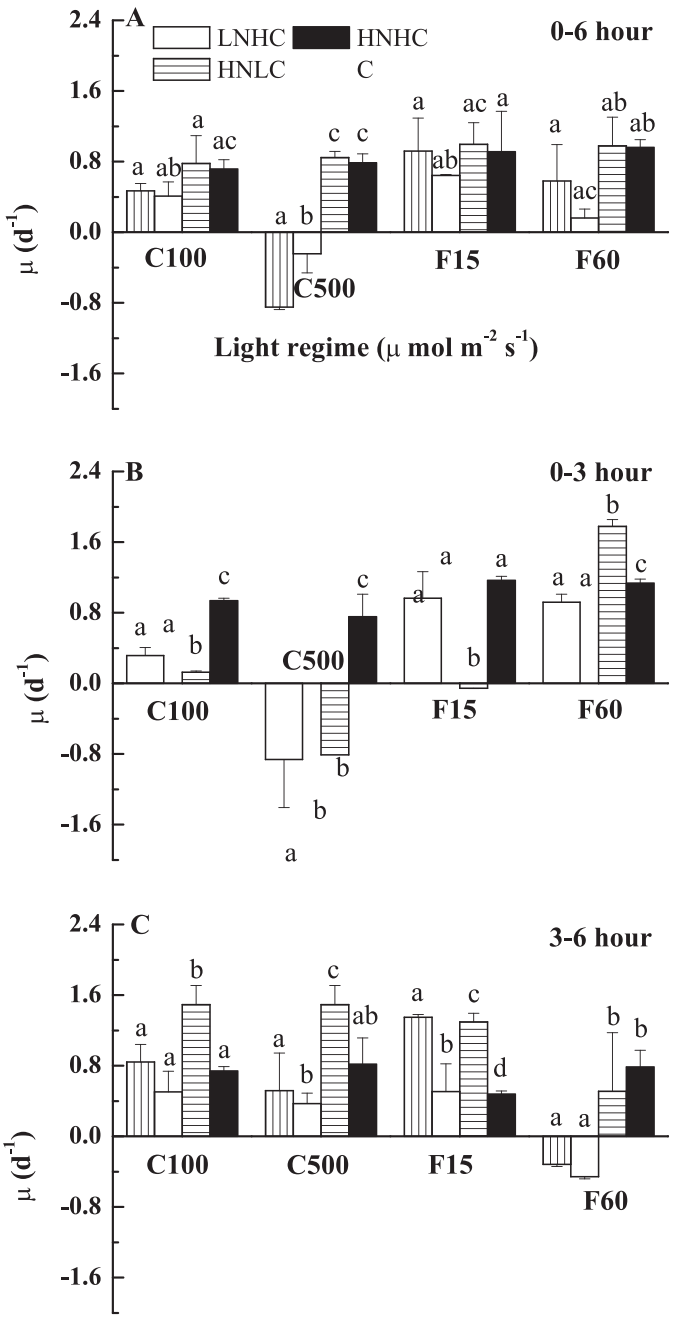


**Fig. 1.** Growth rates (μ, $d^{-1}$) of LNLC- (low $NO_3^-$ low $CO_2$), LNHC- (low $NO_3^-$ high $CO_2$), HNLC- (high $NO_3^-$ low $CO_2$) and HNHC- (high $NO_3^-$ high $CO_2$) cells of *Prorocentrum micans* under C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min) light regimes, over the period of 0–6 h (A), 0–3 h (B) and 3–6 h (C) since illumination. The error bars represent the standard deviation of three or six independent measurements. Different letters indicate significant differences among the four different nutrient ($NO_3^-$ and $CO_2$) cultivations within different light treatments, using Tukey's post hoc tests.

*F15 treatment* – In the F15 treatment, the specific growth rate apparently increased in the initial 3 h under elevated $CO_2$, but decreased in the ensuing 3 h (Fig. 1A and B). The effective quantum yield measured in the fluctuation treatments was consistently lower at 100 μmol $m^{-2}$ $s^{-1}$ than in the C100 treatment and consistently higher at 500 μmol $m^{-2}$ $s^{-1}$ than in the C500 treatments (Fig. 2). The difference in the yield at 500 μmol $m^{-2}$ $s^{-1}$ between cells exposed to continuous (C500) and fluctuating light (regardless of the fluctuation frequency) was usually modest. The difference in the yield at 100 μmol $m^{-2}$ $s^{-1}$ of C100 cells and of cells subjected to light fluctuation was sometimes substantial and increased according to the sequence HNLC < HNHC < LNLC < LNHC (Fig. 2).

In the initial phase (0–3 h) the HNLC F15 cells did not show any growth (Fig. 1B), and the fluorescence data did not seem to show any especially pronounced constraints on these cells at the beginning of the light period. On the contrary, they appeared to have a somewhat higher photosynthetic yield and lower NPQ than cells in all other treatments, when they were exposed to both 100 and 500 μmol $m^{-2}$ $s^{-1}$ (Figs. 2 and S1). In the following three hours, however, the HNLC F15 cells showed the highest growth rate (together with LNLC F15 cells).

In the 3–6 h period, growth of HC F15 cells was depressed, as compared to the preceding period and to the LC cells. This was accompanied by a gradual decline in the effective quantum yield at 100 μmol $m^{-2}$ $s^{-1}$ (especially evident in LNHC cells). This decline in $F'_v/F'_m$ did not correlate with a decline in $F'_o$ (Fig. S2).

*F60 treatment* – The 0–3 h panel of Fig. 1 shows that cells from all nutritional conditions (especially HNLC) grew fairly well in the first part of the F60 treatment. However, growth rates dramatically decreased in the 3–6 h period, especially for the LN-grown cells that were unable to grow at all. At HN, the quantum yield at 100 μmol $m^{-2}$ $s^{-1}$ was very close to that obtained for continuous light at this PFD. Interestingly, the presence of high [$CO_2$] exacerbated the NPQ increase at low N, and appreciably more so in the F60 than in the F15 treatment (Fig. S1).

Even under the control light regimes (C100), different amounts of photoprotective pigments were observed among the four nutrient treatments. HN and HC both increased the contents of chlorophyll *a* and MAAs (Fig. 5B and D); for the carotenoid, HC did not affect it under LN condition but increase it in the presence of high $NO_3^-$ concentration (Fig. 5C).

## Discussion

Our results show that a complex interplay exists between PFD fluctuation and nutrient availability (specifically of inorganic C and $NO_3^-$) in determining the growth and $F'_v/F'_m$ responses of the dinoflagellate *P. micans*. The outcome of our work can be summarized as follows:

(1) Under constant light regimes, the initial portion of the light phase required the concomitant presence of high $CO_2$ and $NO_3^-$ concentrations for maximum growth (in C500, only HNHC conditions allowed growth).
(2) After exposure to light for 3 h, high $CO_2$ exerted a negative effect on growth and $F'_v/F'_m$.
(3) Fluctuating light ameliorated light responses in terms of growth and photosynthetic light reactions, in the first period of illumination. In the second 3 hours of treatment, higher frequency (F15) of fluctuations afforded higher growth rates, whereas lower light fluctuation frequency (F60) had detrimental consequences, especially when $NO_3^-$ concentration was lower.

In the following paragraphs we shall attempt to find explanations for these phenomena, although we are fully aware that our data can only offer a partial picture of cell responses and more experiments, presently beyond our possibilities, are needed to fully clarify the mechanisms through which nutrient availability and light regime interact.

The advantage provided by HNHC conditions in the first three hours of light does not appear to be associated with the overcoming of $CO_2$ limitation: in the longer period, HNLC cells grew faster than their high $CO_2$-grown counterpart. Some hints on the cause of

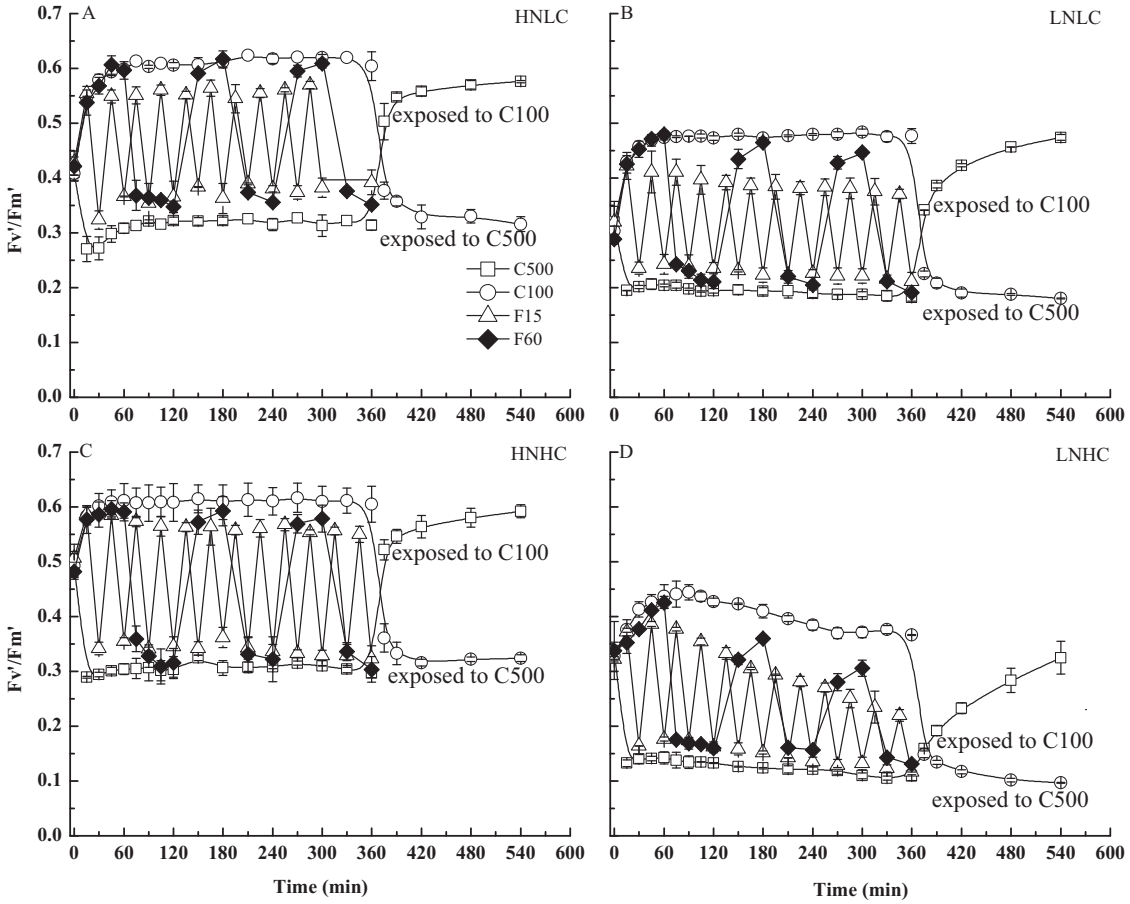


**Fig. 2.** Photosystem II quantum yield ($F'_v/F'_m$) of *Prorocentrum micans* cells grown at (A) HNLC (high $NO_3^-$ low $CO_2$), (B) HNHC (high $NO_3^-$ high $CO_2$), (C) LNLC (low $NO_3^-$ low $CO_2$) and (D) LNHC (low $NO_3^-$ high $CO_2$), under C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min) light regimes, over 360 min. After this period, the C500 cells were exposed to the C100 regime, and the C100 cells to C500 regime, for 180 min. The error bars represent the standard deviation of three or six independent measurements.

this requirement for high $CO_2$ and high $NO_3^-$ during the early light phase may be found in the results for the effective quantum yield of PSII (Fig. 2): in C100 and F60, the $F'_v/F'_m$ values of HNHC cells reached a steady state after about 30 min of exposure to 100 μmol $m^{-2}$ $s^{-1}$; in all other nutritional conditions, over an hour was required for stabilization of $F'_v/F'_m$. "Ad hoc" experiments are needed to precisely identify the mechanism by which this rapid acclimation is obtained.

The requirement for high $CO_2$ and $NO_3^-$ availability may simply be the consequence of the need for promptly available electron sinks, during light acclimation and of incomplete expression of

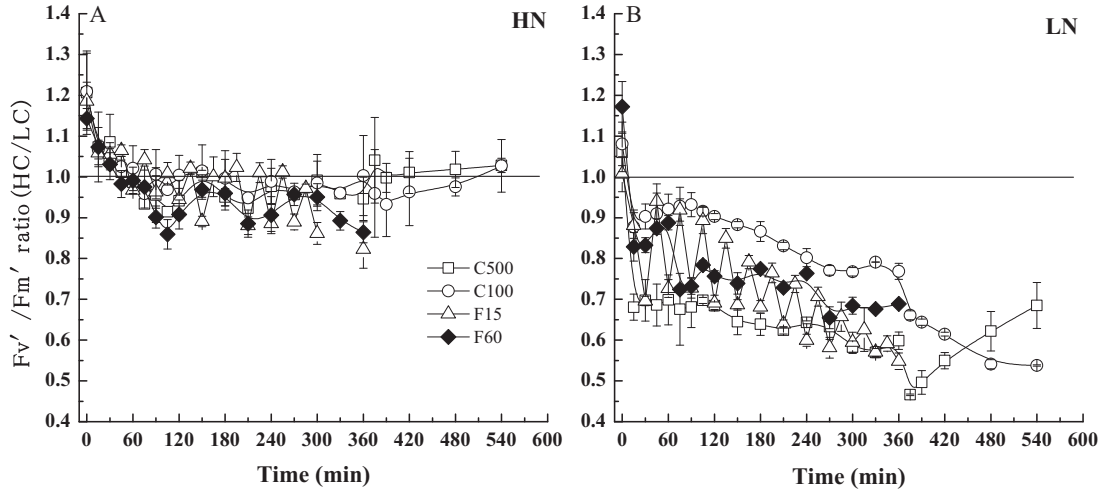


**Fig. 3.** $F'_v/F'_m$ ratio of HC (high $CO_2$) vs. LC (low $CO_2$) grown *Prorocentrum micans* cells at (A) HN (high $NO_3^-$) and (B) LN (low $NO_3^-$) cultivation conditions, under C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min) light regimes, over 360 min. After this period, the C500 cells were exposed to the C100 regime, and the C100 cells to C500 regime, for 180 min. The error bars represent the standard deviation of three or six independent measurements.

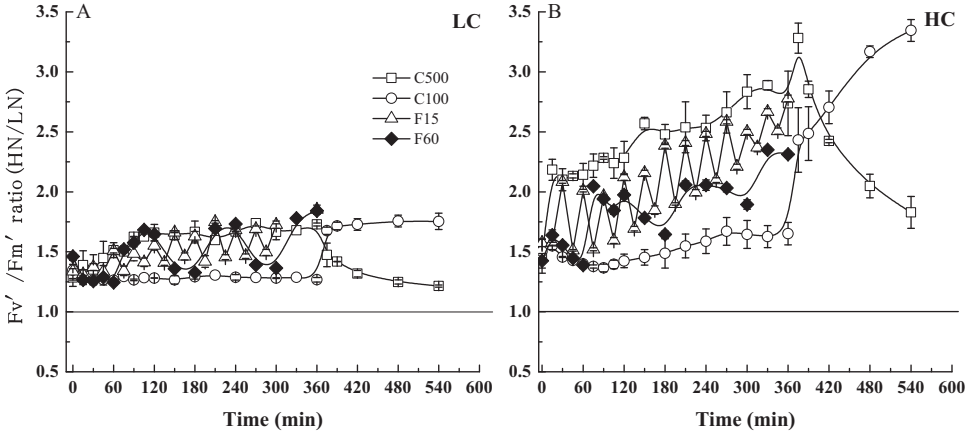


**Fig. 4.** $F_v'/F_m'$ ratio of HN (high $NO_3^-$) vs. LN (low $NO_3^-$) grown *Prorocentrum micans* cells at (A) LC (high $CO_2$) and (B) HC (low $CO_2$) cultivation conditions, under C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$), C500 (constant light of 500 μmol $m^{-2}$ $s^{-1}$), F15 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 15 min) and F60 (fluctuating light between 100 and 500 μmol $m^{-2}$ $s^{-1}$ with a frequency of 60 min) light regimes, over 360 min. After this period, the C500 cells were exposed to the C100 regime, and the C100 cells to C500 regime, for 180 min. The error bars represent the standard deviation of three or six independent measurements.

protection and repair mechanisms (Melis, 1999; Takahashi and Murata, 2008; Raven et al., 2011). It should be considered that dinoflagellates $CO_2$ concentrating mechanisms (CCMs) are rather inefficient (Ratti et al., 2007), thus $CO_2$ passive diffusion becomes rapidly important with increasing ambient $CO_2$. The passive influx of $CO_2$ would, on one hand, reduce energy dissipation through energy-dependent Ci acquisition (but see Raven et al., 2014). On the other hand, it may allow a prompter response, since the complex light-dependent CCMs (Giordano et al., 2005a) would not be required to support $CO_2$ reduction in the Calvin cycle. The slow

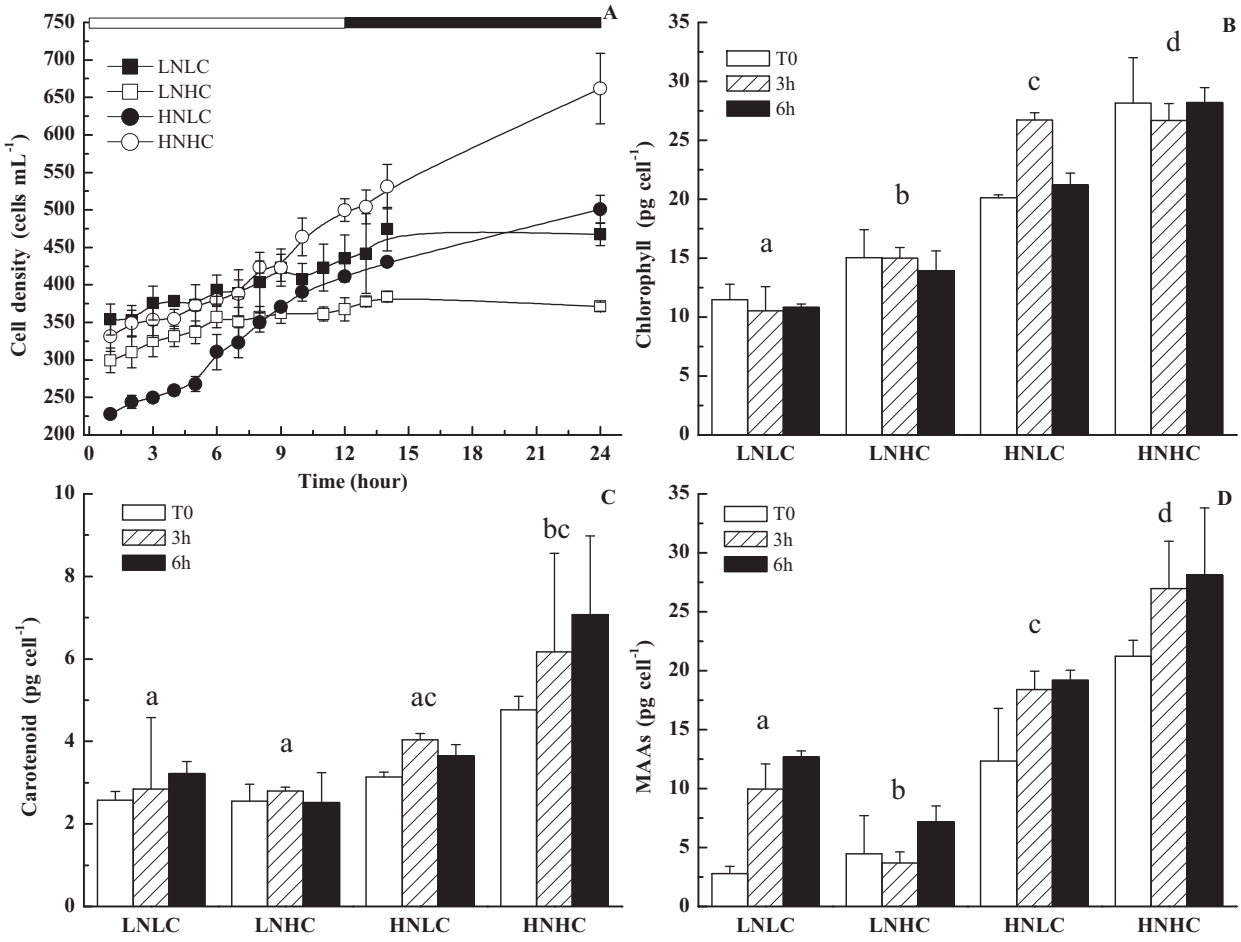


**Fig. 5.** (A) cell concentration (cells $mL^{-1}$) in LNLC (low $NO_3^-$ low $CO_2$), LNHC (low $NO_3^-$ high $CO_2$), HNLC (high $NO_3^-$ low $CO_2$) and HNHC (high $NO_3^-$ high $CO_2$) cultures of *Prorocentrum micans* under C100 (constant light of 100 μmol $m^{-2}$ $s^{-1}$) light regime in 24 h. (B) chlorophyll a content (pg $cell^{-1}$), (C) carotenoids content (pg $cell^{-1}$) and (D) MAAs (pg $cell^{-1}$) of LNLC-, LNHC-, HNLC- and HNHC cultures under the C100 light regime at the beginning of the light phase (T0), and after 3 (3 h) and 6 (6 h) hours in the light. The error bars represent the standard deviation of three independent biological replicates ($n = 3$). Different letters indicate significant differences among the four different nutrient ($NO_3^-$ and $CO_2$) cultivations, using Tukey's post hoc tests.

operation of CCMs, whose full expression typically takes a few hours in microalgae (e.g., Renberg et al., 2010; Brueggeman et al., 2012), may also concur to the poor growth performance of HNLC cells: initially, $CO_2$ supply could limit the utilization of reducing power (i.e., the consumption of electrons) at the Calvin cycle and thus make the accumulation of excess electrons on the photosynthetic apparatus more likely. The observed decrease of the expression of proteins implicated in light capture and photo-protection, during CCM induction (Brueggeman et al., 2012; Fang et al., 2012), may also contribute to the sufferance of the low $CO_2$-grown cells in the initial period of illumination. The requirement for high N in LC cells may be associated with (a) structural deficiencies of the photo-protective apparatus (as suggested by the data reported in Fig. 5), (b) the role of $NO_3^-$ reduction as electron sink (Giordano and Raven, 2014), or (c) the requirement of N for C fixation (Kaffes et al., 2010); also see below). When photo-protection, $CO_2$ fixation and $NO_3^-$ reduction are down-regulated, $F'_v/F'_m$ may become very sensitive to excess electrons, even at relatively low PFD.

In LNHC, although $CO_2$ influx is presumably high, its fixation is presumably down-regulated to match the low supply rate of N (Kaffes et al., 2010). The lower rates of C and N assimilation and the increase of diffusional $CO_2$ influx would lead to a concomitant decrease of the electron fluxes toward $CO_2$ fixation; $NO_3^-$ reduction and photorespiration, which constitute major dissipation pathways for excess electrons. For these reasons, when N is limiting, elevated $pCO_2$ may increase the probability for photo-inhibition, as Hymus et al. (2001) showed for the poacean *Dactylis glomerata*. The observed decline in photosynthetic efficiency ($F'_v/F'_m$) and increase in regulated energy dissipation, which is known to be very important in organisms with diatoxanthin-based xanthophyll cycle (Tóth et al., 2002; Ruban et al., 2004), is compatible with this explanation. In the energetically permissive condition of the C100 treatment, the LN cells are anyway able to attain appreciable growth rate; the sensitivity of LN cells to light becomes more obvious when they are subjected to high light for prolonged times (C500, especially in the initial light phase, and F60, after accumulation of photodamage).

In the second part of the experiment (3–6 h since the onset of illumination), $NO_3^-$ availability was crucial to sustain better $F'_v/F'_m$ and growth performances (especially under constant light regimes and F60); high $CO_2$, instead, exerted a negative effect on growth and photosynthesis. The positive effect of $NO_3^-$ is probably associated, in addition to its obvious requirement for biosynthesis, with the higher effectiveness of the repair processes in HN. In these conditions, the kinetic of recovery from high light stress, upon transfer of the cells to low light conditions, was evidently faster than in LN cells. The slow recovery of LN cells was especially visible at high $CO_2$. The causes of this are probably similar to those for which high N and high $CO_2$ were required during the early phase of the light treatments. In terms of growth, however, no obvious differences could be detected between high and low $NO_3^-$ conditions in C100, C500 and F15, cells at high $CO_2$. It cannot be excluded that, although the fluorescence data points toward a lower stress in HN, even in these conditions 1000 μatm $CO_2$ were not sufficient to saturate the type II rubisco of *P. micans*, as observed for other dinoflagellates (Ratti et al., 2007), and that, regardless of the N availability, the lower demand for electrons by photorespiration was not entirely compensated by a higher electron demand by the Calvin cycle (Hymus et al., 2001). Our results also confirm that growth and photosynthesis, at least when the latter is evaluated through fluorescence, are not necessarily coupled.

When the two fluctuation treatments are compared, interesting patterns emerge. In general, fluctuation of light between the photon flux densities of 100 and 500 μmol $m^{-2}$ $s^{-1}$ attenuated the negative impact on growth of sudden exposure to light. The positive impact of light fluctuation on growth, in the first 3 h, was more evident when the light fluctuation had a periodicity of 60 min than when the period was 15 min (HNLC cells had high growth rate in F60, but did not grow at F15). On the contrary, the F15 treatment was overall more favorable in the longer term (3–6 h period), although, as for the continuous light regime, an inhibitory effect of $CO_2$ on growth was observed, possibly for the same reasons discussed above. The gradual decline of $F'_v/F'_m$ at 100 μmol $m^{-2}$ $s^{-1}$ that accompanied the decrease in growth rate of HC F15 cells (also, although less obviously, at HN) may indicate that under these nutritional conditions, recovery from the high light induced photo damage was incomplete. The advantage offered to cell growth by the F15 treatment vs. the F60 treatment was especially obvious when $NO_3^-$ was scarce (no growth was observed in LN F60 cells).

We are fully aware that our experimental setup does not provide a perfect analog for natural light patterns and for vertical mixing in open oceans (e.g., Ross et al., 2011; Milligan et al., 2012; Neale et al., 2012). However, our results are not deprived of ecological significance: our data provide an evidence of the fact that the impact of atmospheric $pCO_2$ increase on primary production depends (a) on the availability of other nutrients, (b) on the irradiance to which cells are exposed, (c) on the frequency of irradiance fluctuations. The impact of global change on oceanic primary producers would therefore be different in region of the ocean with different chemistry and UML depth. The data obtained for different photon flux densities also show that the interaction between irradiance, $pCO_2$ and N availability are likely to be appreciably different at different times of the day and in different seasons.

## Acknowledgment

National Natural Science Foundation (Grant Nos. 4143000042; 41120164007), Joint project of NSFC and Shandong province (Grant No. U1406403), Strategic Priority Research Program of CAS (Grant No. XDA11020302), Program for Changjiang Scholars and Innovative Research Team (IRT_13R51), SOA (GASI-03-01-02-04) and China–Japan collaboration project from MOST (S2012GR0290) to KG; MIPAF (Bioforme project D.M 23553/7303/2010), Cariverona Foundation (project "Biomasse di oggi e di domani: dai reflui zootecnici e dalle microalghe un contributo all'agricoltura sostenibile e all'energia rinnovabile") and the Assemble program of the European Commission to MG. MG's visit to Xiamen was supported by "111" project from the Ministry of Education of China.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.01.020.

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