Photoinhibition and photosynthetic pigment reorganisation dynamics in light/darkness cycles as photoprotective mechanisms of *Porphyra umbilicalis* against damaging effects of UV radiation

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SUMMARY: *Porphyra umbilicalis* L. Kutzing collected from the upper intertidal zone at Helgoland, north sea, was exposed to different spectral ranges of UV radiation under both 12/12 h light/dark cycles and continuous irradiation. In light/dark cycles, oscillations of the optimal quantum yield ($F_v/F_m$) were observed during the experiments, reaching maximal values at the end of the light phase followed by lower values during the dark phase. Decreased $F_v/F_m$ was observed in thalli illuminated with photosynthetically active radiation (PAR) plus UV-A and PAR+UV-A+UV-B, compared with the PAR control, indicating a certain degree of UV-induced photoinhibition. In addition, a decrease in the percentage of change of the linear initial slope and maximum electron transport rate (ETR) estimated from ETR vs. irradiance curves was induced by UV radiation during the light phase. Recovery during the 12 h dark phase was almost completed in UV-A treated plants. PAR+UV-A seemed not to affect the photosynthesis, measured as $O_2$ production. However, a decrease in $O_2$ production was observed in the PAR+UV-A+UV-B treatment, but it recovered to initial values after 48 h of culture. No changes in total content of photosynthetic pigments were observed. However, thallus absorptance and the in vivo absorption cross-section in the PAR range (400-700 nm) normalised to Chl a (a* parameter) fluctuated during light/dark cycles and were positively correlated with changes in the optimum quantum yield, thus indicating that daily pigment reorganisation in the light-harvesting complex may play a key role in the photosynthetic performance of the algae. Both UV-A and UV-B treatments under continuous irradiation induced a significant reduction in the optimal quantum yield, ETR efficiency and photosynthetic oxygen production during the first 36 h to values around 30% of the initial ones. Thus, different protective mechanisms against UV stress can be observed in *P. umbilicalis*: dynamic photoinhibition when UV-A is combined with PAR, followed by full recovery of photosynthesis during the dark phase, and a more pronounced photoinhibition under UV-B, with only partial recovery after longer time periods, in which photosynthetic pigment reorganisation plays an important role.

Keywords: UV-radiation, chlorophyll fluorescence, thallus absorptance, light/dark cycles, photosynthesis, stress tolerance.

RESUMEN: Dinámicas de fotoinhibición y reorganización pigmentaria bajo ciclos de luz/oscuridad como mecanismos de fotoprotección en *Porphyra umbilicalis* frente a los efectos dañinos de la radiación ultravioleta. – Talos del alga roja *Porphyra umbilicalis* L. Kutzing fueron cultivados bajo diferentes condiciones espectrales de radiación ultravioleta en condiciones de ciclos de luz/oscuridad de 12 horas y bajo luz continua. El rendimiento cuántico óptimo ($F_v/F_m$), estimado a través de la fluoescencia de la clorofila a del fotosistema II, evolucionó con oscilaciones en los ciclos de luz/oscuridad, con valores máximos al final de cada fase de luz seguida de valores bajos a lo largo de la fase de oscuridad. Los cultivos iluminados bajo radiación fotosintéticamente activa (PAR)+UV-A y la PAR+UV-A+UV-B disminuyeron los valores de la relación ($F_v/F_m$) respecto a los cultivos bajo PAR. Los cultivos bajo radiación UV provocaron además una caída, durante la fase de luz, tanto de la pendiente inicial como de los valores máximos de la tasa de transporte electrónico (ETR) estimados a partir de las curvas ETR vs. irradiancia. En el caso de los cultivos bajo PAR + UV-A la recuperación de los valores fotosintéticos fue casi completa durante la fase de oscuridad. Dicho tratamiento no afectó a la producción fotosintética de $O_2$ mientras que el cultivo bajo PAR+UV-A+UV-B disminuyó significativamente dicha tasa, la cual recuperó los valores iniciales tras 48 h de cultivo. No se observaron cambios en el contenido total de los pigmentos fotosintéticos. No obstante, los ciclos de luz/oscuridad afectaron tanto a la absorptancia de los talos como a los valores de corte transversal *in vivo* en el rango del PAR (400-700 nm).
nm) normalized by chlorophyll a (a*), data that were correlated positively with the yield of photosynthesis, which could imply a peak in the maintenance of the activity of the photosynthetic apparatus of the alga. In contrast, the cultivation of the fed algal systems led to a reduction in the yield of photosynthesis, which could be due to photoinhibition and recovery: firstly, for the assimilation of metabolites and the biochemical reactions that lead to cell growth and division (Cuhel et al., 1984); and secondly, for the turnover and reorganization of all damaged photosynthetic machinery from the previous light phase, i.e. the D1 protein (Häder et al., 1998). Therefore, the role of the night period must be as important as that of the light phase for the survival of algae from temperate latitudes. One of the objectives of this work was to determine the degree of participation of the night period in the recovery of Porphyra umbilicalis from photosynthetic damage. In the case of polar algae, which can be exposed to long periods of continuous light in summer, the acclimation strategies observed include the position on the shore, thallus thickness in Fucus species and self-shading in Acrosiphonia species (Aguilera et al., 1999a).

The UV fraction of the solar spectrum has been implicated in photoinhibition and recovery: firstly, the induction of a more pronounced inhibition of photosynthesis in the morning, and secondly, a delay in the recovery process during the afternoon and evening. This double phenomenon has been observed in outdoor experiments with different macroalgae (Hanelt and Nultsch, 1995; Häder and Figueroa 1997; Hanelt 1998; Mata et al., 2006). Exposure of the red alga Porphyra leucosticta in Southern Spain to solar radiation decreased the effective and optimal quantum yield of photosynthesis at noon, as well as the photosynthetic activity measured as O₂ produc-
tion, and led to incomplete recovery in the evening (Figueroa et al., 1997); algae exposed to UV-filtered radiation using selective UV filters were much less affected and full recovery was observed at the end of the day.

In a previous work, Aguilera et al. (1999b) demonstrated that Porphyra shows a high capacity of recovery of the photosynthetic rate (measured as O₂ evolution) and of the effective quantum yield after short exposure to high levels of UV-B radiation. This was due to protective mechanisms against UV stress, one of which was to increase the package effect of the pigments in the thylakoid membranes.

In this study, the long-term effects of continuous UV radiation, combined with PAR, on fluorescence, photosynthesis and pigmentation of P. umbilicalis are compared with the effect of light/dark cycles in order to assess the repair processes during the dark phase.

MATERIAL AND METHODS

Algal material and pretreatment

Porphyra umbilicalis L. Kützing was collected from upper intertidal habitats at Helgoland, North Sea. Algae were pre-cultivated for 30 days in the laboratory at 14 ± 1°C in aerated 5 l beakers in Provasoli’s enriched seawater, and illuminated by cool-white fluorescent light (Osram-L 18W, Germany) at an irradiance of 25 W m⁻² in a light/dark regime of 12/12 h.

Experimental design

Thalli of P. umbilicalis were cultivated in 1 l aerated open flasks and exposed to 25 W m⁻² of UVA and 1.3 W m⁻² of UVB radiation under both 12/12 h light/dark regimes and continuous irradiation. A combination of nine fluorescent lamps (2 X Radium NL 36W/25; 2 X Q-Panel Co. UVA-340; 2 X Osram L36W/32; 2 X Philips TL40 W/12 UVB; 1 X Q-Panel Co. UVA-351) provided UV + PAR from 260 to 700 nm (see Aguilera et al., 1999b, for details). Total PAR+UVA+UVB (295-700 nm) radiation treatment was obtained by covering the flasks with Ultraphan cut-off filter foil (cut-off wavelength <395 nm; Folex, Dreieich, Germany). Appropriate arrangement of the lamps and placement of the samples 60 cm from the light source provided a homogeneous light field for the plants in the different treatments. Spectra were measured using a double monochromator spectroradiometer (Optronic Instruments, model 752, Orlando, FL, USA) with an Ulbricht sphere and a quartz cable.

Oxygen exchange measurements

Photosynthetic determinations in Porphyra were conducted in a transparent 10 ml Plexiglas chamber at time 0 and after 15 min of exposure to 50 W m⁻² of saturating light provided by halogen lamps (Schott KL-1500) in a temperature-controlled (14°C) room. Approximately 100 mg of thallus discs of 1 cm diameter were incubated in each treatment. Saturating, but not photoinhibitory light of 50 W m⁻² was selected after a photosynthetic irradiance curve performed from 0 up to 150 W m⁻². Photosynthetic oxygen production was measured by means of a 5775 microelectrode (Yellow Spring Instruments).

Fluorescence measurements

In vivo chlorophyll fluorescence of photosystem II (PSII) was determined with a portable pulse modulation fluorometer (PAM 2000, Waltz, Effeltrich, Germany). The programmed experimental sequence defined by Hanelt et al. (1997) was used to automatically determine the maximal quantum yield of fluorescence (F_v/F_m). For this purpose, samples were fixed to the end of the optic fibre and placed in a thermostatic chamber. The sequence of commands combined different dark periods with far-red pulses in order to oxidise the first electron acceptor. Then, the initial fluorescence (F_o) from the antenna of fully oxidised PSII was measured, and a saturating flash was applied to obtain the maximal fluorescence level from the fully reduced PSII reaction centre (F_m), and F_v/F_m was obtained (Schreiber et al., 1994). The variable fluorescence (F_v) is the difference between F_m and F_o. Eight replicates were taken for each treatment and time.

In order to relate the fluorescence parameters to the intensity of the actinic irradiance (red light-emitting diode with λ_max = 650 nm), thalli were exposed to a gradient of increasing irradiances between 1 and
79 W m⁻² at intervals of 30 s. At the end of each irradiation period, a saturating white light pulse was applied to calculate the effective quantum yield \( \Delta F/F_m^{'} \), where \( \Delta F \) is the difference between the respective maximal fluorescence (\( F_m^{'} \)) of a light-adapted plant and the fluorescence level at daylight (\( F_d \)) [Genty et al., 1989]. After the saturation pulse, the sample was irradiated with a far-red light pulse (735 nm) for 5 s in order to determine \( F_0^{'} \) (basal fluorescence of light-adapted algae). Measurements were performed in the same dark chambers as described before. The electron transport rate (ETR) was obtained by means of the expression:

\[
ETR = PAR \times A_{680} \times \left( \Delta F/F_m^{'} \right) \times 0.5
\]

where PAR is the irradiance between 400 and 700 nm of the actinic light, \( A_{680} \) is the absorbance of the algae at 680 nm, \( \Delta F/F_m^{'} \) is the effective quantum yield at each irradiance, and 0.5 is a factor that corresponds to the absorption of 2 light quanta (one for each photosystem) needed for the transport of one electron. ETR curves were fitted to non-linear functions according to Jasby and Platt (1976) and then the percentage of change of the linear initial slope of the ETR was calculated.

### Absorption spectroscopy

Absorption spectra (optical density) of thalli of *P. umbilicalis* were measured during exposure in the experimental chamber. Thallus discs of 2 cm diameter were removed from the light, mounted between two slides and placed next to the detector, perpendicular to the light beam of a single beam spectrophotometer (Beckman DU-70, Palo Alto, CA, USA). Average absorbance within the PAR range (\( A \)) was calculated from the in vivo optical density (OD) data obtained by spectrophotometry, after correction for scattering using the equation:

\[
A = 1 - 10^{-1.0 \times OD}
\]

Absorptance data for ETR calculations were obtained from the optical density values at 680 nm.

### Chlorophyll a

The chlorophyll a (Chl a) concentration was analysed at each sampling time under different light treatments. Pigments were extracted from frozen samples kept in liquid nitrogen. Chlorophyll a was extracted in 100% dimethylformamide. Extracts were centrifuged at 10000 X g for 10 min, and the Chl a concentration in the supernatant was determined spectrophotometrically (Inskeep and Bloom, 1985).

From these data, a spectrally averaged in vivo absorption cross-section normalised to Chl a (\( a^* \)) was calculated, which is related to the efficiency of light capture; \( a^* \) is accounted as the ratio between the averaged thallus absorption in the PAR region (400-700 nm) and the total Chl a concentration. Variations in \( a^* \) have been attributed to changes in the degree of packing (package effect) of the pigments in the membranes of the thylakoids [Berner et al., 1989; Mercado et al., 1996].

### Statistics

Mean values and their standard deviations were calculated from the different replicates per treatment and two independent experiments were performed. Measurements obtained for the different UV treatments at different sampling times were grouped and tested for statistical differences in all variables by one-way (UV radiation conditions) model I analysis of variance (ANOVA) of repeated measurements. Homogeneity of standard deviations was assessed by Bartlett’s test. When significance was found, the Fisher test was applied (\( P<0.05 \)) for comparison between treatments. In order to test the statistical differences between light and darkness in all variables, data at the beginning of the light periods were grouped and compared with data at the end of the light periods by means of a Student’s t-test. All the statistical tests were performed in accordance with Sokal and Rohlf (1995).

### RESULTS

Optimal quantum yield \( (F_v/F_m) \) was highest in thalli exposed only to PAR, and lowest under PAR+UV-A+UV-B in 12/12 h light/dark cycles (Fig. 1; see also Table 1 for statistical significance of differences between UV treatments). There was a significant decrease under both PAR+UV-A and PAR+UV-A+UV-B during the first 48 h, after which the optimal quantum yield started to recover. Distinct cycles following the light/dark cycles were detected. In general, especially under PAR and less pro-
Under continuous irradiation (Fig. 1b) both UV-A and UV-B radiation seemed to significantly affect the optimal quantum yield of *P. umbilicalis*. F$_v$/F$_m$ steadily decreased during the first 24 h of exposure to PAR+UV-A, and after this period no significant differences were found. Under PAR+UV-A+UV-B this decrease of F$_v$/F$_m$ lasted for 36 h, after which it did not change for the rest of the experiments. The decrease in optimal quantum yield was always more pronounced under PAR+UV-A+UV-B than under PAR+UV-A.

The electron transport rate (ETR) was calculated from the variation of the effective quantum yield (ΔF/F$_{m'}$) under increasing irradiance of actinic white light (400-700 nm). ETR followed a linear pattern at irradiances of 0-10 W m$^{-2}$ in all cases, and a rapid saturation was observed at increasing irradiances (data not shown). From the ETR curves, the percentages of change of the linear initial slope and the maximum ETR were calculated and plotted (Fig. 2). The initial slope of ETR reveals the ETR efficiency at low irradiance; it did not significantly vary during the experiments in the PAR treatment under both light/dark cycles and continuous light (Fig. 2a, b). Treatments with PAR+UV-A and PAR+UV-A+UV-B in light/dark cycles significantly reduced the initial slope of ETR, reaching only 55% of the initial value at the end of the experiments in the latter treatment. It is interesting to note that dark periods induced a recovery of the capacity for light utilisation by the photosynthetic apparatus, especially under PAR+UV-A. Thus, a partial recovery of the thalli from the deleterious effects of UV-A radiation may be expected during the dark periods. However,
the initial slope of ETR gradually decreased after exposure to PAR+UV-A and PAR+UV-A+UV-B under continuous light (Fig. 2b).

Similar results were found for maximal ETR under the different light treatments (Fig. 2c, d). Under PAR, maximal ETR values were significantly increased after 24 h, returning to initial figures at the end of the experiments under both light/dark cycles and continuous light. UV radiation plus PAR induced a gradual decrease in maximal ETR under both light/dark cycles and continuous irradiation, although the effect of PAR+UV-A+UV-B was more pronounced.

Maximal net photosynthetic O$_2$ production measured at 50 W m$^{-2}$ (saturating irradiance for the photosynthesis of $P$. umbilicalis) decreased rapidly in thalli cultured under PAR+UV-A+UV-B after the first illumination period of 12 h, but started to recover to initial levels after 36 h (Fig. 3a). A significant recovery during the dark period was also observed during the first two light/dark cycles. UV-A did not seem to affect the capacity for photosynthetic O$_2$ production in $P$. umbilicalis, since no significant differences appeared when the thalli were exposed to PAR or PAR+UV-A under both light/dark cycles

Fig. 2. – Influence of exposure to UV radiation on the initial slope and the maximal electron transport rate vs irradiance curves under light/dark cycles of 12/12 h (a,c) and continuous light (b, d). Initial values (t=0) in a) were arbitrarily set to 100%. Standard deviation of less than 10% (n=6) was avoided for clarity.

Fig. 3. – Influence of exposure to UV radiation on O$_2$ production of thalli of $P$. umbilicalis under a) light/dark cycles of 12/12 h and b) continuous illumination. Data are mean ± SD (n=6).
and continuous light. Moreover, O₂ production under these two treatments remained constant during the first 48 h, followed by a significant increase at the end of the experiments.

Under continuous irradiation (Fig. 3b), UV-B radiation significantly reduced the photosynthetic oxygen production, while no significant differences were found in O₂ production between the PAR and PAR+UV-A treatments. O₂ production under these two light treatments did not change during exposure, showing values of about 0.6 mg O₂ min⁻¹ g⁻¹ FW. It can be seen that Fₘₐₓ/Fₘₐₜ (Fig. 1b) and ETR (Fig. 2b,d) decreased in PAR+UV-A, while oxygen evolution did not change (Fig. 3b).

Average absorbance of the thalli in the range 400-700 nm, namely total absorbance, also fluctuated following light/dark cycles (Fig. 4a). It was maximal at the end of the light periods and minimal at the end of the dark periods. No significant differences appeared between PAR and PAR+UV-A treatments. Averaged absorbance under PAR+UV-A+UV-B did not show a fluctuating tendency fol-
owing the light/dark cycles. It decreased gradually during the experiments, with a final decay of 25-30% in comparison with the initial level after 72 h. However, this differential tendency of the light absorption characteristics of the thalli under UV radiation was not reflected by changes in the concentration of photosynthetic pigments. The chlorophyll a content, as observed in Figure 4b, showed no significant changes under different UV treatments and no oscillations were observed in light/dark cycles. No changes in phycoerythrin and phycocyanin were observed either (data not shown). The spectrally averaged in vivo absorption cross-section normalised to Chl a (a*) fluctuated again following the light/dark cycles. The chlorophyll a content, as observed in Figure 4b, showed no significant change under different UV treatments and no oscillations were observed in light/dark cycles. No changes in phycoerythrin and phycocyanin were observed either (data not shown). 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pigment reorganisation has been described for different algae and is correlated with the thallus thickness and cell layers (Agustí et al., 1994). In the case of Porphyra, a* values are very high due to the low thallus thickness (with only one cell layer), and variations are due mainly to changes in the content of Chl a. No changes were observed in the Chl a concentration, so variation in the packing of the pigments plays an important role in this alga. Not only pigment reorganisation in the antenna but also chromatophore displacements in the thallus could be responsible for a* values, as observed in the case of the brown alga Dystiota dichotoma (Hanelt and Nultsch, 1990). However, no changes in chromatophores have been found in red algae (Nultsch and Pfau, 1979).

In a previous work, we demonstrated that PAR, PAR+UV-A and PAR+UV-A+UV-B resulted in differential thallus absorption and package effect in short-term experiments (Aguilera et al., 1999b). Exposure for 24 h to PAR+UV-A induced a clear increase in total absorption, with a significant decrease in the packing of chlorophyll a and carotenoids. Nevertheless, thallus absorption decreased under UV-A+UV-B radiation, but this decrease was not due to photodestruction of the pigments but to the increasing package effect. Longer time exposure, as observed in this work, follows the same tendency, with no change in pigment composition but with variation in their packing in the thylakoids, especially under continuous light under PAR+UV-A+UV-B.

ETR curves vs. irradiance were also affected by the UV radiation treatment. The degree of inhibition of the efficiency of ETR was similar under PAR+UV-A and PAR+UV-A+UV-B after the light phase. However, while the dark phase promoted only a very small increase in ETR in UV-A+UV-B treated plants, recovery of efficiency was almost complete after the dark period in UV-A treated plants. Thus, it can be postulated that P. umbilicata is photoregulated under UV-A stress by a dynamic photoinhibition process (in the terms defined above), while regulation of photosynthesis under UV-B is made via chronic photoinhibition, in which recovery and repair mechanisms may take many hours or even days, and results in higher recovery of photosynthetic capacity than that of photosynthetic efficiency (Aro et al., 1993; Necchi, 2005). The resistance to the photoinhibition damage has been described in Porphyra perforata as the main photoprotective mechanism during the severe desiccation and exposure to full sun that simultaneously occurs during low tides (Herbert, 1990).

In this work, photosynthetic rate measured as O₂ evolution at saturating irradiance did not agree with the ETR measurements, since UV-A did not promote significant changes in comparison with the PAR control. In the case of PAR+UV-A+UV-B, there was a strong decrease in photosynthetic rate measured by O₂ production in thalli cultivated under continuous light. However, under light/dark cycles, O₂ production gradually recovered to initial values, again indicating a clear recovery of photosynthetic performance during the dark phase. It is thus demonstrated that night plays an important role in the maintenance of physiological parameters of the plant, and in this case, at least for periods of 12/12 h light/darkness, minimal doses for photodamage are not reached. In contrast, absence of darkness indicated that non-repaired damage occurs after 48 h, since no recovery was observed. This means that other processes apart from a decrease in photosynthetic performance may occur as protein breakdown (Lao and Glazer, 1996), production of reactive oxygen species (Rijstenbil et al., 2000) and DNA damage (van de Poll et al., 2002). Although there are no references for minimal doses of photodamage in algae, UVB irradiance (1.3 W m⁻²) used in this work leads to a biologically effective dose of 9200 J m⁻² and 15300 J m⁻² when the DNA action spectrum of Setlow (1974) and the generalised plant damage action spectrum of Caldwell (1971) are used, respectively. A UV-weighted dose ten times lower than that of this work was used by van de Poll et al. (2001) to promote high cyclobutane-pyrimidine dimers (CPDs) in various temperate red macroalgae. Thus, as expected, Porphyra species must have different photoprotection strategies apart from photoinhibition and pigment reorganisation in order to survive under natural radiation conditions in the intertidal system. Very low CPD formation has been found in Antarctic Porphyra propagules after UVB irradiation (Zacher et al., 2007). One reason for this may be related to an efficient repair of photoproducts in this species, not only via photolysis during the light phase, but also by night induction of other DNA repairing enzymes, or directly due to the presence of high content of UV absorbing substances, namely mycosporine-like aminoacids. Porphyra has almost the highest concentrations of MAAs among macroalgae (Korbee-Peinado et al., 2005), and their role in photoprotection in red algae has been extensively described (Korbee-Peinado et al., 2006), including an important DNA natural screen (Misonou et al., 2003).
Only continuous UV radiation significantly affected the photosynthetic performance. Therefore, culture under stressful continuous light (PAR+UV-A+UV-B) includes a down-regulation of the PSI activity (photoinhibition) that may have consequences for primary production, with a significant decrease in the O₂ production rate. These physiological changes under continuous PAR+UV-A+UV-B light were reflected in the growth rate after one week, with a significant decrease down to 9% day⁻¹ compared with 15% day⁻¹ in PAR, while no differences were observed between light treatments under light/darkness cycles. In any case light is necessary for repair, as has been reported for different macroalgae (van Poll et al., 2002; Roleda et al., 2004, 2006).

In conclusion, Porphyra species are photoregulated against the damaging effects of UV radiation in photosynthesis through dynamic photoinhibition in response to UVA stress, and through chronic photoinhibition after exposure to UVB. These two processes seem to be very effective for photoprotection of primary production machinery. The dark period leads to a complete recovery of photosynthetic performance mediated by a reorganisation of pigments in the photosynthetic apparatus.

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