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Ultraviolet Radiation-Induced Changes in Mycosporine-Like Amino Acids and Physiological Variables in the Red Alga *Lemanea fluviatilis*

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ABSTRACT

Samples of *Lemanea fluviatilis* were cultured in the laboratory under three different radiation regimes, which included the absence and presence of ultraviolet (UV) radiation. Photosynthetic pigment composition, photosynthetic activity, and mycosporine-like amino acids (MAAs) were analyzed after 16 and 64 h of exposure to the radiation. Two MAAs were identified as porphyra-334 and mycosporine-glycine. Mycosporine-glycine was specifically induced by UV-B radiation, but porphyra-334 was degraded by this radiation. The level of UV irradiance used in the induction experiment produced damages in the alga as revealed by reductions in photosynthetic activity and photosynthetic pigments (chlorophyll, phycocyanin, and total carotenoids). Our results indicate that the MAAs failed to fully protect the photosynthetic machinery against UV-B under the specific conditions used in this study.

INTRODUCTION

Anthropogenic depletion of the stratospheric ozone layer has led to an increase in the solar ultraviolet-B radiation (UV-B: 280-315 nm) in the biosphere. The most frequent response of algae and vascular plants to high levels of UV-B radiation is the increase in UV-absorbing compounds (Day and Neale 2002, Searles et al. 2001). Mycosporine-like amino acids (MAAs) absorb UV-B radiation and act as protecting compounds against excessive UV radiation in algae, especially rhodophytes (Karsten et al. 1998, Gröniger et al. 2000, Sinha et al. 2001, Häder 2001). Many marine red algae have been surveyed in search of MAAs, whereas freshwater ones have received much less attention.

Lemanea fluviatilis is a red alga common in turbulent acidic streams, where it may contribute significantly to primary production. Given that the alterations caused by UV-B in primary producers affect nutrient cycles and food webs (Rogers et al. 2001), any potential risk suffered by this species could be also a risk for the whole ecosystem. In addition, this alga can grow at relatively high altitudes and thus it may be especially exposed to the adverse effects of UV-B radiation, since 1) the biologically active UV-B radiation increases between 5% and 20% per 1000 m in altitudinal increase (Björn et al. 1998); 2) mountain streams are often oligotrophic and shallow, and UV-B radiation can penetrate oligotrophic waters (Häder 1997); and 3) low temperatures which prevail during most of the year may limit the development of protection and repair mechanisms against UV-B radiation (Caldwell et al. 1998).

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The objective of our work was to establish the physiological responses of the freshwater red alga *Lemanea fluviatilis* to UV radiation under laboratory conditions. In addition, the presence and identification of MAAs in this species and their role in the defence against UV radiation were evaluated.

METHODS AND MATERIALS

Samples of the red alga *Lemanea fluviatilis* (L.) C. Agardh were collected at 1450 m altitude from an unshaded region of the first-order stream Lavieja, which is located in the upper basin of the River Iregua (La Rioja, northern Spain) within the limits of the Natural Park of Sierra Cebollera (42°02' N, 02°35' W). Samples were collected in October 2003, placed in polythene bottles with stream water, and transported on ice (<5°C) to the laboratory within 48 h. The material was then rinsed with distilled water, and healthy apices were selected.

For mycosporine-like amino acids (MAAs) extraction, clumps of healthy apices (75-85 mg fresh mass) were homogenized in a mortar with 5 ml of five different extraction solvents: (a) 100% tetrahydrofuran; (b) chloroform : methanol : acetic acid : water (170 : 30 : 20 : 7); (c) light petroleum : diethylether : acetic acid (80 : 20 : 1); (d) methanol : water : 7 M HCl (70 : 29 : 1), and (e) 100% methanol. After grinding, one set of samples was incubated at 4°C overnight (Method 1) and another set was kept at 45°C for 2 h (Method 2). Samples were centrifuged at 6000 x g for 15 min at 10°C, and absorption spectra of supernatants were recorded between 250 and 700 nm (DU 70 spectrophotometer, Beckman, Palo Alto, USA). MAA amounts were estimated in triplicate by measuring the area under the absorption curve between 290 and 350 nm ($AUC_{290-350}$) per mg dry mass (DM). Three more samples were weighed to obtain the dry mass/fresh mass ratio by drying the samples at 80°C for 24 h.

For MAAs induction under laboratory conditions, the samples were pre-cultured for three days, and the induction experiment was performed over the next four days using a SOL 1200 unit (Dr. Hönle AG, Gräfelfing, Germany) which provided the following irradiance values: 300 W m^{-2} for photosynthetically active radiation (PAR), 27.9 for UV-A, and 1.3 for UV-B. Irradiances were measured by an Eldonet dosimeter (Lebert et al. 2002). Cut-off filters of different transmittance characteristics were used to impose three different radiation regimes:

- P (PAR alone), using Ultraphan 395 (Digefra GmbH, Munich, Germany), which eliminates all UV radiation. This regime was regarded as control.
- PA (PAR + UV-A), using Folex 320 (Folex GmbH, Dreieich, Germany), which eliminates UV-B and UV-C radiation.
- PAB (PAR + UV-A + UV-B), using Ultraphan 295 (Digefra GmbH, Munich, Germany), which eliminates UV-C radiation.

Samples were exposed to a photoperiod of 16 : 8 (light : dark). This exposure provided a total UV daily dose of 1682 kJ m^{-2} d^{-1}, which included 1607 kJ m^{-2} d^{-1} of UV-A and 75 kJ m^{-2} d^{-1} of UV-B.

Physiological variables were measured at the beginning of the induction experiment and after 16 and 64 h of exposure. For photosynthetic pigments and MAAs analysis, 80-100 mg of fresh material were ground in a mortar with 2 ml 100% methanol and were kept overnight at 4°C (Method 1) for further extraction. After centrifugation at 6000 x g for 15 min at 10°C, the supernatant was collected for spectrophotometric and chromatographic analysis. Absorption spectra between 250 and 750 nm were obtained for the simultaneous quantification of photosynthetic pigments and MAAs. Photosynthetic pigments were estimated by the evolution of their maximum absorption peaks (Sinha et al. 2000). The

ratio between total carotenoids and chlorophyll *a* (OD_{471}/ OD_{665}) was also determined. The amounts of MAAs were calculated in arbitrary units as $AUC_{290-350}$ per mg DM. All the measurements were obtained in triplicate.

For high-performance liquid chromatography (HPLC) analysis of MAAs, 1 ml of supernatant was evaporated to dryness at 40°C, and the extract was redissolved in 500 µl distilled water, filtered through 0.2 µm filters, and analyzed with a Waters HPLC system following the method of Sinha et al. (1999). MAAs were separated on a LiCrospher RP 18 column (5 µm, 250 x 4 mm I.D.). The mobile phase was 0.02% acetic acid run isocratically at a flow rate of 0.750 ml min^{-1}. MAAs were detected with a Waters photodiode array detector at 334 and 310 nm, and absorption spectra were recorded in the wavelength range between 250 and 400 nm. Quantification was made by calculating the area of each peak per g DM. Area values were obtained at the maximum absorption wavelength of each MAA. The MAAs were identified by comparing their absorption spectra and retention times to those of different standards from the MAAs library at the Institut für Botanik und Pharmazeutische Biologie, Friedrich-Alexander Universität, Erlangen, Germany. Measurements were conducted in triplicate.

In vivo chlorophyll fluorescence of PS II was measured with a portable pulse amplitude modulation fluorometer (PAM 2000, Walz, Effeltrich, Germany). Light was applied to a group of alga apices through an optical fiber of 0.6 cm diameter positioned at an angle of 60° (relative to the sample plane) with the aid of a distance clip (Walz). The apices were placed into a shallow thermostatted cuvette filled with stream water and were fastened below the hole of the distance clip, which was fitted between two guides so that the fiberoptics was situated in the same position during all measurements. Minimal and maximal fluorescence (F_0 and F_m) were measured in dark-adapted samples for 20 min following the protocol described by Hanelt (1998), using a 600 Hz modulated beam with white saturating flashes of ~ 9200 μmol m^{-2} s^{-1} photon flux density (PFD) and 400 ms duration. The maximum quantum yield of PS II was given by the ratio F_v/F_m, where $F_v = F_m - F_0$ (Schreiber et al. 1995). Measurements were replicated five times.

To test the effect of the type of solvent on MAAs extraction, a one-way ANOVA was conducted once the normality and homoscedasticity of the data were proven. In the case of significant differences, means were then compared by the least significant differences test. Differences between the two extraction methods for each solvent were

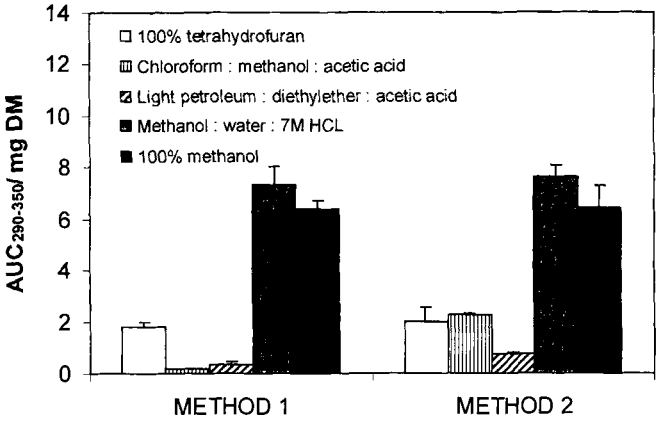

Figure 1. Extraction of MAAs by five different solvents using Method 1 (overnight at 4°C) and Method 2 (2 h at 45°C). MAAs are expressed in relative units as the area under the absorption curve per mg dry mass ($AUC_{290-350}$ mg^{-1} DM). Mean values of three replicates are given with standard errors.

assessed using Student's *t* tests. The effects of the radiation regime and exposure time on the physiological variables were tested using a two-way ANOVA if the data met the assumptions of normality and homoscedasticity. If not, a Kruskal-Wallis test was used. In the case of significant differences, means were then compared by the least significant differences or Mann-Whitney tests, respectively. All statistical procedures were performed with SPSS 9.1 for Windows.

RESULTS

Extraction experiment

The efficiency of MAA extraction showed significant differences between solvents for each method ($p<0.01$) but not between methods for each solvent (except for chloroform : methanol : acetic acid : water). Aqueous acidified methanol and 100% methanol showed similar high efficiencies as compared with the rest of solvents (Fig. 1). Thus, for the induction experiment, 100% methanol was chosen due to its ability to extract simultaneously photosynthetic pigments and MAAs.

Induction experiment

Spectra of the 100% methanol crude extracts of *Lemanea fluviatilis* showed five main peaks (Fig. 2) at 334 (MAAs), 436 (chlorophyll *a*), 471 (carotenoids), 618 (phycocyanin), and 665 (chlorophyll *a*) nm. The radiation regime ($p<0.05$) and the exposure time ($p<0.01$) influenced significantly the evolution of the absorbance of the photosynthetic pigment peaks. After 16 h of exposure, the chlorophyll peaks showed, with respect to the initial condition, significant decreases in the PAB samples, significant increases in the PA ones, and no changes in the P ones; differences between P and PA samples were not significant (Table 1). The carotenoids peak decreased strongly in the PAB samples, whereas it showed no change in the PA ones and a slight reduction in the P ones. The phycocyanin peak also showed a significant reduction after 16 h of exposure under PAB regime, whereas values under P and PA regimes kept stable. After 64 h of exposure, there were significant and similar declines of chlorophyll, carotenoid, and phycocyanin peaks in all the radiation regimes with respect to the initial condition. The ratio OD_{471}/ OD_{665} remained stable after 16 h of exposure under every treatment as compared with the initial value and increased significantly after 64 h in the treatments P (45% higher than at the beginning of the experiment) and PA (44%); the increase in the treatment PAB (25%) was not significant.

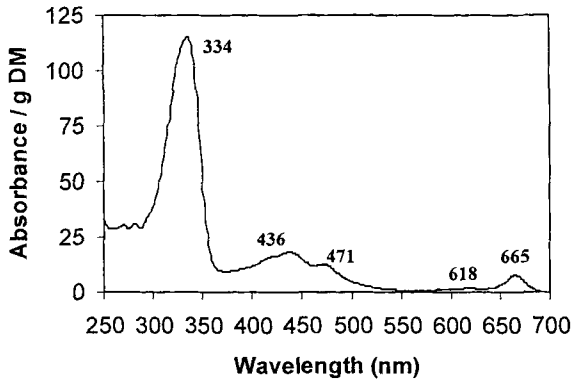

Figure 2. Absorption spectrum of a 100% methanol crude extract of *Lemanea fluviatilis* at the beginning of the experiment. The five main peaks occur at 334 (MAAs), 436 (chlorophyll *a*), 471 (carotenoids), 618 (phycocyanin) and 665 (chlorophyll *a*) nm.

Table 1. Values of the physiological variables measured in *Lemanea fluviatilis* at the beginning of the experiment (0 h) and after 16 and 64 h of exposure to the three radiation regimes: P (PAR alone, control); PA (PAR + UV-A); and PAB (PAR + UV-A + UV-B). Mean values of three replicates (five for F_v / F_m) are given with standard errors. For each variable, different letters indicate significant differences ($p<0.05$). OD = optical density; DM = dry mass.

Variable	0 h	16 h exposure			64 h exposure		
		P	PA	PAB	P	PA	PAB
OD_{665} g^{-1} DM	7.59±0.25^{a}	8.53±1.02^{ab}	9.28±0.33^{b}	4.10±0.42^{c}	3.14±0.30^{c}	3.26±0.05^{c}	3.79±0.25^{c}
OD_{436} g^{-1} DM	18.2±0.5^{a}	18.8±1.1^{ab}	21.2±0.5^{b}	10.1±0.6^{c}	11.9±0.6^{c}	12.0±0.4^{c}	10.8±1.0^{c}
OD_{471} g^{-1} DM	12.5±0.6^{a}	10.7±0.1^{b}	13.9±0.2^{a}	6.7±0.6^{c}	9.4±0.3^{bd}	9.6±0.1^{bd}	8.4±1.0^{bcd}
OD_{618} g^{-1} DM	1.86±0.20^{a}	1.85±0.21^{a}	1.91±0.17^{a}	1.09±0.21^{b}	0.82±0.16^{b}	0.84±0.13^{b}	0.93±0.06^{b}
OD_{471} / OD_{665}	1.64±0.03^{a}	1.60±0.03^{a}	1.50±0.06^{a}	1.63±0.03^{a}	3.00±0.25^{b}	2.94±0.03^{b}	2.20±0.19^{ab}
F_v / F_m	0.624±0.017^{a}	0.575±0.020^{ab}	0.501±0.030^{bc}	0.439±0.045^{cd}	0.509±0.033^{bc}	0.480±0.038^{bc}	0.387±0.032^{d}
$AUC_{290\text{-}350}$ mg^{-1} DM	4.60±0.19^{a}	3.98±0.38^{a}	4.27±0.25^{a}	2.26±0.24^{b}	3.97±0.29^{a}	3.72±0.45^{a}	2.41±0.24^{b}
Porphyra-334 (peak area g^{-1} DM)	266.0 ±21.1^{a}	237.2±30.4^{ab}	213.1±24.4^{ab}	96.1±20.1^{c}	198.4±18.1^{ab}	154.5±27.4^{bc}	94.4±6.8^{c}
Mycosporine-glycine (peak area g^{-1} DM)	15.3±0.9^{a}	8.3±1.4^{b}	9.2±0.1^{b}	22.5±1.2^{c}	7.4±0.5^{b}	12.4±1.0^{a}	13.5±1.1^{a}

F_v/F_m was influenced significantly by the radiation regime ($p<0.01$) and the exposure time ($p<0.001$). F_v/F_m significantly decreased in all the regimes except P after 16 h of exposure, and the decrease continued until 64 h (Table 1). At this point, F_v/F_m had decreased by 18% in the radiation regime P, 23% in PA and 38% in PAB as compared to the beginning. At the end of the experiment, F_v/F_m values under the PAB regime were significantly lower in comparison to P and PA.

The MAA amount, expressed as $AUC_{290\text{-}350}\ mg^{-1}$ DM, was significantly affected by both the radiation regime ($p<0.01$) and the exposure time ($p<0.001$). MAAs in the samples cultured under the regime PAB showed a strong significant decrease after 16 and 64 h of exposure as compared to the rest of the samples, which had fairly homogeneous values (Table 1).

Chromatograms obtained by HPLC at 310 nm revealed that the samples contained a mixture of three compounds (P1, P2, and P3: Fig. 3), whose absorption spectra are shown in Figure 4. In the chromatograms obtained at 334 nm, P3 disappeared due to its particular absorption spectrum. The two main compounds could be identified as porphyra-334 (fraction P2, RT 4.4 min) with a maximum absorption at 334 nm and mycosporine-glycine (fraction P3, RT 9.2 min) with a maximum absorption at 310 nm. The peak at 3.0 min (fraction P1) had an absorption maximum at the same wavelength as porphyra-334 (334 nm). In both chromatograms porphyra-334 appeared as the main compound.

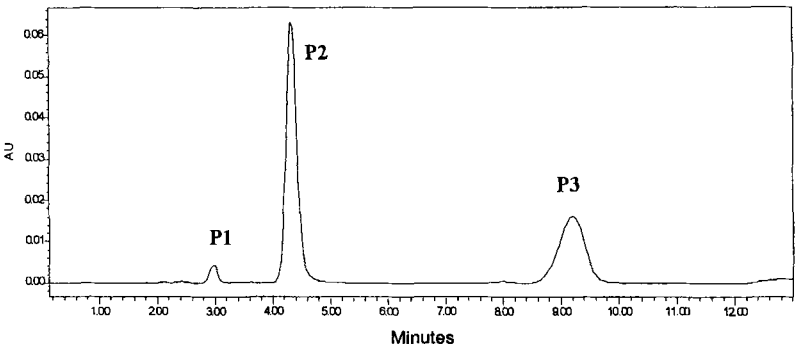

Figure 3. HPLC chromatogram of a 100% methanol extract from *Lemanea fluviatilis* after 16 hours of exposure to the PAB radiation regime (PAR + UV-A + UV-B). LiCrospher RP 18 column (5 µm, 250 x 4 mm I.D.); mobile phase 0.02% acetic acid; flow rate 0.750 ml min^{-1}. The signal was recorded at 310 nm, where three main peaks (P1, P2 and P3) were detected. AU: absorption units.

Samples at the beginning of the exposure contained higher amounts of porphyra-334 than of mycosporine-glycine (Table 1). Porphyra-334 showed a significant reduction under the PAB regime after 16 h exposure compared with the beginning and the P and PA regimes, which showed non-significant changes. After 64 h, values hardly changed except in the PA samples, in which porphyra-334 decreased. A substantial induction of mycosporine-glycine ($p<0.001$) was observed under the PAB treatment after 16 h of exposure in comparison with the initial value, whereas values under the P and PA regimes were significantly lower than the initial one. However, after 64 h of exposure, values decreased under the PAB regime, increased under the PA one and remained unchanged under the P one.

DISCUSSION

The marked reduction in the absorbance of photosynthetic pigments under the PAB regime indicated early photodamage caused by UV-B radiation, since the other regimes

only caused reductions in photosynthetic pigments after 64 h of exposure. Chlorophyll, carotenoids, and phycocyanin are recognized molecular targets of UV-B radiation (Jansen et al. 1998, Sinha et al. 2000), and thus it is not surprising that both types of pigments were affected early by the PAB regime. The concomitant decrease of chlorophyll and carotenoids was also expected, since the degradation of photosystems leads to the breakdown of both types of pigments. The reduction in photosynthetic pigments under all the radiation regimes at the end of the experiment could be caused by photooxidation processes induced by UV and high PAR absorption of endogenous chromophores (Sinha et al. 2000). UV-B radiation caused similar degradations of both chlorophyll and carotenoids in *Lemanea fluviatilis*, and thus the ratio OD_{471}/ OD_{665} remained stable over the culture period, whereas PAR and PAR+UV-A radiations caused a preferential degradation of chlorophyll, although only after 64 h of exposure. The persistence of carotenoids *vs.* chlorophyll under these conditions could indicate that carotenoids may serve as photoprotectors against high PAR and UV-A radiations. The biochemical pathway for this function is still obscure, since it could be linked with the induction of the xanthophylls cycle (Häder and Figueroa 1997), which is doubtful to occur in red algae (Figueroa et al. 2003).

Damage under the PAB regime was also revealed early by the drastic decline of F_v/F_m after 16 h of exposure. F_v/F_m is used extensively as a vitality index since it declines under stress conditions (Maxwell and Johnson 2000), and comparatively it may be more sensitive to UV-B radiation than photosynthetic pigments (Martínez-Abaigar et al. 2003). A slight reduction in F_v/F_m was also observed under P and PA radiation regimes after 16 h of exposure, but it remained subsequently stable under both regimes. Our results agree with those found in *Porphyra leucosticta* (Figueroa et al. 1997), where F_v/F_m decreased in the treatment including UV-B radiation. In addition, F_v/F_m decline should be expected in all of our treatments because of the high photosynthetic, UV-A, and UV-B irradiances applied and the long photoperiod used. Similar reductions in F_v/F_m after 72 h of exposure to P, PA, and PAB regimes were recorded in the green alga *Dasycladus vermicularis* (Pérez-Rodríguez et al. 1998).

The biological role of MAAs as protective compounds against UV radiation has been widely studied in organisms from different habitats, but the red alga *Lemanea fluviatilis* is virtually unknown regarding this aspect (Karentz et al. 1991, Häder et al.

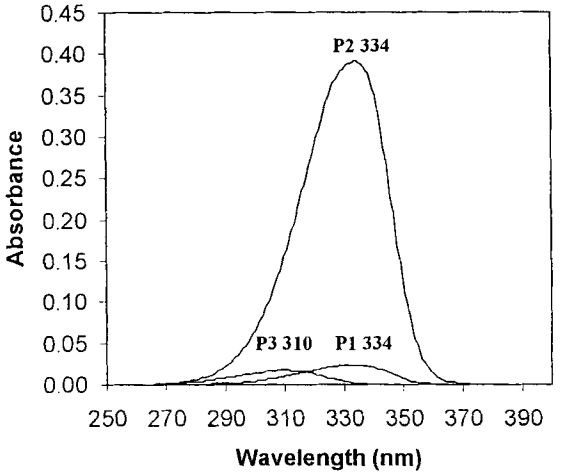

Figure 4. Absorption spectra (between 250 and 400 nm) and maximum of each purified fraction separated by HPLC in Figure 3.

1998, Gröniger et al. 2000). Our results reveal that this species contains two main MAAs, porphyra-334 and mycosporine-glycine, which have been previously found in marine red algae (Karsten et al. 1998, Gröniger et al. 2000, Figueroa et al. 2003). MAAs were observed in remarkably high amounts in *Lemanea fluviatilis* before starting the UV treatment, and thus it could be placed into the physiological group of red algae with a constant relatively high MAAs concentration (Hoyer et al. 2001). The occurrence of high concentration of MAAs in organisms exposed to intense solar radiation has been described to provide protection as UV-absorbing compounds (Sinha et al. 2000 and references therein); therefore, *Lemanea fluviatilis* should be expected to be a UV-tolerant organism.

MAAs were not equally induced by photosynthetic, UV-A, or UV-B radiations under the specific conditions used in our study. MAA concentrations ($AUC_{290-350}$ per DM) remained stable under P and PA regimes, and decreased under the PAB one. This could be explained by the apparent universal sensitivity of MAAs to UV-B radiation (Sinha et al. 2000, Ryan et al. 2002). Our results show that MAAs are more stable than chlorophylls and phycocyanin under photosynthetic and UV-A radiation, and therefore they could have great importance as photoprotectors against these radiations in natural conditions (Karsten et al. 2000).

The most abundant MAA found in *Lemanea fluviatilis* is porphyra-334, whose evolution showed a similar pattern to that observed for total MAAs per DM. In our study, porphyra-334 was not induced by any radiation regime, and was degraded strongly in the PAB and slightly in PA. The induction of this MAA may depend not only on the radiation wavelength (Franklin et al. 2001, Kräbs et al. 2002) but also on the species and the culture conditions. Porphyra-334 was induced remarkably only by UV-B radiation in three strains of the cyanobacterium *Nodularia* (Sinha et al. 2003), but in the red alga *Palmaria palmata* both full solar spectrum and natural solar radiation without UV-A and UV-B could greatly induce it (Karsten and Wiencke 1999). The lack of induction of porphyra-334 observed in our study could be explained by the high initial content of this compound, as it has been found in several species of *Porphyra* (Figueroa et al. 2003). This high content would be enough to develop the photoprotective role assigned to porphyra-334 in other red algae (Conde et al. 2000). In addition, degradation of porphyra-334 has been found in the marine dinoflagellate *Gyrodinium dorsum* under short wavelength UV radiation (Klisch and Häder 2002).

Although UV-B radiation did not increase the total MAAs concentration in *Lemanea fluviatilis*, it stimulated strongly the induction of mycosporine-glycine after 16 h of exposure. The fact that mycosporine-glycine has the shortest wavelength absorption maximum (310 nm) for any described MAA is in line with this specific induction by UV-B, which might contribute to protection against these wavelengths. Induction of mycosporine-glycine by UV-B radiation has been also found in the brown alga *Laminaria saccharina* (Apprill and Lesser 2003). Additionally, mycosporine-glycine may be induced under high solar UV radiation (Dunlap and Yamamoto 1995, Sommaruga and Garcia-Pichel 1999, Suh et al. 2003). After being induced by 16 h of exposure to UV-B radiation, mycosporine-glycine decreased after 64 h of exposure; this could be due to the generally weakened physiological state of the alga, as is revealed by the decrease in photosynthetic pigments and F_v/F_m. Caution should be taken when global MAAs induction by UV radiation is estimated spectrophotometrically through the measurement of $AUC_{290-350}$, since the degradation of certain MAAs may mask the real induction of other ones.

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