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Effects of light intensity and nitrogen starvation on growth, total fatty acids and arachidonic acid in the green microalga *Parietochloris incisa*

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Abstract The effects of light and nitrogen deficiency on biomass, fatty acid content and composition were studied in *Parietochloris incisa*, the unicellular freshwater chlorophyte accumulating very high amounts of arachidonic-acid-rich triacylglycerols. *P. incisa* cultures grown on complete nutrient medium and under high light (400 μmol photons m^{-2} s^{-1}) showed the highest rate of growth in comparison to medium (200 μmol photons m^{-2} s^{-1}) and low (35 μmol photons m^{-2} s^{-1}) light intensity. Cultures grown under high light (on complete BG-11 medium) attained higher volumetric contents of total fatty acids and arachidonic acid due to greater increase in biomass. Nitrogen starvation brought about a strong increase in the arachidonic acid proportion of total fatty acids. Thus, adjustments to cultivation conditions could serve as an efficient tool for manipulation of yield and relative content of arachidonic acid in *P. incisa*. The significance of the changes in lipid metabolism for adaptation of *P. incisa* to high-light stress and nitrogen deficiency is also discussed.

Keywords Alga cultivation · Lipids · Microalgal biotechnology · Photoadaptation

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Abbreviations

AA	Arachidonic acid
DW	Dry weight
FA	Fatty acids
PFD	Photon Flux Density
TAG	Triacylglycerols
TFA	Total fatty acids
PUFA	Polyunsaturated fatty acids

Introduction

Photoautotrophic microalgae are often considered as potential producers of valuable polyunsaturated fatty acids (PUFA) suitable for large-scale photobiotechnology (Thompson 1996; Cohen 1999; Molina-Grima et al. 1999; Sukenik 1999). Under nitrogen starvation, many algal species were reported to accumulate lipids (mostly triacylglycerols [TAG]) (Shifrin and Chisholm 1981), which generally contain saturated and monounsaturated fatty acids (FA) (Piorreck et al. 1984; Cohen 1986; Henderson and Sargent 1989; Bigogno et al. 2002a, b). The oleaginous freshwater alga *Parietochloris incisa* comb. nov (Chlorophyta, Trebouxiophyceae) is one of the very few microalgae accumulating PUFA-rich TAG and is known as the richest plant source of arachidonic acid (AA). Under nitrogen-starvation, *P. incisa* enhances TAG biosynthesis and TAG account for over 30% of dry weight (more than 95% of total lipids), with AA comprising as much as 60% of total fatty acids (TFA) (Khozin-Goldberg et al. 2002; Merzlyak et al. 2007). AA is accumulated mainly in TAG deposited in cytoplasmic oil bodies (Bigogno et al. 2002a, b; Khozin-Goldberg et al. 2002).

The optimization of cultivation conditions from the standpoint of both AA and biomass production turned out to be a nontrivial problem. Thus, experiments with outdoor cultivation of *P. incisa* showed that light intensities of 250 μmol photons m^{-2} s^{-1} were limiting for algal growth but beneficial for AA accumulation, whereas high light (2,500 μmol photons m^{-2} s^{-1}) facilitated rapid growth but with low AA content in biomass (Cheng-Wu et al. 2002). Therefore the responses of lipid (FA) metabolism to combined high light and nitrogen-deficiency stresses are of particular interest for establishing the photobiotechnology of this alga, as AA is a valuable nutraceutical, being one of the major FA of brain cell phospholipids and precursor of eicosanoids (Koletzko and Braun 1991; Hansen et al. 1997).

The goal of the study was to determine the influence of light intensity and nitrogen starvation on the yield of biomass TFA and AA production under controlled conditions by *P. incisa* with particular attention paid to the effects of high light intensities (400 μE m^{-2} s^{-1}) and availability of N. Investigation of these problems will facilitate the search for an approach to optimize *P. incisa* cultivation conditions.

Experimental

Cultivation conditions

P. incisa was isolated from Mt. Tateyama in Japan (Watanabe et al. 1996). The cultures were batch cultivated on complete (+N) and nitrogen-free (-N) BG-11 medium (Stanier et al. 1971), in 1-L glass columns under constant illumination (by daylight fluorescent lamps) at three different intensities (35, 200, and 400 μmol photons m^{-2} s^{-1}) and with constant bubbling of CO_2:air mixture (1:99, v/v) at 25°C. Prior to the experiment, cultures were diluted daily to maintain logarithmic growth. In all cases, initial chlorophyll and dry weight (DW) content were maintained at 30 mg L^{-1} and 1 mg mL^{-1}, respectively. The nitrogen content in the medium was checked during the experiment using the nitrate assay kit (Merckoquant 1.10020.001, Merck, Germany). The +N cultures had retained at least half of the initial nitrogen content of the medium by the 14th day of the experiment. For nitrogen-starvation, cells were washed three times with sterilized distillated water and resuspended in -N BG-11.

Fatty acid analysis

Capillary gas chromatography was used for fatty acid quantification; the analysis was performed according to Cohen et al. (1993). Freeze-dried biomass was transmethylated with 2% H_2SO_4 in the dry methanol:toluene mixture (90:10, v/v) at 80°C for 1.5 h. Heptadecanoic acid was added as an internal standard. FA methyl esters were identified by co-chromatography with authentic standards (Sigma) and by comparison of their equivalent chain length (Ackman 1969). The data shown represent mean values with a range of less than 5% for major peaks (over 10% of fatty acids) and 10% for minor peaks, of at least two independent samples, each analyzed in duplicate.

Chlorophyll measurements

Chlorophyll content was measured in acetone extracts spectrophotometrically (Lichtenthaler 1987).

Statistical treatment

Average values of the results of three independent experiments (with two analytical replications in each) and their standard errors are presented in the figures. The significance of difference was tested using ANOVA.

Results

The time course of *P. incisa* culture growth under different conditions is plotted in Fig. 1. Cultures grown under low light intensity (LL, 35 μmol photons m^{-2} s^{-1}) showed relatively slow linear growth regardless of the presence of nitrogen (0.26 and 0.30 mg DW day^{-1} for LL-N and LL+N, respectively) and attained DW of ca. 4.2 mg ml^{-1} by the end of cultivation period (14 days), as shown in Fig. 1a. Under photon flux densities (PFDs) of 200 μmol photons m^{-2} s^{-1} (medium light, ML), the cultures grown on complete BG-11 medium (+N) possessed higher final biomass (ca. 7 mg ml^{-1} at an average growth rate of 0.39 mg DW day^{-1}) than the nitrogen-deprived (-N) cultures. The latter reached a DW similar to that of cultures grown under LL; however, in the first days their growth rate was higher (Fig. 1b). Under high light (HL, 400 μmol photons m^{-2} s^{-1}) the +N cultures reached the highest biomass concentration (ca. 8 mg ml^{-1}; Fig. 1c) and displayed the highest grow rate, 0.47 mg DW day^{-1}, whereas the biomass and the growth rate of the nitrogen-depleted culture under HL did not differ significantly from that grown under ML (Fig. 1b) or LL (Fig. 1c).

In all cultures, the proportion of AA increased with time but was always higher in the nitrogen-starved cultures. In the latter, the proportion of AA was inversely related to light intensity, reaching ca. 60% under low light. However, among the +N cultures, those grown under ML had the highest proportion of AA (47% TFA). In the HL+N cultures (Fig. 2i), the AA proportion reached a plateau after 3 days,

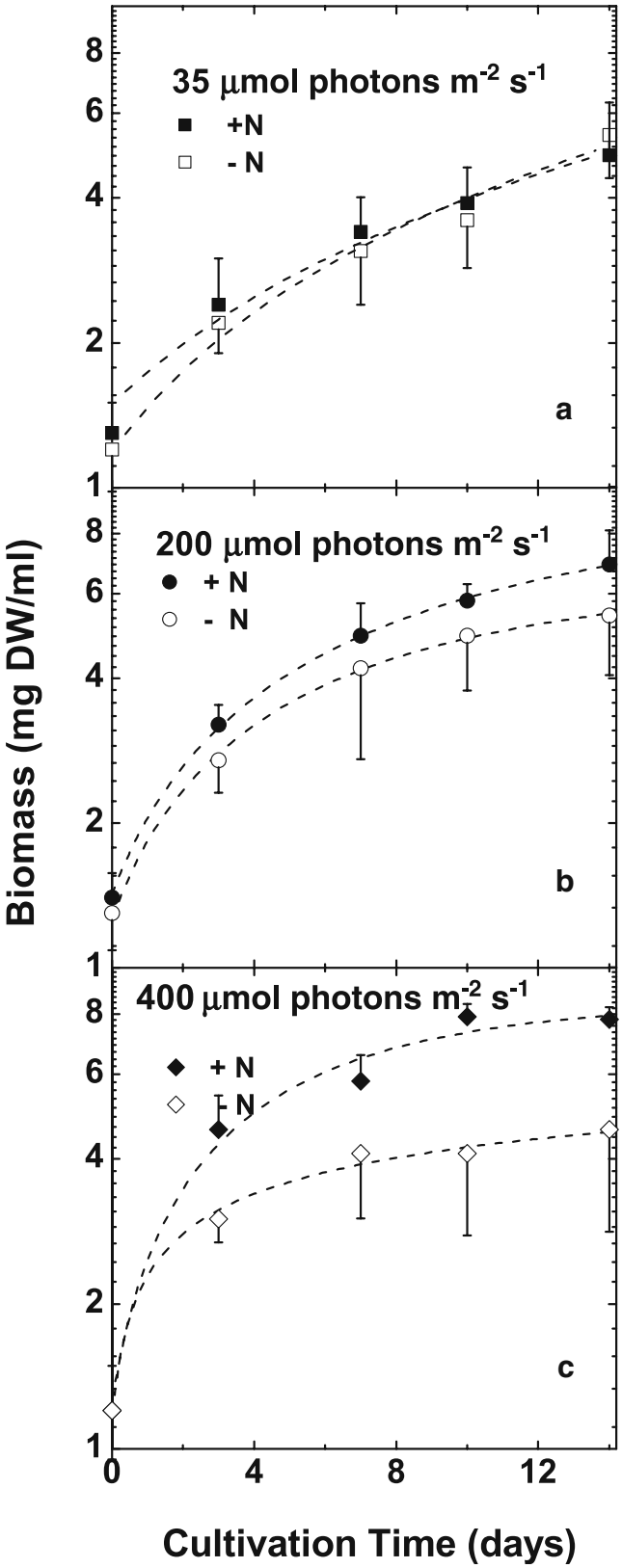

Fig. 1 Growth of *P. incisa* cultures under low (**a**), medium (**b**) and high (**c**) illumination intensity

resulting in the lowest proportion of AA (<40%). At the same time, the share of both 18:1 and 18:2 increased at the expense of 16:0 and 18:3ω3. The highest level of C18 fatty acids was achieved by the HL+N cultures (data not shown).

The TFA content also increased with time. Under LL and ML, the -N cultures accumulated more fatty acids than the +N cultures, whereas under HL, the accumulation was similar for the first 10 days, at which point it leveled off in the -N cultures but continued to increase linearly in the +N cultures (Fig. 2a,d,g). In contrast to the pattern observed for the AA proportion, TFA content increased with light intensity in all cultures, with the exception of the last days of the -N (HL) cultures. It should be noted also that nitrogen-deficient cultures grown for at least 10 days under high PFDs underwent in some experiments a drastic decrease in TFA and pigment content, which eventually led to the culture's death (data not shown).

Due to higher growth rates (Fig. 1), cultivation under HL resulted in higher contents of both TFA (ca. 250 mg l^{-1}, Fig. 3a) and AA (88 mg l^{-1}, Fig. 3b) from total biomass (i.e., volumetric contents) in the cultures grown on complete medium. Notably, HL+N cultures accumulated ca. twice as much TFA and AA as the corresponding nitrogen-depleted cultures (Fig. 3). However, under ML and LL both the TFA and the AA volumetric contents were higher in the -N cultures.

Discussion

The pattern of growth (Fig. 1) showed that under light-limiting conditions, *P. incisa* cultures exhibit a slow linear growth regardless of the presence of nitrogen (Fig. 1a). Cultivation under HL+N led to higher growth rates which changed with time, resulting in transition to curvilinear growth curves characteristic of early stationary phase by the end of cultivation period (Fig. 1b,c). The similarity of the curves in Fig. 1a suggests that at low light intensity, light rather than nitrogen availability is limiting the growth of *P. incisa*.

The data obtained for the cultures grown on complete medium (Fig. 2) are generally compatible with previous observations of aging of nitrogen-replete cultures (Khozin-Goldberg et al. 2002), which are characterized by enhanced proportion of AA and TFA content. The increase in the AA proportion during aging is believed to be, at least partially, a result of a shift from ω3 to ω6 PUFA in chloroplastic lipids that ensues after a transfer from logarithmic to stationary phase, which has been reported to occur in many microalgae, including *P. incisa* (Cohen et al. 1988; Khozin-Goldberg et al. 2002).

Previously it was shown that both the TFA content and the composition are changed along with culture growth, however,

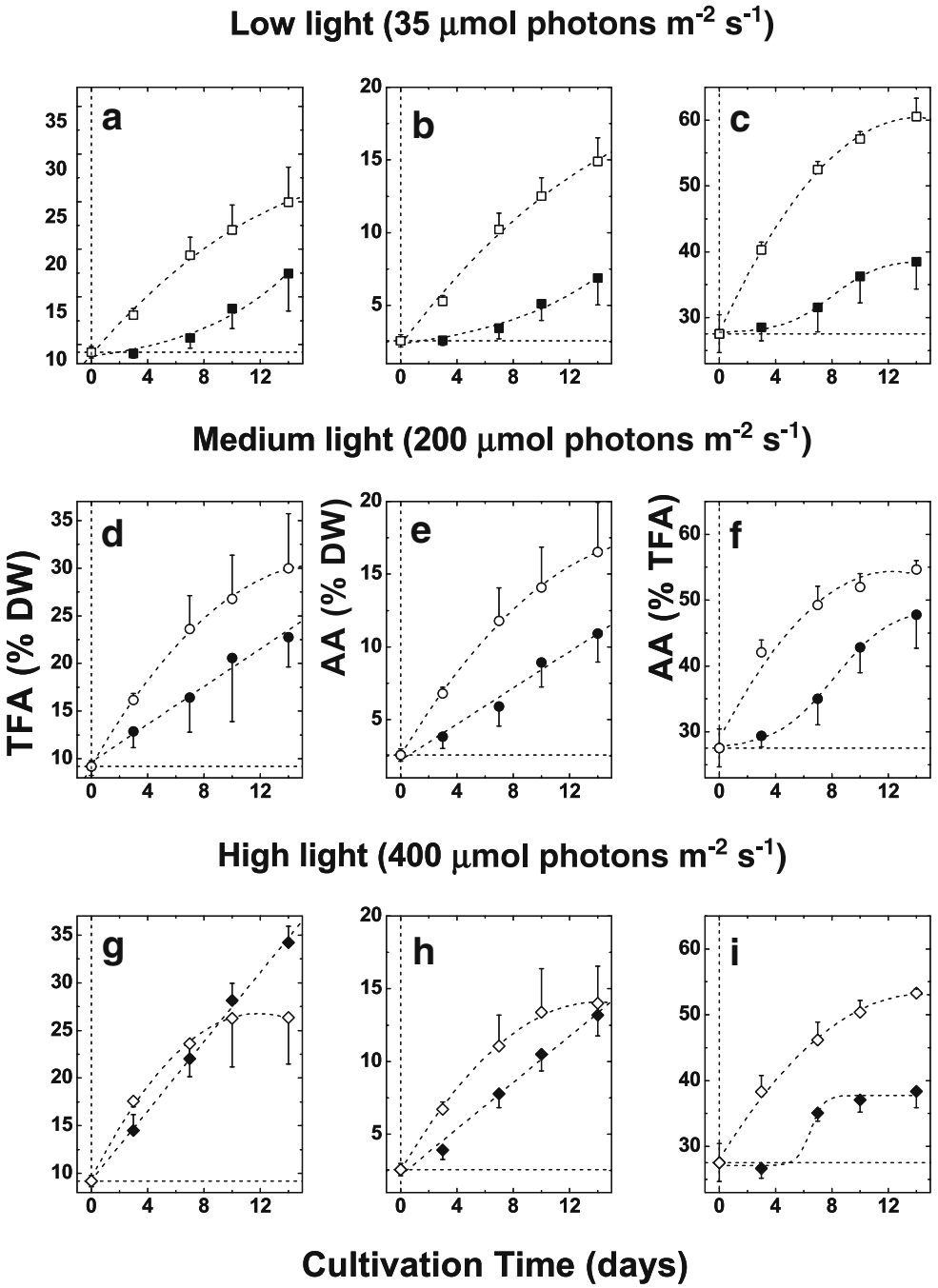

Fig. 2 Dynamics of total fatty acids (**a**, **d**, **g**) and arachidonic acid (**b**, **e**, **h**) content and arachidonic acid percentage (**c**, **f**, **i**) in *P. incisa* cells grown with (*closed symbols*) and without (*open symbols*) nitrogen, under low (**a**–**c**), medium (**d**–**f**) and high (**g**–**i**) illumination. Data presented relate to cultures described in Fig. 1

nitrogen starvation induces a larger increase in FA content than the culture growth itself (Khozin-Goldberg et al. 2002). Thus, nitrogen-deficient cultures of *P. incisa* grown under very low-light conditions for 60 days accumulated AA up to 62% of TFA (Merzlyak et al. 2007). Under conditions used by Khozin-Goldberg et al. (2002), the proportion of AA in TFA of *P. incisa* was as high as 58.9%. Therefore, in spite of a slowing of cell division or even a complete cessation, which is possible under such conditions, biomass could still increase due to net production of TAG and TFA. The increase in the TFA content was predominantly due to the accumulation of neutral lipids, which are the major depot of AA in the cell, as shown earlier (Khozin-Goldberg et al. 2002). High irradiance considerably enhanced TFA accumulation to the level achieved by the corresponding nitrogen-deplete culture (Fig. 2g).

Illumination conditions exert a considerable effect on algal FA content and composition (Figs. 2 and 3, see also

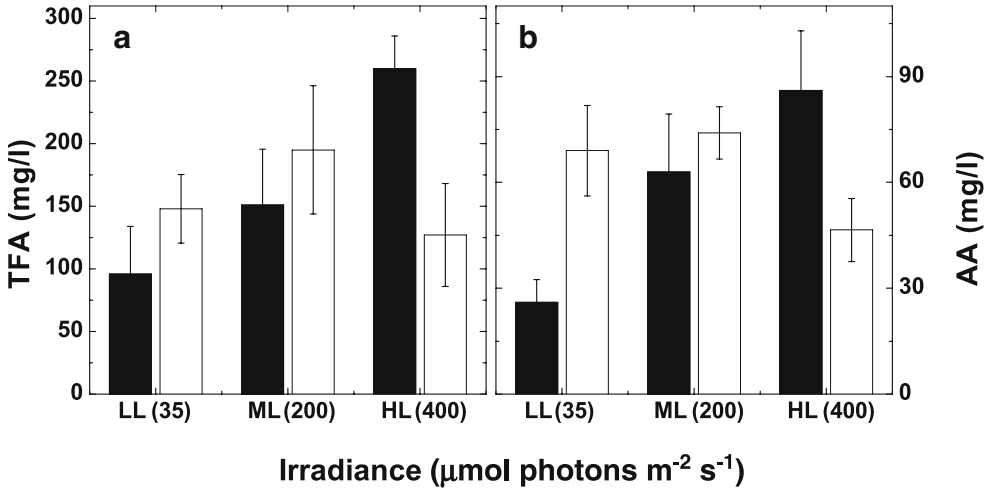

Fig. 3 Volumetric contents of total fatty acids (**a**) and arachidonic acid (**b**) in *P. incisa* culture after 14-day cultivation on nitrogen-containing and nitrogen-free medium (*filled* and *open bars*, respectively) under different illumination intensities

Cohen 1999 and Bigogno et al. 2002b). In our experiments, production of TFA by nitrogen-supplemented cultures under HL reached and surpassed that of the nitrogen-depleted cultures (Fig. 2g). This may be a consequence of the production of excessive photoassimilates that can then be stored in the form of FA within TAG, probably as a means to convert excess light to chemical energy in order to avoid photooxidative damage (Asada 1994; Rabbani et al. 1998; Mendoza et al. 1999; Niyogi 1999). The accumulation of AA per dry weight unit was also enhanced by intensive illumination (Figs. 2b,e,h) but to a lesser extent in comparison with TFA accumulation. Notably, high light did not exert as prominent an effect on FA production in nitrogen-starved cultures, which accumulated high amounts of TFA and AA regardless of the illumination conditions; at the same time high illumination brought about a small decrease in TFA accumulation (cf. Fig. 2a–c with g–i).

Fatty acid desaturation is considered to be an important factor for promoting microalgal tolerance to strong light, especially at low temperatures, by accelerating the synthesis of the D1 protein (Gombos et al. 1998). Klyachko-Gurvich et al. (1999) suggested that PUFA are necessary for the maintenance of photosynthetic membrane function and also play an important role in acclimation to low light conditions. This finding may explain the higher AA proportions recorded under HL-N conditions in comparison to HL+N (Fig. 2i). Bigogno et al. (2002b) have recently shown that the capability to use TAG in a buffer capacity for PUFA enables the organism to swiftly adapt to the rapidly changing environment. It is generally accepted that TAG serve in some algae as a sink of excessive energy absorbed by photosynthetic apparatus (Rabbani et al. 1998; Mendoza et al. 1999). At low temperatures or high irradiation, PUFA-producing algae can utilize AA of TAG for rapid biosynthesis of the eukaryotic-like molecular species of monogalactosyldiacylglycerols, which contain AA or eicosapentaenoic acid (Khozin et al. 1997; Khozin-Goldberg et al. 2000). One can suggest that, under harsh environmental conditions (high light, lack of N, low temperature) algae could have difficulties in increasing their chloroplastic PUFA content by de novo synthesis. The translocation of AA, accumulated under high light, from TAG to membrane lipids (Khozin-Goldberg et al. 2000) could represent a mechanism for a quick response of the cells to conditions requiring a higher level of AA in the membranes. This also makes the algae less dependent on the de novo synthesis of PUFA.

The higher rates of growth observed in the +N cultures allowed the cultures to attain the highest volumetric contents of TFA and AA under high light (Fig. 3); however the AA proportion among the TFA of the HL+N cultures was ca. one-third lower than in the HL-N culture. This observation is compatible with the results of outdoor experiments by Cheng-Wu et al. (2002) that showed that although higher radiation intensity was associated with lower AA proportion, the volumetric content of AA of *P. incisa* was still higher.

The data on biomass and TFA volumetric content suggest that under high light, cultures grown on complete medium are more efficient in net production of photoassimilates, especially the energy-rich PUFA. The lower proportions of AA under these conditions may result from the discrepancy between the rates of carbon fixation and the terminal steps of PUFA synthesis (desaturation) (Rodrigues et al. 2002). Under extremely high PFDs (2500 μE m^{-2} s^{-1}), when culture growth was even faster, a further decrease in the proportion of AA was recorded (Cheng-Wu et al. 2002). Therefore, it is possible to obtain higher amounts of biomass rich in FA with lower proportion of AA or lower yields of biomass with higher FA content but

enriched in AA. Selection of the preferable approach for mass production will depend on the cost and complexity of the AA purification.

However, one should keep in mind that high light intensities, especially under nitrogen-starvation conditions, slow down growth of *P. incisa* and cause damage (presumably photooxidative) to the cells. Therefore, lower light per cell achieved by lower light intensities or higher cell density should be considered when *P. incisa* cultures are maintained on nitrogen-free medium.

Conclusion

Due to higher biomass yield, the volumetric contents of both TFA and AA are higher in nitrogen-deplete cultures than in nitrogen-deficient cultures under high light. In contrast, under nitrogen-depleted conditions, biomass yield was half as much, but AA enrichment was ca. 30% higher. Special care should be exercised for the selection of light intensity for *P. incisa* cultivation since low illumination decreases growth rate but excessive illumination exceeding the photoadaptive potential of the alga readily causes photooxidative damage to the culture. Adjustment of both light environment and nutritional conditions is necessary for manipulation of the biomass and TFA and AA yields in *P. incisa*. Additional investigations are needed to reveal mechanisms of the high-light tolerance of *P. incisa* under stressful conditions that may further enhance the production of valuable PUFA by this alga.

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