Plant, Cell and Environment (2001) **24,** 1113–1118

Sub-optimal morning temperature induces photoinhibition in dense outdoor cultures of the alga *Monodus subterraneus* (Eustigmatophyta)

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ABSTRACT

Diel changes in photosynthetic oxygen evolution and several photochemical parameters measured by chlorophyll fluorescence quenching and induction were measured in outdoor dense cultures of the alga *Monodus subterraneus* (Eustigmatophyta). Cultures were maintained under two temperature regimes. In one, a rise in temperature was initiated in the morning by the increase in solar radiation up to the optimal temperature of 28 °C; in the other, a heating device was used to increase the rate of warming up in early morning.

Although the two cultures were maintained at the same temperature and light intensity for most of the day, cultures exposed for only a short time to suboptimal morning temperature showed a larger decrease in almost all the photosynthetic parameters. By comparing the diel changes in maximal photochemistry efficiency of photosystem II, the electron transport rate and the photochemical and non-photochemical chlorophyll fluorescence quenching of the cultures, we concluded that even a relatively short exposure to suboptimal morning temperatures induced photoinhibitory damage. The higher photochemical activity of the heated culture was also reflected in a significant increase in productivity, which was 60% higher in the morning heated cultures than in the non-heated cultures.

Key-words: Fluorescence; low temperature stress; outdoor cultures; oxygen evolution; photosynthesis.

Abbreviations: ETR, electron transport rate; F_0, F_v, F_m, minimum variable and maximum fluorescence in the dark; F, F'_m, steady-state and maximum fluorescence in the light; PQ, plastoquinone; PSII, photosystem II; Q_A, primary electron acceptor of PSII; RC, reaction centre.

INTRODUCTION

Outdoor algal cultures are exposed to diurnal changes in environmental conditions, particularly irradiance and temperature, which may fluctuate during the day between limiting and potentially inhibiting levels for photosynthesis. While fluctuations in light intensity occur in a range of 1–2 h, the increase in temperature is a much slower process that takes about 4–5 h. This kind of de-synchronization between the two most important environmental factors affecting photosynthesis and the growth of outdoor algal cultures results in a unique stress condition under which photoinhibition may be induced at relatively low light intensity because of the suboptimal temperature conditions. Hence, algal cells grown outdoors have to adapt their photosynthetic apparatus to environmental changes in order to utilize effectively the light energy harvested by the photosynthetic pigments.

The effect of environmental factors on the growth of the alga *Monodus subterraneus* (Eustigmatophyta) under laboratory conditions was first reported by Miller & Fogg (1957, 1958). *Monodus subterraneus* has been suggested as a potential source for eicosapentaenoic acid (EPA), a therapeutic and nutritional agent of ω3-polyunsaturated fatty acids (PUFA) (Iwamoto & Sato 1986). The effect of environmental factors such as irradiance and temperature on the production rate of total fatty acids and EPA content were studied previously (Cohen 1994; Hu *et al*. 1997). However, a commercially viable production system should provide the means to control the different growth conditions required for the induction of EPA accumulation. However, not enough information concerning the growth and productivity of *M. subterraneus* under outdoor conditions is available.

Chlorophyll fluorescence represents a non-invasive, reliable and powerful technique to assess the changes in the functioning of the photosynthetic apparatus, especially under stress conditions. Fluorescence induction kinetics (the so-called Kautsky curve) of all photosynthetic organisms show a polyphasic rise between initial (F_0) and maximum (F_m) fluorescence (Schreiber & Neubauer 1987; Strasser *et al*. 1997; Srivastava *et al*. 1999). These phases were designated as O, J, I and P, and can be visualized using a logarithmic time scale. By monitoring fluorescence transients and quenching, it is possible to obtain information on the absorption, transfer and dissipation of energy by PSII.

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The objective of this paper was to evaluate the effect of suboptimal morning temperature on the diurnal course of photochemical activities, growth and productivity of *M. subterraneus* grown outdoors.

MATERIALS AND METHODS

Organism and cultivation conditions

Laboratory cultures of the microalga *Monodus subterraneus* (Eustigmatophyta), re-named as *Monodopsis subterraneus* by Hibberd (1981), were cultivated photoautotrophically in a mineral medium, BG11 (Rippka *et al.* 1979), at 25 °C. Outdoor cultures were grown in a 140 L tubular photobioreactor made of 10 parallel plastic tubes (length 25 m, internal diameter = 28 mm). The photobioreactor was placed on the ground and water sprinklers were used to prevent heating up of the cultures above a preset temperature of 28 ± 1 °C. For more details on the reactor design, see Richmond *et al.* (1993). The pH value of the cultures was controlled by a flow of CO_2 and maintained at a range between 7·1 and 7·3. The circulation of algal culture was maintained by an airlift pumping system that induced a fully turbulent flow (Re ≅7000) at a speed of 0·5 m s^{-1}.

Photosynthetic active radiation (PAR) was measured with a Li-185B quantum sensor (Li-Cor, Lincoln, NE, USA).

Analytical procedures

Chlorophyll *a* was extracted by dimethyl sulfoxide (DMSO) and assayed spectrophotometrically (Bennet & Bogorad 1973). Dry weight (DW) was determined in duplicates using 50 mL samples filtered through nitrate cellulose filters with a 3 μm pore size (Sartorius, Göttingen, Germany).

Oxygen and fluorescence measurements

Photosynthetic oxygen evolution was measured under a saturating irradiance of 1200 μmol photons m^{-2} s^{-1} in a stirred glass chamber using a Clark-type oxygen electrode. Temperature was kept constant at 28 °C. Samples withdrawn from outdoor algal cultures were diluted to a constant chlorophyll concentration of 2 mg L^{-1} with fresh medium in order to minimize self-shading.

Fluorescence measurements

Variable chlorophyll fluorescence $F_v = (F_m - F_0)$ was used for the detection of changes induced by stress conditions in the photosynthetic apparatus (F_0 and F_m are the minimum and maximum fluorescence yields of a dark-adapted sample, with all PSII reaction centres fully open or closed, respectively). The ratio of the variable to maximal fluorescence (F_v / F_m) is considered as a measure of the maximal quantum efficiency of PSII photochemistry (fluorescence nomenclature and definitions follow van Kooten & Snel 1990). F_v / F_m was determined on triplicate dark-adapted (10 min) algal samples (5·0 mg Chl *a* L^{-1}) using an induction fluorometer plant efficiency analyser (PEA, Hansatech, Norfolk, UK), equipped with a liquid sample holder. Chlorophyll fluorescence induction kinetics (OJIP transient) was recorded within a time span of 50 μs to 1 s with a data acquisition rate of 10^5 readings s^{-1} for the first 2 ms; it was then switched to 10^3 readings s^{-1} (Strasser *et al.* 1997; Srivastava *et al.* 1999). Measurements were carried out at room temperature.

Outdoor monitoring of chlorophyll fluorescence quenching was carried out using a PAM-2000 fluorometer (K. Walz GmbH, Effeltrich, Germany). The fibre-optic light guide was placed directly on the surface of the photobioreactor (Torzillo *et al.* 1996). The fluorometer was connected to a portable chart recorder (model L 120 E, Linseis, Selb, Germany) to record the steady-state values of F_o, F and F'_m. The minimum fluorescence F_o was measured using modulated light from a light-emitting diode (< 0·3 μmol photons m^{-2} s^{-1}, peak wavelength at 655 nm, 600 Hz) under a black plastic cover. The parameters F and F'_m represent steady-state and maximum fluorescence measured in the light; F'_m was measured after a saturating light pulse (5500 μmol photons m^{-2} s^{-1}, 0·8 s in duration), which closed all PSII reaction centres (when all Q_A, the primary electron acceptor of PSII, was reduced). The steady-state levels of F and F'_m were usually reached after few minutes. At least five flashes were administrated to the culture to record the average value of F'_m. The electron transport rate (ETR) of the cultures during the day was calculated as:

$$ETR = [(F'_m - F) / F'_m] \times PFD \times 0{\cdot}85 \times 0{\cdot}5 \times 0{\cdot}98,$$

where $(F'_m - F) / F'_m$ is the effective quantum yield of PSII (Genty *et al.* 1989), PFD is the photon flux density (400–700 nm) in μmol m^{-2} s^{-1}, 0·5 is a multiplication factor (because the transport of one electron requires absorption of two quanta, as two photosystems are involved), 0·85 is the transmittivity of plexiglass tube reactors and 0·98 is the fraction of incident radiation absorbed by dense microalgal cultures. The value of 0·98 was used because dense microalgal cultures absorb all the incident light and reflect about 2% of it (Gitelson *et al.* 1999). The resulting ETR is measured in μmol electrons m^{-2} s^{-1}.

The non-photochemical quenching coefficient (NPQ) was calculated according to the Stern–Volmer equation, $NPQ = F_m - F'_m / F'_m$. In order to compensate for the increase in F_m due to increase in chlorophyll concentration during the day, the F_m values were re-calculated according to $F_m = F_o / (1 - F_v / F_m)$, where F_o are the values measured at different times of the day and F_v / F_m is the maximum value measured in the early morning. The experiments were repeated three times on independently grown cultures during three consecutive sunny days with very similar pattern and total light irradiances.

RESULTS

Outdoor algal cultures were exposed to two temperature regimes. In the first culture, the maximum temperature was set not to exceed 28 ± 1 °C; otherwise, the culture experienced a drop in temperature in late afternoon and an increase in the morning that was initiated by the increase in solar irradiance up to the optimum growth temperature of 28 °C (Fig. 1). This culture is hereafter referred to as non-heated. In the second culture (subsequently designated as heated), a heating device was used to increase the rate of warming up in the early morning hours, up to 28 °C. A typical daily fluctuation in irradiance is depicted in Fig. 1. The two cultures were exposed to a relatively steep increase in irradiance between 0600 and 0900 h (that is, from almost complete darkness to 1300 μmol photons m^{-2} s^{-1}, respectively; Fig. 1). It must be pointed out that after the initial differences in early morning temperatures, both cultures were exposed to the same light and temperature conditions for the rest of the day.

In order to evaluate the effect of the different temperature regimes on the photosynthetic apparatus of *M. subterraneus*, the diurnal changes in the maximum photochemical efficiency of PSII (F_v / F_m) were followed in the heated and non-heated cultures. In both cultures, a decline in the F_v / F_m ratio was observed, starting at sunrise. The decline was faster and to a greater extent in the non-heated cultures, reaching a midday value of 0·48, compared with 0·58 in the heated cultures. A recovery in the maximum photochemical efficiency of PSII was observed in both cultures in the afternoon, as reflected in the increase in the F_v / F_m values (Fig. 2).

The light-saturated oxygen evolution rate of algal cells withdrawn from the outdoor cultures at different times during the day was measured under laboratory conditions (Fig. 3). At sunrise, the rate of oxygen evolution was significantly higher in the heated cultures than in the non-heated ones. This difference was even bigger at midday. However, by sunset, both cultures showed similar oxygen evolution rates.

The time course of the ETR and the changes in the NPQ of the cultures were measured *in situ* (Figs 4 & 5). A fast inactivation of PSII activity, reflected in slower electron transport rate and an increase in the NPQ, was observed in the non-heated cultures. In these cultures, the daily integrated value of ETR was 32% lower than that measured in the heated cultures. The NPQ values were lower in the heated cultures (Fig. 5). It is worth noting that the differences in ETR and NPQ between the two cultures remained throughout the day, although the cultures reached the same temperature at about 0900 h. This indicated greater demand by the carbon assimilation reactions for reducing

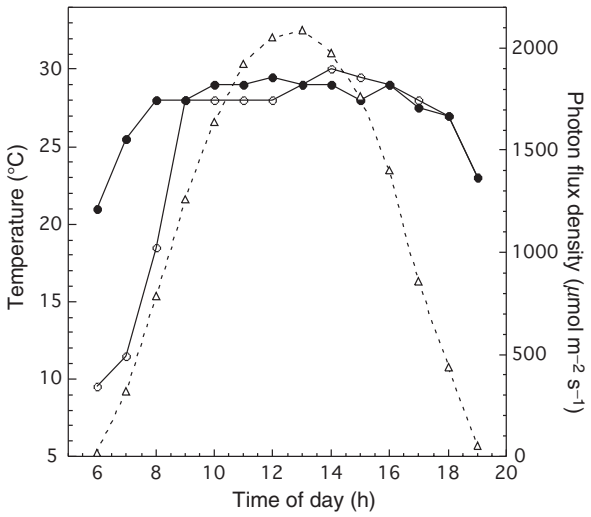

Figure 1. Diurnal changes in solar irradiance (△) and temperature in non-heated (○) and heated (●) *M. subterraneus* cultures grown outdoors in photobioreactors. Data are mean values recorded during three days' measurements.

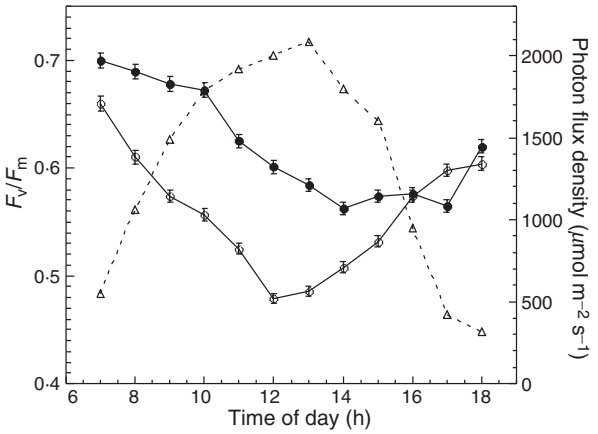

Figure 2. Diurnal changes in the F_v / F_m ratio in non-heated (○) and heated (●) cultures of *M. subterraneus*. The dashed line describes the diurnal changes in light intensity. Bars represent SE (not shown if smaller than symbol); n = 3.

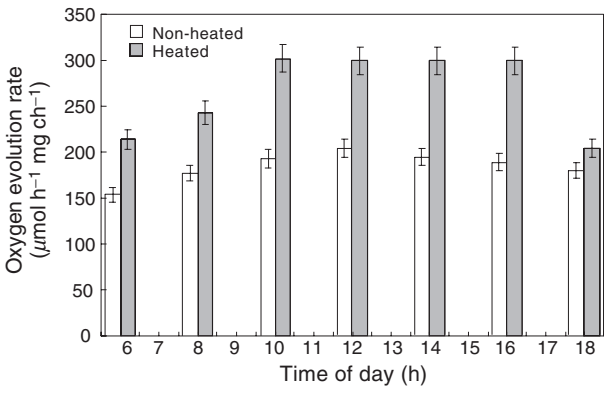

Figure 3. Diurnal changes in the light-saturated oxygen evolution rates in *M. subterraneus* cells withdrawn from non-heated (open) and heated (filled) cultures. Samples were diluted with fresh medium to a constant chlorophyll concentration of 2·0 mg L^{-1}. The oxygen evolution rate was measured at 28 °C and a PFD of 1200 μmol m^{-2} s^{-1}. Bars represent SE (n = 3).

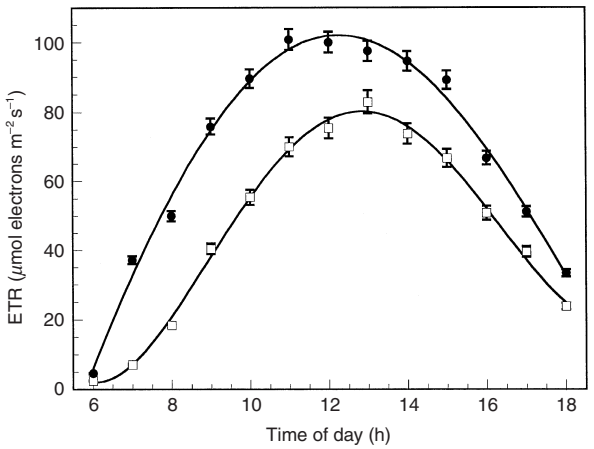

Figure 4. Diurnal changes in the electron transport rate in non-heated (○) and heated (●) cultures of *M. subterraneus* grown outdoors in photobioreactors. Bars represent SE (n = 3).

power, as there was apparently little change in NPQ in heated cultures but more energy appeared to be dissipated via non-photochemical quenching in the non-heated ones.

The changes induced by the early morning suboptimal temperature on the photosynthetic apparatus were further analysed using the JIP test as described by Srivastava *et al.* (1999). The polyphasic rise of chlorophyll fluorescence transients (induction curves recorded by the PEA fluorometer) measured at various times of the day revealed significant differences between the heated and non-heated cultures. Fluorescence induction curves of morning, midday and afternoon samples (0600, 1200 and 1800 h) showed a clear O to J step for the non-heated and heated cultures (Fig. 6), corresponding to the net photochemical reduction of Q_A to Q_A^- (Srivastava *et al.* 1999). In the non-heated culture, there was no distinctive step between the I and P points as compared with the heated one.

A decline in the induction curve beyond the P point indicates the reoxidation of the PQ pool. Such a decline was

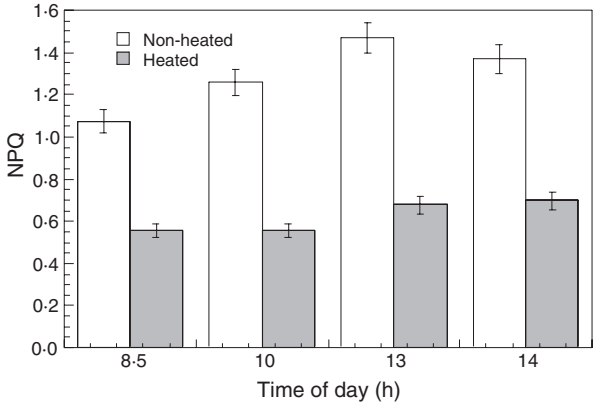

Figure 5. Diurnal changes in the non-photochemical quenching in non-heated (open) and heated (filled) cultures of *M. subterraneus* grown outdoors in photobioreactors. Bars represent SE (n = 3).

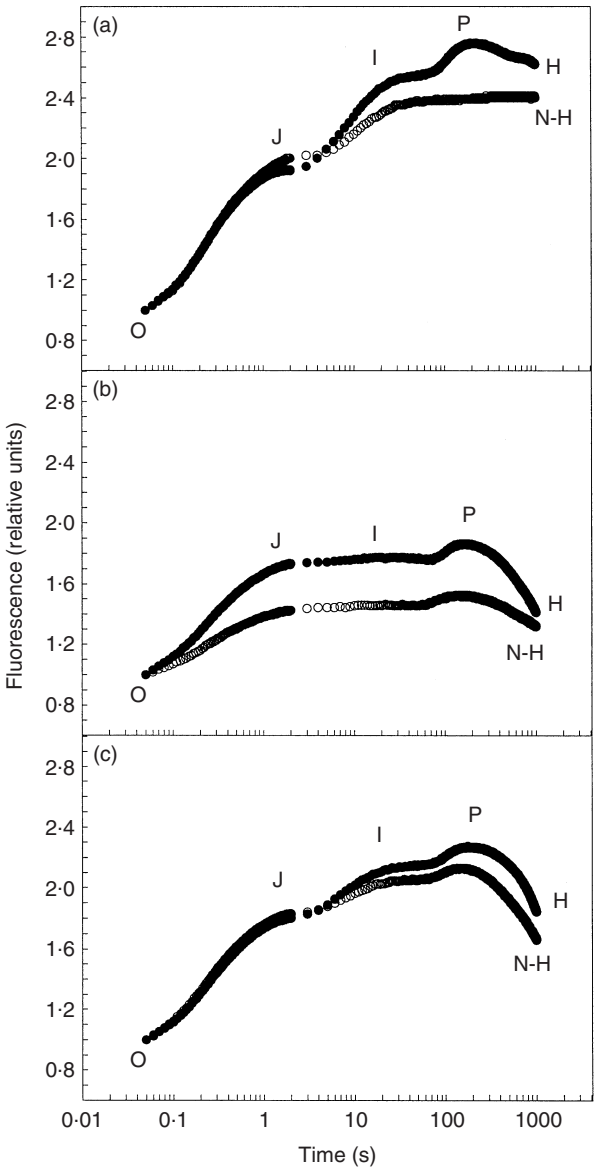

Figure 6. Chlorophyll *a* fluorescence induction kinetics (from 50 μs to 1 s) of *M. subterraneus* cells taken at 0600 (a), 1200 (b) and 1800 (c) h from non-heated (○) and heated (●) cultures. Cells were dark-adapted for 10 min before measurements were taken. Bars represent SE (n = 3).

evident in the early morning samples (0600 h) from the heated cultures, while in samples withdrawn from the non-heated cultures, no decline was observed (Fig. 6a). At midday (Fig. 6b), both induction curves showed a decrease in the maximum fluorescence yield (average 32%) compared with the morning values. At 1800 h (Fig. 6c), the value of F_m in the non-heated cultures recovered to 90% of the initial morning value and fast reoxidation of the PQ pool was observed.

The differences observed in the photochemical parameters between the two cultures are also reflected in the daily biomass productivity – from 0·52 ± 0·07 g (DW) L^{-1} d^{-1} in

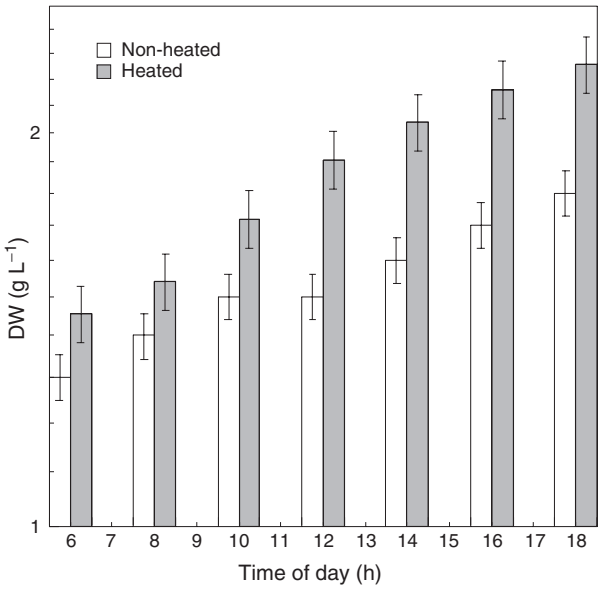

Figure 7. Increase in DW in non-heated (open) and heated (filled) cultures of *M. subterraneus* grown outdoors in photobioreactors. Bars represent SE (n = 3).

the non-heated culture to 0·83 ± 0·06 g (DW) L^{-1} day^{-1} in the heated culture. This reflects an increase of 60% in the daily volumetric productivity (Fig. 7).

DISCUSSION

The results presented here show that the exposure of outdoor *Monodus* cultures to suboptimal temperatures in the early morning caused a considerable reduction in PSII activities, reflected by the decrease in maximum efficiency of PSII photochemistry (F_v / F_m), the decrease in ETR and the increase in non-photochemical quenching. The diurnal pattern changes in F_v / F_m exhibited a midday depression corresponding to the maximum irradiance, indicating the inactivation of PSII reaction centres. Recovery started in the afternoon, with the decline in solar irradiance.

In the polyphasic induction curve, the intermediate step I and the final step P reflect fast and slow plastoquinol-reducing reaction centres as well as different redox states of the PSII reaction centre (Strasser *et al*. 1997). The fact that there was no distinctive step between the I and P stages in the non-heated cultures suggests that the Q_B-non-reducing centres prevailed. In addition, no drop in the induction curve beyond the P phase was observed, indicating that the PQ pool remained reduced because of the low demand for reduction equivalents from the Calvin cycle. The midday decline in the maximal photochemical efficiency of PSII in the cultures has been observed widely in nature, even in plants grown under optimal conditions (Ögren & Evans 1992). This decline was referred to as diurnal photoinhibition and was correlated to similar diurnal patterns in the quantum yield of photosynthesis (Neale 1987; Long *et al*. 1994).

Despite the significant reduction in the F_v / F_m ratio at midday, the light-saturated oxygen evolution rates did not show any reduction and even increased in the heated culture. This contradictory phenomenon of midday maxima in both photoinhibition and photosynthesis had already been observed by Behrenfeld *et al*. (1998). The effect of photoinhibition on photosynthesis depends upon which step of the electron transport chain is rate-limiting at a given incident irradiance. Photosynthetic activity at subsaturating light irradiance is rate-limited by light absorption; as a result, energy transfer to PSII reaction centres is linearly dependent on light intensity. Conversely, at light saturation, the acceptor side of PSII (which reflects the enzymatic processes in the Calvin cycle) limits photosynthesis. The tolerance of light-saturated photosynthetic oxygen evolution to photoinhibition observed in our experiments suggests that photoinhibition does not directly affect the rate-limiting step of photosynthesis at light saturation. It was found that in *Thalassiosira weissglogii* cultures, a decrease in the population of functional PSII reaction centres of up to 50% could be compensated by an increase in the rate of electron transport through the functional PSII reaction centres (Behrenfeld *et al*. 1998).

Algal productivity depends primarily on light energy conversion efficiency, i.e. the absorption and utilization of light by the photosynthetic apparatus to assimilate CO_2 into dry matter. Dense algal cultures are predominantly grown at light limitation and, consequently, their photosynthetic performance would be more dependent on the initial slope rather than on the light-saturated portion of the P/I curve. Hence, photoinhibition in outdoors dense algal cultures will result in a decrease in the photosynthetic rate.

Outdoor dense microalgae cultures may experience large variations in light intensity because of the changes in daily irradiance and mixing. Although turbulent mixing is aimed at exposing the cells to average, uniform irradiance, relatively long exposure of cells to excess light cannot be avoided. On the other hand, a strategy striving to counteract photoinhibition at midday through an increase in cell concentration would lead to acclimation of the cells to low irradiance, which may result in an increase in the PSII antenna size and thus to an increased risk of over-excitation (Falkowski & Raven 1997; Neidhardt *et al*. 1998). Moreover, the increased fraction of cells deprived of light in the deeper layers would bring about an increase of energy dissipated through respiration (Torzillo *et al*. 1991).

The energy cost of photoinhibition in the *Monodus* cultures increases substantially if additional stress (e.g. suboptimal temperatures) is superimposed. Thus, a larger proportion of the radiation absorbed by the photosynthetic apparatus is dissipated through non-photochemical pathways.

In conclusion, our results suggest that in algal cultures maintained at suboptimal temperature, photoinhibition may be induced even at relatively low light intensities. Such a condition can be very common in desert areas where the morning temperature of the culture is very low.

ACKNOWLEDGMENTS

J.M. and G.T. were awarded fellowships of the Access to Research Infrastructures Programme of the European Commission to work in Israel. J.M. was also supported by project No LN00A145. The authors thank Prof. Reto Strasser and Dr Ronald Rodriguez for discussions and interpretation of fluorescence transient.

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Received 6 February 2001; received in revised form 31 May 2001; accepted for publication 25 June 2001