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Photoinhibition in the Mediterranean Green Alga *Halimeda tuna* Ellis et Sol Measured *in situ*

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ABSTRACT

Photoinhibition of photosynthesis was investigated in the Mediterranean green alga *Halimeda tuna* measuring pulse amplitude modulation (PAM) chlorophyll fluorescence and oxygen evolution *in situ* under solar radiation. Exposure to solar radiation at the surface caused a drastic decline in the photosynthetic quantum yield accompanied by a decline in the photochemical quenching, while the nonphotochemical quenching dramatically increased. The algae recovered from these effects within a few hours indicating that these are mainly due to reversible photoinhibition and only to a smaller extent to nonreversible photodamage. Individuals harvested from deeper waters were more affected than those from shallower waters. Photoinhibition occurs in this alga even in its natural habitat when the sun is at high angles as shown by measuring the fluorescence parameters at hourly intervals during the course of the day. Photoinhibition was less pronounced when the short wavelength band was increasingly removed from solar radiation using cut-off filters. After exposure of thalli to solar radiation at the water surface, oxygen production decreased drastically within 30 min; this inhibition was even more pronounced in algae harvested from deeper waters. Oxygen measurements at different depths showed optimal photosynthesis at a depth of 1 m. Also for photosynthetic oxygen production inhibited by high solar irradiance at least partial recovery could be observed within several hours. Despite the fact that UVB accounts for a very small fraction of solar radiation, it has a considerable effect on photosynthesis, whereas UVA seems to contribute only little to photoinhibition in *H. tuna*.

INTRODUCTION

In the past, most ecophysiological work on photosynthesis has been restricted to higher plants (1,2) while there is relatively little information on algae. This unbalance is contrasted by the fact that about 50% of the primary biomass production on our planet is based on aquatic ecosystems (3,4). Most of the productivity is due to phytoplankton, but also macroalgae are major contributors of biomass in aquatic ecosystems.

In contrast to phytoplankton, which can move vertically in the water column by active or passive displacement, most macroalgae are sessile and thus exposed to the natural fluctuations of solar radiation. There is a pronounced vertical distribution of species on sea shores: some organisms inhabit the supralittoral above the tidal range while others populate the eulittoral defined by its regular pattern of tidal action. Still others are restricted to the sublittoral and are thus rarely affected by tidal changes. Probably the most important factor controlling the species distribution on the shore is sunlight exposure. Some algae are adapted to full solar radiation at the surface, *e.g.* in rock pools, whereas others are adapted to habitats under overhanging rocks or in crevices with very low irradiances. The differences in the irradiances measured in the various habitats are enormous, and fluence rates range from 400 W m^{-2} (photosynthetic active radiation, PAR,† 400–700 nm) on clear days at the surface to 0.001% of the surface irradiance measured at a depth of 268 m in the Bahamas, which sustains survival and growth in some red algae (5).

High solar irradiance has been found to be a stress factor: higher plants respond by the phenomenon of photoinhibition, with the photosynthetic yield decreasing dramatically upon exposure to extreme fluence rates (6,7). Also macroalgae face a serious light stress when exposed to high irradiances (8,9). Excess irradiation causes reversible photoinhibition (10–13) or even irreversible photodamage. Photoinhibition

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†*Abbreviations*: F_o, initial fluorescence in the dark-adapted state, all reaction centers are open (oxidized); F_m, maximal fluorescence in the dark-adapted, all reaction centers are closed (reduced); F_o' and F_m', the same for the light-adapted state; F_v, variable fluorescence, calculated as $F_m - F_o$; F_t, current steady-state fluorescence; LED, light-emitting diode; PAM, pulse amplitude modulated fluorometer; PAR, photosynthetic active radiation; qN, nonphotochemical quenching of chlorophyll fluorescence; qP, photochemical quenching of chlorophyll fluorescence; UVA, ultraviolet A radiation (315–400 nm); UVB, ultraviolet B radiation (280–315 nm).

serves as a protective mechanism by which the photosynthetic electron transport chain is interrupted and excessive irradiation is thermally dissipated (2,14). The key reaction is the proteolytic removal of the D1/D2 protein complex in the photosynthetic reaction center of photosystem II after undergoing a structural change of the protein complex by excessive radiation.

Photoinhibition can be detected either as a decrease in the photosynthetic oxygen production or using PAM (pulse amplitude modulated fluorescence) measurements (15,16). The PAM measurements are based on the still unexplained fact that chlorophyll fluorescence arises almost exclusively from photosystem II. Energy harvested by photosystem II can be either funneled into photochemistry or dissipated by fluorescence and nonradiative processes in the form of heat. When less energy is allocated to photochemistry the other two components experience a relative increase. In addition, changes in fluorescence emission and nonradiative processes have different time kinetics. Therefore measurement of the fluorescence parameters allows the determination of photochemical and nonphotochemical quenching of the photosynthetic apparatus (2,17–21). The molecular mechanism of nonphotochemical quenching is still under debate; it includes all nonradiative processes dissipating excitation energy (22). In contrast to the optimal quantum yield defined above, the overall effective quantum yield of photochemical energy conversion can be determined from $(F_m' - F_t)/F_m'$ (23).

While in the past PAM measurements were almost exclusively carried out in the laboratory, recent developments of readily portable instruments allow easy computer-aided determinations in the field (15). In addition, submersible devices have been developed for assessing oxygen exchange in the field under natural conditions of solar radiation and temperature (24,25). This has the advantage that the measurements can be performed in the natural habitat of the plants. Especially algae face serious stress situations when transported into the laboratory due to changes in temperature, light and salinity.

Not only visible radiation (PAR 400–700 nm) but also UVA (315–400 nm) and UVB (280–315 nm) are important stress factors for both terrestrial and aquatic plants (9,26). The relative efficiency of the UV bands exceeds by far their energy contribution in the solar spectrum (27–29). Inhibition of photosynthesis and chlorophyll fluorescence by solar UVB have been described in several species of marine benthic algae as well as in phytoplankton (16,30,31). The targets of short-wavelength radiation in the photosynthetic apparatus are still under debate: UVB seems to impair the D1 protein associated with photosystem II (32,33) and to damage the water-splitting site of photosystem II (34,35).

The aim of this paper is to describe the effects of exposure to solar radiation in the field on photosynthetic oxygen production and fluorescence induction in the Mediterranean green alga, *Halimeda tuna*.

MATERIALS AND METHODS

Plant material. The common Mediterranean green alga *H. tuna* Ellis et Sol was used for all experiments. The thalli were collected from a depth range of 0–6 m on a rocky shore facing east and on submerged boulders of Saronikos Gulf, near Korinth, Greece (37°58'N, 23°0'E). The thalli were transported in a light-tight container and immediately subjected to the measurements. The experiments were carried out during the summers of 1994 and 1995.

Measurements of oxygen exchange. A submersible device has been developed to measure oxygen exchange under solar radiation at the surface or in the water column (24,25). A Clark-type electrode assays the oxygen concentration, and simultaneously PAR irradiance, temperature and depth are measured. After appropriate amplification the signals are relayed to an analog/digital card housed in a laptop computer. A computer program was developed to measure the data at frequent intervals, to determine mean values, to display the data and to store them on the hard disk drive. Another part of the program calculates the regression line to determine oxygen produced or consumed per unit time.

In one type of experiment, the kinetics of photoinhibition was determined in thalli immediately after harvest. The thalli were transferred into the chamber, and dark respiration was determined first. Subsequently, net oxygen production was measured until it ceased. In another experiment the thalli were first exposed at various depths in the water column; then photoinhibition was determined just below the water surface. Then the samples were stored at 5 m depth for a predefined period of time in a translucent container for regeneration of their photosynthetic capacity, and finally oxygen production was measured again. At the end of all experimental runs the areas of the thalli were measured to determine the cell number as well as the dry weight.

Measurements of fluorescence induction. Rapid fluorescence induction kinetics are based on the determination of the concentration of open reaction centers (36). In practice, first the ground fluorescence F_o is measured, which is induced by low irradiation of a dark-adapted sample (all reaction centers in the open state). Subsequently, a single saturating flash is applied which elicits maximal fluorescence, F_m. By this pulse all reaction centers are closed. The so-called variable fluorescence, F_v, can be calculated from the difference between F_o and F_m. The ratio F_v/F_m defines the optimal quantum yield for the photosynthetic electron transport chain (37). In contrast, the effective quantum yield is calculated in light-adapted organisms from F_v'/F_m'.

Light adaptation of a previously dark-adapted sample causes a decrease of F_m, which is then called F_m', and either an increase or decrease in F_o, termed F_o'. The photochemical quenching qP, *i.e.* the amount of energy allocated to photochemistry, can be calculated from these values in combination with the currently measured steady-state fluorescence F_t (22).

$$qP = (F_m' - F_t)/(F_m' - F_o').$$

The nonphotochemical quenching qN is calculated from

$$qN = 1 - (F_m' - F_o')/(F_m - F_o).$$

A portable PAM 2000 (Waltz, Effeltrich, Germany) was employed to determine *in vivo*-induced chlorophyll fluorescence on site (15). Freshly harvested thalli were mounted in open frames made from UVB translucent Plexiglas (GS 2458, Röhm and Haas, Darmstadt, Germany; transmission >85% at all wavelengths 280–700 nm) and kept in a shallow rock pool under a shading cover for 30 min, where irradiance was less than 10% of direct sunlight. After this dark adaptation the fluorescence parameters were measured and the optimal quantum yield was determined. Subsequently, the algae were exposed to solar radiation and the fluorescence parameters evaluated again to document the degree of photoinhibition. Thereafter the samples were transferred back into the shade, and the recovery of the photosynthetic quantum yield was determined periodically during the following hours (up to 6 h).

In a different type of experiment, four samples were exposed in parallel under different cut-off filters (WG 295, WG 335, WG 360 and GG 400, 2 mm, all from Schott & Gen., Mainz, Germany; transmission curves in the company documentation). These filters remove short-wavelength radiation below their nominal value (*e.g.* a WG 295 filter has a 50% transmittance at 295 nm). The experimental procedure was similar to the one described above.

To determine the time course of the quantum yield over the day at their natural growth site, thalli were collected every hour from sunrise to sunset, and the fluorescence parameters (F_o', F_m') were measured immediately after harvest. From these the effective yield was calculated.

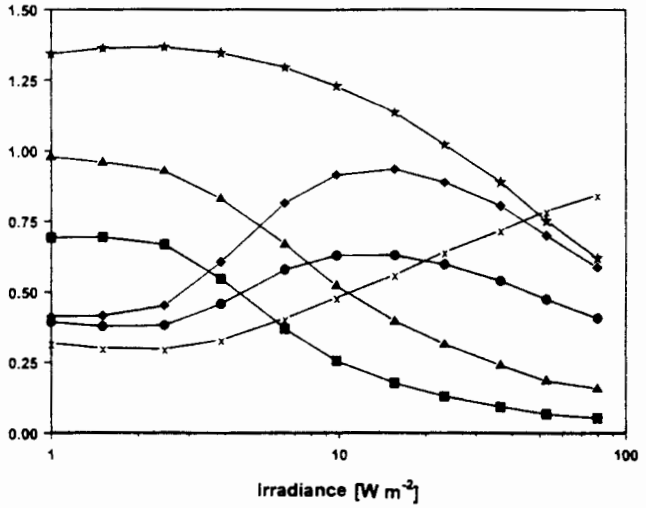

Figure 1. Fluorescence parameters measured in *H. tuna* harvested from 5 m depth dependent upon the fluence rate of the actinic red light produced from the PAM instrument. Before the experiments the thallus was adapted to an intermediate fluence rate of 23 W m^{-2} for 10 min and then exposed to increasing irradiances for periods of 6.5 min each. At the end of each period the fluorescence parameters were determined. Diamonds, F_t; squares, effective photosynthetic quantum yield; triangles, photochemical quenching; crosses, nonphotochemical quenching; asterisks, F_m' and circles, F_o'.

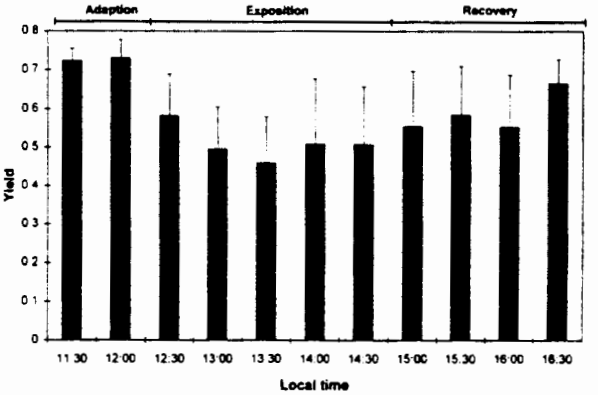

Figure 2. Photosynthetic quantum yield of *H. tuna* measured immediately after harvesting and again after 30 min of adaptation in the shade (<10% of full solar radiation), during exposure to solar radiation in a rock pool and during recovery (in the shade). For each data point at least eight measurements were averaged and the standard deviation calculated.

The PAM 2000 fluorometer allows to run preprogrammed experimental sequences. Thus it is *e.g.* possible to determine the dependence of the fluorescence parameters on the irradiance provided by a red light-emitting diode (LED). Before the run, F_m and F_o were measured in 30 min dark-adapted samples. During the run the sample was first exposed to an intermediate irradiance for 10 min for activation of the Calvin cycle enzymes. Then a series of 11 levels of irradiation, starting from the lowest, was applied, lasting 6.5 min each. At the end of each irradiation period a white light-saturating light pulse was applied to determine the fluorescence parameters, F_t, F_m', as well as the effective photosynthetic quantum yield. For correct determination of the quenching parameters, qP and qN, a far red light pulse was given before the saturating light pulse in order to determine F_o'.

Statistics. At least eight individual measurements were taken during the PAM measurements (except for the actinic irradiance series) from which mean values and standard deviation were calculated. Photosynthetic oxygen exchange was measured at least three times for each irradiation step. All experimental runs were repeated several times and Student's *t*-tests were performed where appropriate.

Measurement of solar radiation. Irradiance of solar radiation was measured in parallel to the experimental exposures in three wavelength bands (UVB: 280–315 nm; UVA: 315–400 nm; PAR: 400–700 nm) using a permanently installed filter instrument developed recently (ELDONET, Real Time Computer, Möhrendorf, Germany). The machine takes readings at 1 s intervals in each channel that are integrated over 1 min. The data are graphically displayed and stored on a computer after amplification and analog/digital conversion. Doses of irradiation are calculated on an hourly and daily basis for each channel. Typical irradiances under clear skies were 390 W m^{-2} for PAR, 38 W m^{-2} for UVA and 0.95 W m^{-2} for UVB at local noon. All experiments were carried out under cloudless skies.

RESULTS

In the first experiment the dependence of the fluorescence parameters on the actinic light was determined in a preprogrammed automatic run on the PAM instrument using samples freshly harvested from 1 m depth (Fig. 1). In preparation of the run the sample was dark adapted for 30 min and F_o and F_m measured. Then the thallus was allowed to adapt to light using the built-in red LED at an irradiance of 23.3 W m^{-2}. After these initial measurements the actinic light irradiance was increased in 11 steps from 1 to 79 W m^{-2}. The steady-state fluorescence, F_t, increased from an intermediate value to an optimum at an irradiance of 15.6 W m^{-2} and subsequently fell again. The F_o' followed a similar pattern but at lower values. In contrast, F_m' had an optimum at a low irradiance of 2.47 W m^{-2} and declined gradually thereafter. The photosynthetic yield had an optimal value of about 0.65 at low irradiances and declined with increasing irradiances, almost dropping to zero. The photochemical quenching followed a similar pattern starting with values close to 1 indicating optimal energy exploitation for photosynthesis and decreased to values below 0.2. In contrast, nonphotochemical quenching rose from values near 0.3 to about 0.9.

Thalli were harvested from 1 m and kept in a shallow rock pool suitable for on-site measurements with the PAM instrument (Fig. 2). The quantum yield was determined immediately after harvesting and again after 30 min of dark adaptation (<10% of unattenuated solar radiation). Then the thalli were exposed to solar radiation for a total of 150 min and the yield was measured every 30 min. During this time the yield first drastically decreased and then stayed constant. After exposure the thallus was shaded again and recovery measured also at 30 min intervals. In this experiment the algae were kept free floating in the rock pool so that different parts of the thallus were exposed to solar radiation.

In the following experiment the thalli were exposed in a UV-transmitting Plexiglas container that kept the algae in place so that the algal surface area exposed to solar radiation could be controlled while sea water circulated through the container. After 30 min of dark adaptation the algae were exposed for 30 min to solar radiation and then placed in the shade again for recovery (Fig. 3). The initially high yield of 0.65 dropped dramatically to 0.2 and then gradually rose again to almost its initial value before exposure within 6 h of recovery. In order to determine whether there were any other stress factors besides high solar irradiance, affecting the yield, a control thallus was subjected to the same experimental conditions except for the light treatment. The high

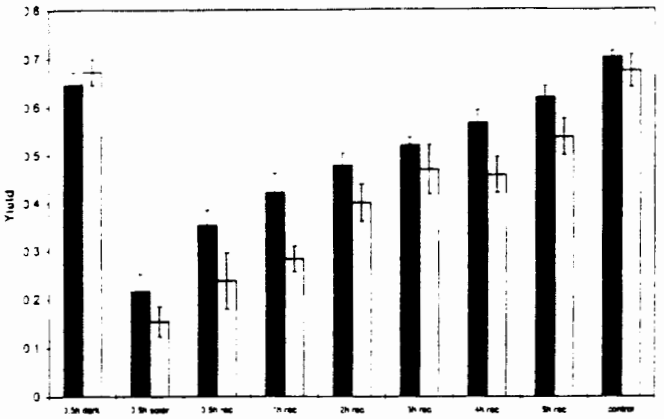

Figure 3. Photosynthetic quantum yield of *H. tuna* harvested from just below the water surface (closed columns) and 5 m (open columns) was measured after 30 min of adaptation in the shade, after 30 min of exposure to solar radiation in a rock pool and during recovery (in the shade). The rightmost bars (controls) indicate the yield for thalli subjected to the same experimental procedure except for solar radiation. For each data point at least eight measurements were averaged and the standard deviation calculated.

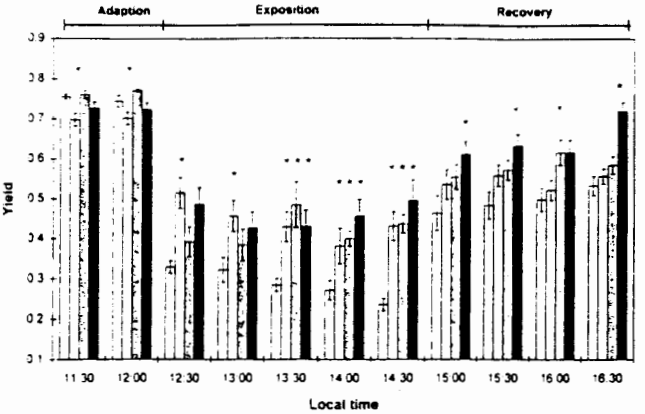

Figure 4. Photosynthetic quantum yield of *H. tuna* harvested from just below the water surface was measured before and after 30 min of dark adaptation, during 2.5 h of exposure to solar radiation in a rock pool and during recovery (in the shade). The thalli were exposed under the following cut-off filters (100 × 100 mm, 2 mm thick): WG 295 (open bars), WG 335 (hatched bars), WG 360 (dotted bars) and GG 400 (solid bars). For each data point at least eight measurements were averaged and the standard deviation calculated. Asterisks indicate those values that significantly ($P < 0.05$) deviate from the values measured for the WG 295 filter in each time group.

yield value indicates that the experimental handling had not affected the photosynthetic capacity of the algae.

Halimeda tuna can be found in the range from the surface to 25 m of depth. Though regarded as the same species, individuals from the surface and from depth differ in their morphology and phenomenology; therefore it has been suggested, that they belong to different strains, adapted to different depths (38). In order to determine whether these two strains also behave differently during exposure and recovery, the same experiment was repeated in parallel to the one described above using thalli harvested from 5 m (Fig. 3). These algae showed the same trend but significantly lower yield values both during exposure and recovery.

The following experiment aimed at determining the role of different UV wavelength bands of solar radiation. For this purpose thalli were harvested from 5 m and exposed under cut-off filters that removed increasing portions of the short-wavelength radiation (Fig. 4). The sample under the WG 295 filter can be regarded as a control because solar radiation reaching the earth hardly contains any radiation below 295 nm. The yield values of the thalli exposed under the WG 295 filter were significantly lower during both exposure and recovery than those of the samples exposed under the other filters as indicated by the Student's *t*-test. In contrast, the values of the algae exposed under the other filters did not show a clear-cut trend.

In the experiments described above the thalli were exposed to solar radiation in a shallow rock pool where they received higher solar radiation than at their growth sites. In order to determine whether photoinhibition occurs at their natural site, algae were harvested from dawn to dusk at 1 h intervals and the yield was determined immediately after harvest. Again both forms adapted to either 1 m or 5 m water depth were used. The first sample, which was harvested before sunrise, showed a maximal yield of about 0.8 (Fig. 5). In the subsequent samples the value gradually decreased as solar irradiance increased at the habitat and increased again when the east-exposed shore experienced shading. The algae harvested from 5 m showed a significantly smaller photoinhibition than those from 1 m; but both strains regained their full photosynthetic capacity at the end of the day.

Oxygen exchange measurements

A thallus of *H. tuna* harvested from a rock pool showed substantial respiration when transferred into darkness (Fig. 6a). When exposed to solar radiation just below the water surface oxygen production (integrated over 10 min intervals) commenced and increased to about 60 fmol per cell and minute. Shortly afterward oxygen production decreased sharply and at 30 min a negative value was observed that increased thereafter. The same experiment was repeated with thalli harvested from 1 m (Fig. 6b). The result followed a similar pattern but even at 20 min slightly negative values were encountered. The final examples show the behavior of

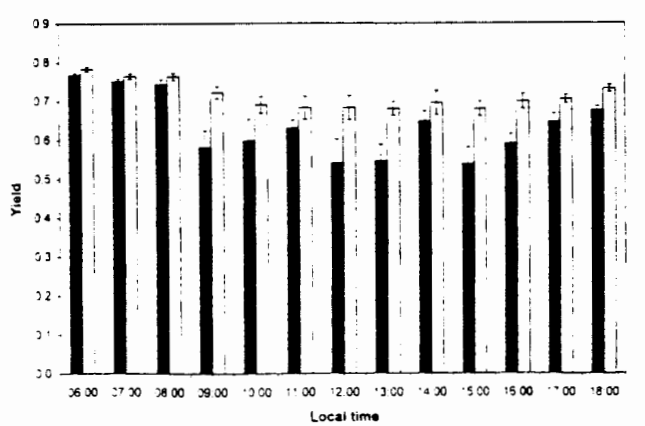

Figure 5. Photosynthetic quantum yield from dawn to dusk of *H. tuna* harvested from 1 m (closed columns) and 5 m (open columns). Thalli were retrieved from their growing site and measured immediately after harvest. For each data point at least eight measurements were averaged and the standard deviation calculated.

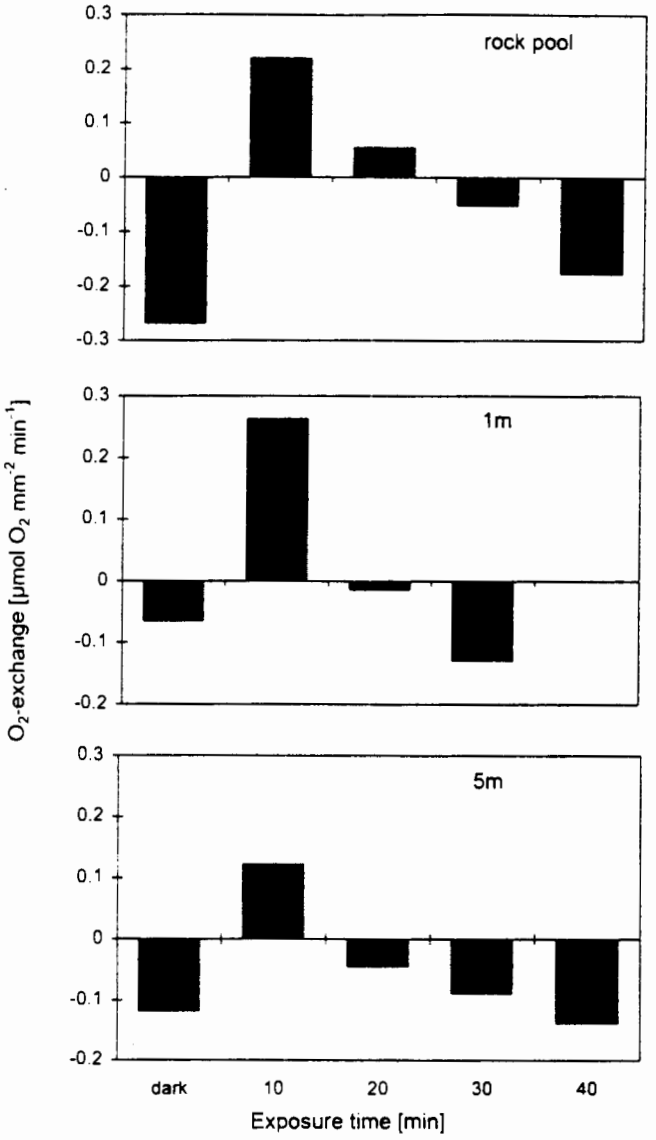

Figure 6. Photosynthetic oxygen exchange of *H. tuna* harvested from 0 m (rock pool), 1 m and 5 m measured under solar radiation at the surface. Before exposure, dark respiration was determined and then oxygen exchange measured integrated over 10 min periods each. Representative result from three independent repetitions.

algae harvested from 5 m (Fig. 6c); in these samples photoinhibition was even more pronounced.

In another type of experiment a sample was harvested from 5 m depth and after measuring dark respiration it was exposed at different depths between 5 m and 1 m starting at the lowest level (Fig. 7). It is interesting to note that the highest net photosynthetic oxygen production could be found at 0 m, even though the sample was harvested from 5 m depth. Exposure time at the surface was too short (4 min) to induce photoinhibition.

In the final experiment freshly harvested algae (from 5 m) were exposed at decreasing depths from 5 m to 1 m for 4 min each (Fig. 8). Subsequently, the chamber was installed just under the water surface and oxygen production was recorded until photoinhibition was observed after about 40 min of exposure. The sample was then stored at 5 m in a translucent container for 2 h to recover and finally, the oxygen production determined at decreasing depths from 5 m to 2 m. The sample had not fully recovered from photoinhibition and showed negative values at 4 m and values around zero in shallower water. Similar results were obtained with samples harvested from different depths (data not shown).

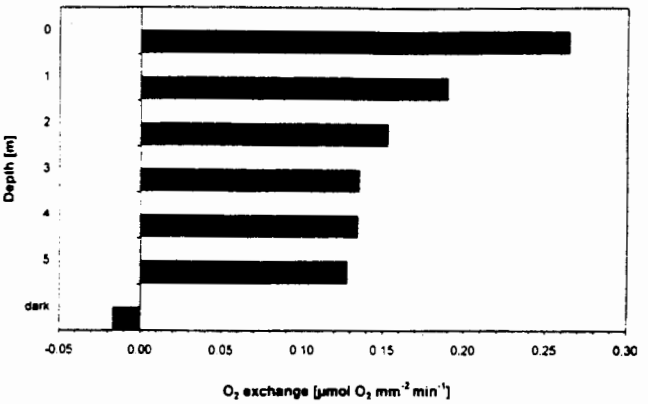

Figure 7. Oxygen exchange of *H. tuna* harvested from 5 m in darkness and subsequently at increasing depths from 5 m to 0 m for 4 min each.

DISCUSSION

Photoinhibition after exposure to solar irradiation of high fluence rates has been observed in higher plants (22,37), macroalgae (12,13,39) and phytoplankton (40–42). The mechanism of photoinhibition is still controversial; however, it can be regarded as an active physiological regulatory process to protect the photosynthetic apparatus from excessive radiation. It is characterized by a decrease in photosynthetic quantum yield and photochemical quenching. In the current study, photoinhibition in the green alga *H. tuna* was determined using PAM fluorescence and oxygen exchange measurements. It is interesting to note that even though the two approaches measure different photosynthetic parameters they show similar kinetics for inhibition.

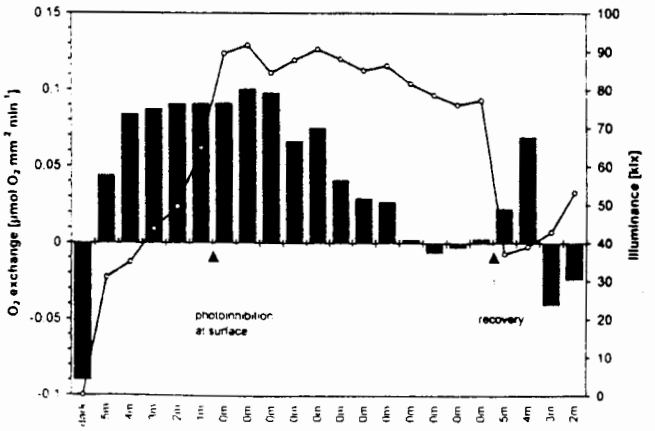

Figure 8. Photosynthetic oxygen exchange of *H. tuna* harvested from 5 m at increasing depths from 5 m to 0 m. After this treatment the thalli were kept at the surface until photoinhibition occurred and then allowed to recover at a depth of 5 m for 2 h. Subsequently, photosynthetic oxygen exchange was determined again at depths of 5, 4, 3 and 2 m.

The preliminary experiments indicated optima for both the photosynthetic quantum yield and photochemical quenching at rather low irradiances of about 1 W m^{-2}. This is in support of the fact that the algae grow to great depth in the water column. However, some individuals were also found at the surface exposed to bright sunshine. Are these thalli photoinhibited during most times of the day or do they use different mechanisms for adaptation and protection from excessive radiation?

When exposed to unfiltered solar radiation the photosynthetic quantum yield in *H. tuna* decreased drastically. Depending on the degree of photoinhibition recovery took between one and several hours. It is interesting to note that thalli harvested from 5 m were more affected and recovered more slowly than those from the surface. The algae from 5 m seem to be adapted to greater depths where fluence rates of solar radiation are lower due to the attenuation in the water column. This confirms the hypothesis that thalli growing at the surface and at greater depths, respectively, represent different physiological strains. At the test site the irradiance at a depth of 5 m was measured to be less than 50% of the surface irradiance. Despite this there was some photoinhibition even at the natural growth site of the organisms when the sun was at high angles. In contrast to the hypothesis of different physiological strains, thalli were more affected at 1 m depth than at 5 m.

Inhibition of the photosynthetic quantum yield was significantly less dramatic when the UVB component of solar radiation was removed by a cut-off filter. In contrast, the organisms exposed to full solar radiation also recovered almost as fast as those exposed under the longer wavelength cut-off filters. However, even if they are not highly significant, there is a tendency in those samples exposed to increasingly shorter wavelengths to recover slower and to lower yield values. These results seem to indicate that the yield is more affected by UVB than by UVA, especially when taking into account that the former wavelength band represents less than 1% of solar radiation, whereas the UVA is about 50 times higher than UVB. Thus, UVA does not seem to be very effective in *H. tuna*. Similar results were reported before for other algae (30,40). This result is in contrast to observations in phytoplankton, where inhibition of $^{14}CO_2$ incorporation elicited by UVA had about the same or even higher efficiency than that induced by UVB (43).

Photoinhibition of oxygen production followed the pattern found for the fluorescence parameters. A decrease was observed even after a few minutes, and thalli harvested from greater depth were more sensitive than those harvested from the surface. After photoinhibition, recovery of oxygen production was only partial within a few hours. Photoinhibition resulting from exposure at the surface up to a point where no net photosynthetic oxygen production is observed could not be fully repaired within more than 2 h, indicating some photodamage by this treatment. After 2 h of recovery the yield is about 75% of initial values.

The primary targets of UV radiation in the photosynthetic apparatus are still under debate. Algae seem to differ in several respects from higher plants in their regulatory mechanisms and capacity (21). Further investigations, including inhibition and recovery kinetics, are necessary to study the mechanisms of photoinhibition and photodamage.

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