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Light and oxygen stress in *Spirulina platensis* (cyanobacteria) grown outdoors in tubular reactors

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As the effects of light and oxygen stress in algae on mass culture has not been intensively studied, we investigated them in *Spirulina platensis* under outdoor conditions in controlled tubular reactors where the respective roles of each stress can be distinguished. It was observed that exposure of this cyanobacterium at two oxygen concentrations (ca 20 and 53 mg l^{-1}) caused very little change in the ratio between variable and maximum fluorescence (F_v/F_m) during the day even when the culture was grown at higher oxygen concentration (about 7% lower in the evening than in the morning). Vice-versa, when the photochemical efficiency of PSII (photon yield, Φ_e) was measured, a reduction of about 20% was observed. Neither the F_v/F_m ratio nor the Φ_e of the culture grown at the lower oxygen concentration changed significantly during the day. The daily productivity of the culture exposed to the higher oxygen concentration was reduced by about 20%. Laboratory cultures bubbled with air or pure oxygen under continuous light showed a similar response; i.e., a smaller decrease in F_v/F_m (17%) than in the Φ_e (56%) after 4 h. After 32 h of culture in pure oxygen, a total lysis of the cells occurred. Our results support the hypothesis that photoinhibition and photooxidation, two traditionally linked terms, although often closely associated under similar environmental conditions, may comprise two types of stress with different sites of inhibition.

Key words – Cyanobacteria, fluorescence, oxygen, photoinhibition, photooxidation, photosynthesis, PSII, *Spirulina*.

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Introduction

Oxidative stress in photosynthetic organisms is traditionally associated with excessive absorption of light energy by the reaction centers. The greater photosynthetic activity under high light is associated with exposure to increased oxygen levels, resulting in the inhibition of photosynthetic activity at the first stage and leading to what has been described as photooxidative death at a later stage (Abeliovich and Shilo 1972, Krause 1994). Photooxidation is usually considered to be a secondary phenomenon, occurring after a distinct lag phase during which there is a time- and light-dependent decline in photosynthesis due to photoinhibition (Powels 1984). Current work on photoinhibition would seem strongly to suggest that PSII is the most sensitive site for this stress. Some work has suggested that the reaction of dioxygen (O_2) and active oxygen species cause photoinhibition of photosynthesis (Powels 1984, Krause and Cornic 1987, Krause 1994).

Present work carried out with the cyanobacterium *Spirulina platensis* indicates that photoinhibition and photooxidation phenomena, although closely associated under similar environmental conditions, may be parallel processes under stress conditions and should be considered as two types of stress with different sites of inhibition.

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Variable chlorophyll fluorescence is a useful tool for investigating mechanisms of stress damage in photosynthesis in higher plants (Schreiber and Bilger 1987, Baker and Horton 1987, Strand and Öquist 1988). However, because of distinct physiological differences between higher plants and cyanobacteria, special attention is required in the evaluation of fluorescence data gathered with the latter organisms (Ting and Owens 1992, Büchel and Wilhelm 1993, Schreiber et al. 1995). We have applied this methodology to study the effect of high oxygen concentrations on the photosynthetic activity of *Spirulina platensis* cultures grown outdoors in closed reactors.

Abbreviations – F_m, F_v, F_0, maximal, variable, and minimal fluorescence yield of dark-adapted cultures; F'_m, maximal fluorescence intensity in light-adapted cultures (closed PSII centers); F_s, fluorescence intensity in light-adapted cultures (open PSII centers); Φ_e, photochemical efficiency of PSII per absorbed photon (or photon yield), calculated as $(F'_m - F_s)/F'_m$.

Materials and methods

Organism and culture conditions

Spirulina platensis Geitler (strain M2) from the Culture Collection of the Centro di Studio dei Microrganismi Autotrofi of Florence, Italy, was used. The cyanobacterium was grown in Zarrouk's medium, containing 200 m*M* sodium bicarbonate (Vonshak et al. 1982) at 35.0 ± 0.5°C. The pH was maintained at 9.4 ± 0.1 by automatic addition of CO_2. In the outdoor experiments, the concentration of dissolved oxygen was monitored continuously using polarographic electrodes connected to control units (IL 533 and IL 534; Instrumentation Laboratory S.p.A., Milan, Italy). The oxygen concentration in the culture was maintained at 20.8 ± 1.8 mg l^{-1} by automatic addition to the culture of pure nitrogen through air stone spargers. In another reactor, oxygen concentration was uncontrolled and reached 53.0 ± 9.4 mg l^{-1} as a result of photosynthetic activity. The culture speed was 0.46 m s^{-1}, corresponding to a Reynolds number of about 11 000. Total solar radiation was measured with a Kipp and Zonen pyranometer sensor (model CM6; Delft, The Netherlands). Laboratory cultures were grown in bubbled air in vertical glass tubes (5 cm diameter) placed in a water bath at 35°C. The cultures were exposed to a photon flux density of 100 µmol m^{-2} s^{-1} (Philips TLD 30 W fluorescent lamps; Eindhoven, The Netherlands). At the beginning of the experiment, light intensity was increased to 350 µmol m^{-2} s^{-1} and the cultures were then bubbled with air or pure oxygen. Gas flow rates were set at 1.6 l min^{-1}. The oxygen concentration was 10 mg l^{-1} in the culture bubbled with air and 36 mg l^{-1} in that bubbled with pure oxygen.

Outdoor culture equipment

The photobioreactors were described by Bocci et al. (1987). Each reactor consisted of a loop made of 10 parallel Pyrex tubes (length 2 m, i.d. 4.85 cm, volume 51 l) connected to PVC U-bends with watertight flanges. The cultures were recycled by PVC pumps.

Analytical procedures

Dry weight was determined in duplicate using 10-ml samples (Torzillo et al. 1993). Light intensity was measured with a quantum sensor (model LI-185 B; Li-Cor Inc., Lincoln, NE, USA). Chlorophyll concentration was measured according to Bennet and Bogorad (1973).

Fluorescence measurements

Fluorescence measurements were performed on 0.5-ml samples using a portable pulse-amplitude-modulation fluorometer (PAM-2000; H. Waltz, Effeltrich, Germany). Algal samples were withdrawn from outdoor and laboratory cultures at intervals and incubated in the dark for 15 min to remove any energy-dependent quenching. In addition, one far-red light (above 700 nm) pulse of 10 s duration (10 W m^{-2}), supplied by the PAM-2000, was applied. The ratio between variable and maximum fluorescence, F_v/F_m, was then measured to determine the extent of photoinhibition (Björkman and Demmig 1987, Adams et al. 1990, Vonshak et al. 1994). Both dark adaptation and fluorescence measurements were carried out in a liquid sample cuvette, type KS-101, at 35°C. The photochemical efficiency of PSII per absorbed photon, or photon yield (Φ_e), was measured under the actinic light of the PAM-2000 (150 µmol m^{-2} s^{-1}) and calculated as $(F'_m - F_s)/F'_m$ (Genty et al. 1989). Each measurement was performed in triplicate.

Results

The time course of the oxygen concentrations measured in the outdoor cultures of *Spirulina* is shown in Fig. 1. It

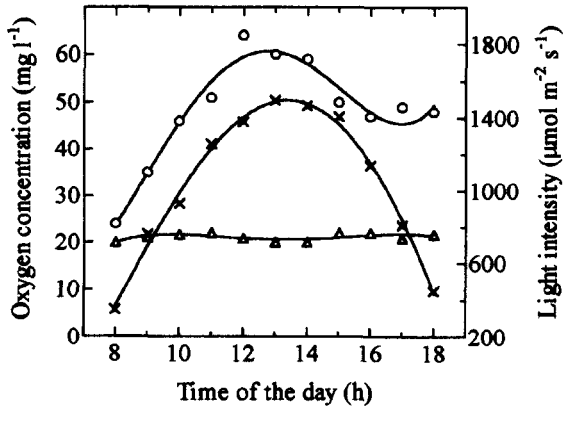

Fig. 1. Diurnal changes in the oxygen concentrations (△, 20.8±1.8 mg l^{-1}; ○, 53.0±9.4 mg l^{-1}) in the two photobioreactors. Changes in light irradiance (PAR) recorded during the day are indicated (×).

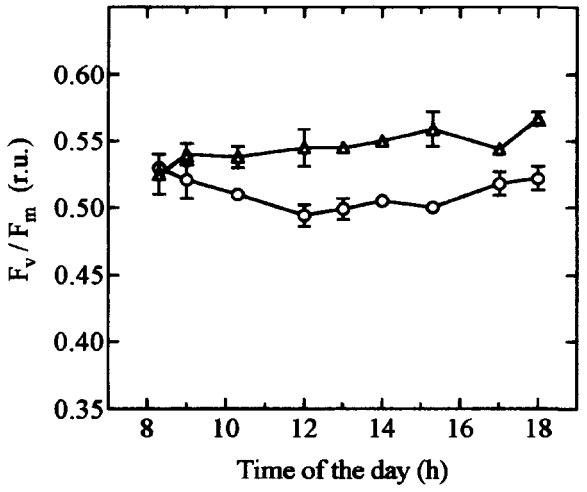

Fig. 2. Diurnal changes in the F_v/F_m ratio of *Spirulina* cultures grown at 20.8±1.8 mg l^{-1} (△) or 53.0±9.4 mg l^{-1} (○) of oxygen. Bars represent SD; not shown where smaller than the symbols. n=3.

reached 55–60 mg l^{-1} at midday in the uncontrolled oxygen culture as a result of photosynthetic activity. When no efficient degassing was applied, the oxygen concentration in the reactor remained very high, even after the level of irradiation and photosynthetic activity of the culture decreased to almost zero. For example, at 18:00 light intensity was < 200 µmol $m^{-2}s^{-1}$ and the O_2 concentration was ca 50 mg l^{-1}.

To distinguish between the effect of high oxygen levels (oxygen stress) on the photosynthetic apparatus and the effect of high light intensity (photoinhibitory stress), we maintained an optimum temperature and grew the culture at a high biomass concentration of 1.4 g (dry weight) l^{-1}, equivalent to 26 mg Chl l^{-1}. Under these conditions, photoinhibition in *Spirulina* can be greatly reduced (Vonshak et al. 1994). Measurement of the photochemical yield of open reaction centers (F_v/F_m), revealed only a small decrease (6–7% in initial activity) in the culture grown at 53.0±9.4 mg l^{-1} O_2 at midday (Fig. 2). A different pattern was observed when the photon yield (Φ_e) was measured. As shown in Fig. 3, Φ_e was reduced by about 20% in the early afternoon in the high-oxygen culture; only insignificant changes were observed during the course of the day in the low-oxygen culture.

Tab. 1. Effect of oxygen concentration on the daily productivity of *Spirulina platensis* M2 grown outdoors in tubular reactors. Data are mean values ± SD of a 4-day experiment. Total solar irradiation throughout the experiment was 22.0 ± 1.6 MJ m^{-2} day^{-1}.

Oxygen concentration (mg l^{-1})	Net productivity (mg l^{-1} day^{-1})
20.8±1.8	522±20
53.0±9.4	417±32

The output rate of the *Spirulina* biomasses grown at the two oxygen concentrations is shown in Tab. 1. The productivity of the culture grown at the higher O_2 concentration was about 20% lower than that grown at the lower, results that agree with the effect of oxygen on photon yield (Fig. 3).

To study the effect of high oxygen concentrations on the photosynthetic apparatus further *Spirulina* cultures were grown under laboratory conditions and bubbled with pure oxygen so that the concentration reached 36 mg l^{-1} O_2. Figure 4 shows the changes in the fluorescence parameters. The F_v/F_m ratio decreased by 17% during the first 4 h, while that of Φ_e decreased by 56%.

To evaluate the effect of extended exposure to oxygen on growth and the ability to recover, laboratory cultures

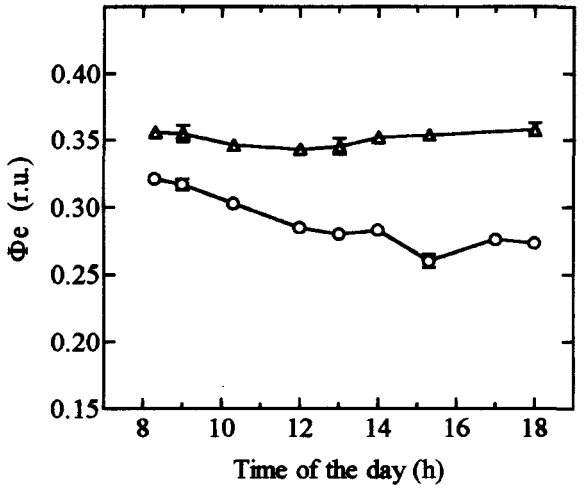

Fig. 3. Diurnal changes in the $\Phi_e = (F'_m - F_s)/F'_m$ of *Spirulina* cultures grown at 20.8±1.8 mg l^{-1} (△) or 53.0±9.4 mg l^{-1} (○) of oxygen. Bars represent SD; not shown where smaller than the symbols. n=3.

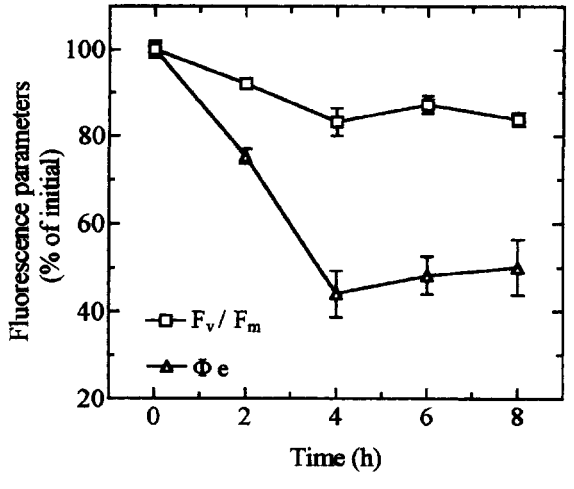

Fig. 4. The effect of oxygen on F_v/F_m (□), and $\Phi_e=(F'_m-F_s)/F'_m$ (△), in laboratory grown cultures of *Spirulina*. Initial values of F_v/F_m and Φ_e were 0.607±0.012 and 0.380±0.007, respectively. Bars represent SD; not shown where smaller than the symbols. n=3.

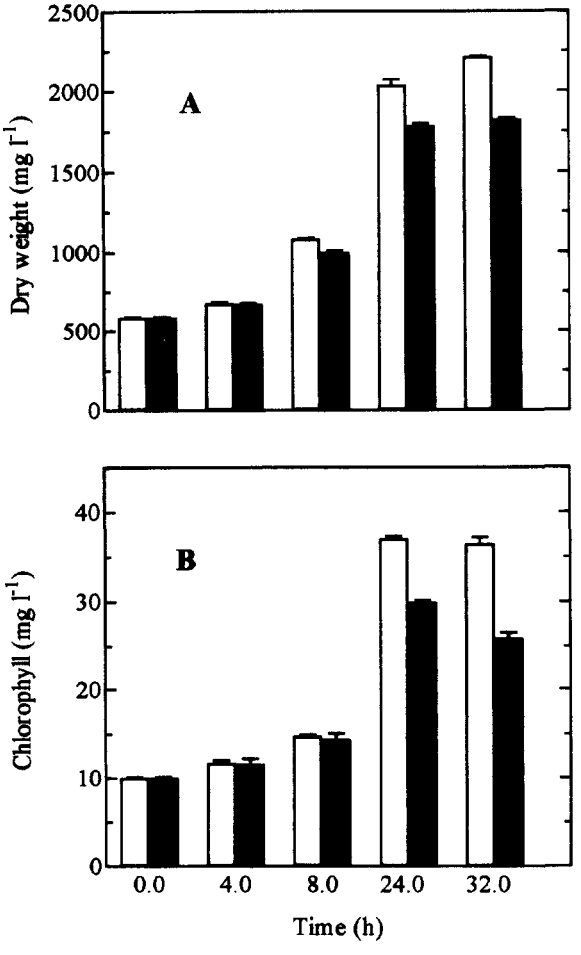

Fig. 5. Increase in dry weight (A) and chlorophyll (B) in *Spirulina* cultures grown in the laboratory with bubbling of air (open) or oxygen (closed). Bars represent SD, n=3.

were grown under air- or oxygen-bubbled conditions for a period exceeding 32 h. After 24 h, the biomass concentration of the culture grown under pure oxygen was 12.5% lower than that grown in air (Fig. 5A). After 32 h, the difference increased to 18%. The chlorophyll growth rate of the culture grown in pure oxygen, measured after 24 h, was about 20% lower and after 32 h the difference increased to about 30% (Fig. 5B). Between 24 and 32 h, a decline in chlorophyll was observed in the oxygen-grown culture, indicating destruction of chlorophyll. During the following hours the culture became chlorotic. Microscope observation of the cultures showed that at this stage the *Spirulina* trichomes appeared deprived of pigments and gas vacuoles but were still intact. The culture was diluted with fresh medium 1:10 (v/v) and incubated at 30°C under dim light in order to determine whether it could still recover. After one day of incubation the trichomes appeared totally lysed. It may be concluded that irreversible damage took place after 30–32 h of exposure to the oxygen stress.

Discussion

Comparison of the kinetics of the changes in F_v/F_m ratio and photon yield, Φ_e, under conditions of oxygen stress provides information on the site(s) and mechanisms of such inhibition. The fact that the decline in Φ_e could not be entirely explained by a similar decrease in the F_v/F_m ratio may indicate that photoinhibition and photooxidation occurred in *Spirulina* as parallel processes and that the site of oxygen stress could be distinct from PSII, supporting a similar observation by Adams et al. (1990). Adams et al. concluded that, when factors other than high light intensity alone result in a depression of the photon yield of O_2 evolution due to inhibition at sites other than the PSII reaction centers, the photon yield may be much more depressed than the F_v/F_m ratio measured in dark-adapted samples, due to the regulation of PSII, that is, to an increase in radiation-less energy dissipation in response to restrictions in electron flow beyond PSII. However, it cannot be excluded that the difference between the two chlorophyll fluorescence parameters reflects light-induced changes in PSII antenna, e.g., modulation of the phycobilisome structure (size) or of the attachment of phycobilisomes to the PSII complex.

Extended exposure of laboratory cultures of *Spirulina* to pure oxygen resulted in chlorophyll destruction. Since the growth medium was sufficient to sustain an increase in biomass concentration of about 4 g l^{-1}, nutrient starvation can be ruled out. It may be concluded that the decrease in chlorophyll was therefore the result of photooxidative damage. After 32 h of exposure, irreversible damage took place, resulting in photooxidative death of cells.

Our results suggest that fluorescence parameters may be used as a tool to detect the role of different stress conditions in *Spirulina* cultures. It was observed that oxygen stress is better assessed by Φ_e than by F_v/F_m, since the former appears to be better correlated to culture growth.

Although *Spirulina* is grown in industrial-scale units, knowledge of its growth physiology under outdoor conditions is scarce. The development of a closed reactor that may be easily scaled up to a commercial level would represent a potential improvement in algal biotechnology. The choice of an efficient degassing system to prevent increased oxygen concentration in the culture to over the harmful level of 20 mg l^{-1} represents an important issue for successful photobioreactor design.

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