Influence of temperature on the effects of artificially enhanced UV-B radiation on aquatic bryophytes under laboratory conditions

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Abstract

We examined, under laboratory conditions, the influence of temperature (2 ºC *vs.* 10 ºC) on the physiological responses of two aquatic bryophytes from a mountain stream to artificially enhanced UV-B radiation for 82 d. These organisms may be exposed naturally to relatively low temperatures and high levels of UV-B radiation, and this combination is believed to increase the adverse effects of UV-B radiation. In the moss *Fontinalis antipyretica*, UV-B-treated samples showed severe physiological damages, including significant decreases in chlorophyll (Chl) and carotenoid (Car) contents, Chl *a/b* and Chl/phaeopigment ratios, Chl *a* fluorescence parameters F_v/F_m and Φ_{PS2}, electron transport rate (ETR_{max}), and growth. In the liverwort *Jungermannia cordifolia*, UV-B radiation hardly caused any physiological change except for growth reduction. Thus, this liverwort seemed to be more tolerant to UV-B radiation than the moss under the specific experimental conditions used, maybe partly due to the accumulation of UV-B absorbing compounds. The influence of temperature on the effects of UV-B radiation depended on the species: the higher the UV-B tolerance, the lower the influence of temperature. Also, different physiological variables showed varied responses to this influence. Particularly, the lower temperature used in our study enhanced the adverse effects of UV-B radiation on important physiological variables such as F_v/F_m, growth, and Chl/phaeopigment ratios in the UV-B-sensitive *F. antipyretica*, but not in the more UV-B-tolerant *J. cordifolia*. Thus, the adverse effects of cold and UV-B radiation were apparently additive in the moss, but this additiveness was lacking in the liverwort. The Principal Components Analyses (PCA) conducted for both species with the physiological data obtained after 36 and 82 d of culture confirmed the above results. Under natural conditions, the relatively high water temperatures in summer might facilitate the acclimation of aquatic bryophytes from mountain streams to high levels of UV-B radiation. This may be relevant to predict the consequences of concomitant global warming and increasing UV-B radiation.

Additional key words: carotenoids; chlorophylls; *Fontinalis*; *Jungermannia*; net photosynthetic rate; pheopigments; respiration.

Introduction

Ultraviolet-B radiation (UV-B; radiation in the wavelength range 280–315 nm) in the biosphere has increased as a consequence of the anthropogenic depletion of the stratospheric ozone layer. At northern mid-latitudes, biologically effective erythemal levels of UV-B have increased 4–7 % since 1980 (Madronich *et al.* 1998). In photosynthetic organisms, enhanced UV-B may cause alterations in DNA, photosynthesis, growth, and development, together with an increase in UV-screening compounds (Jansen *et al.* 1998, Day and Neale 2002). However, some controversy still persists about the ecological relevance of these effects (Searles *et al.* 2001). The deleterious effects of UV-B may be shielded by UV-A (Gartia *et al*. 2003).

Much of the research regarding the effects of UV-B on photosynthetic organisms has focused on terrestrial and marine environments (Franklin and Forster 1997, Häder 1997, Searles *et al.* 2001, Day and Neale 2002, Šprtová *et al*. 2003), while freshwater ecosystems, especially running waters, have received less attention (Germ and Gaberščik 2003), in line with their minor contribution to the global biomass and primary production. However, rivers and streams have outstanding ecological importance as local systems, and mountain streams and the

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organisms inhabiting them might be particularly exposed to the effects of ozone depletion, since (*1*) the biologically active UV-B increases between 5 and 20 % per 1 000 m altitudinal increase (Björn *et al.* 1998); and (*2*) UV-B can easily reach the organisms because they live at relatively low depths or even emersed, and UV-B radiation can penetrate deeply into oligotrophic waters (Häder 1997), which are typical in mountain streams.

Bryophytes prevail over other primary producers (algae, vascular macrophytes) in mountain streams and play a relevant ecological role in nutrient cycles and food webs, support periphyton, provide a refuge, and occasionally direct food for macroinvertebrates, amphibian, and fish (Bowden *et al.* 1999). Thus, bryophytes should be considered when estimating the impact of enhanced UV-B radiation on mountain streams at the ecosystem level, but only two works, to our knowledge, have been devoted to this issue (Rader and Belish 1997, Martínez-Abaigar *et al.* 2003). Bryophytes as a group might be sensitive to UV-B (Gwynn-Jones *et al.* 1999), due to a low potential of protective pigment formation and their structural simplicity: their leaves have only a single layer of cells and they lack the structural defences which higher plants use against UV-B, such as thick cuticles, multilayered epidermis, and hairs. However, the effects of UV-B radiation on bryophytes from terrestrial environments and peat lands, which have been much more studied than strictly aquatic bryophytes, are contradictory in many aspects (for details, see Martínez-Abaigar *et al.* 2003). Also, there is an important lack of knowledge about the effects of UV-B radiation on liverworts, which might possess a higher diversity of protecting compounds than mosses.

In vascular plants, algae, and lichens, the effects of UV-B may be influenced by environmental factors, such as temperature, water status, or availability of mineral nutrients. In this respect, temperature seems to be crucial, since low temperatures may exacerbate the adverse effects of UV-B on several physiological processes, such as photosynthesis, photochemical reactions of photosystem 2 (PS2), ribulose-1,5-bisphosphate carboxylase/oxygenase activity, photosynthetic pigment composition, and the repairing capacity of previously UV-B-damaged DNA (Van de Poll *et al.* 2002). In bryophytes, the effects of this combination are virtually unknown. Only Sonesson *et al*. (2002) pointed out that the growth of *Sphagnum fuscum* responded negatively to increased UV-B under increased temperature (2 °C over ambient) at the peak of the growing season, but they also suggested that higher temperatures in the field might have reduced the sensitivity of mosses to UV-B. It would be interesting to know the interaction of UV-B and temperature in aquatic bryophytes from mountain streams, since they are often exposed to both high UV-B and relatively low temperatures.

The objective of our work was to test the influence of temperature on the physiological responses of aquatic bryophytes from mountain streams to UV-B under laboratory conditions. To this aim, we cultured two bryophytes under enhanced UV-B and two different temperatures (10 and 2 °C) that are found naturally over the annual cycle (Núñez-Olivera *et al.* 2001). The two bryophytes studied were the moss *Fontinalis antipyretica* and the liverwort *Jungermannia exsertifolia* subsp. *cordifolia*, which show a different tolerance to enhanced UV-B radiation under laboratory conditions (Martínez-Abaigar *et al.* 2003). Their physiological responses were analyzed in terms of: photosynthetic pigment composition, some variables of chlorophyll (Chl) fluorescence, net photosynthesis (P_N) and dark respiration (R_D) rates, protein concentration, UV-absorbing compounds, sclerophylly, and growth. The question under consideration was whether the tolerance of the two species to enhanced UV-B radiation increases with increasing temperature.

Materials and methods

Plants: Specimens of two aquatic bryophytes, the moss *Fontinalis antipyretica* Hedw. and the liverwort *Jungermannia exsertifolia* Steph. subsp. *cordifolia* (Dumort.) Váña (hereafter *J. cordifolia*) were collected at the first-order stream Senestillos, in the upper basin of the River Iregua (La Rioja, northern Spain). The stream flows over sandstones and quartzites (Purbeck-Weald facies, Jurassic-Cretaceous) and the prevailing vegetation of the catchment is a sparse *Fagus sylvatica* L. forest. The coordinates of the sampling site, which is located at 1 350 m a.s.l., are 42º02'N, 02º37'W. The material of each species was taken from a single population. Populations of the two species were permanently submerged and 1 m apart, under similar irradiance conditions (un-shaded). Samples were collected on 15th June 2001, rinsed with stream water, stored in ice-covered polythene bottles, and transported to the laboratory in a portable icebox (temperature ever below 5 °C). The material was then rinsed again with stream water and green healthy shoots of each species were selected and pre-cultured separately in 40 000 cm^3 plastic containers filled with air-bubbled stream water. The plants were maintained at 10 ºC in a growth chamber with a 10 : 14 h photoperiod (light : dark) for 5 d. The photosynthetic photon flux density (PFD), which was provided by *True-Lite* full spectrum fluorescent tubes (*True Sun*, Steubenville, Ohio, USA), was around 98 μmol m^{-2} s^{-1} at the water surface (*LI-190SA* quantum sensor; *LI-COR*, Lincoln, NE, USA).

Experimental design: After the pre-treatment, 12 groups of 5-cm shoot apices of *F. antipyretica* and 12 mats of *J. cordifolia*, each one with 10 g of fresh mass (FM), were placed into separate plastic tubes with a basal net which prevented material losses. Six tubes of each

species were placed in a circulating bath system filled with stream water at a constant temperature of 10 ºC, and another set of 12 tubes was placed in a second similar bath at 2 ºC. The plants were cultivated in a growth chamber regulated at 10 ºC, and an immersion chiller was used to maintain 2 ºC in the appropriate bath. The bryophytes were submerged at 1–2 cm depth, after checking that photosynthetic and UV wavelengths were hardly attenuated by this shallow water column (attenuation was lower than 0.01 %). The radiation was provided by a combination of two types of lamps: photosynthetically active radiation (PAR) lamps (*True-Lite*) and UV-B lamps (*Philips TL 40W/12*, *Philips Lighting*, Eindhoven, The Netherlands). The lamp frames were placed 90 cm above the plants. Three replicates of the two following radiation regimes were set for each species and each temperature by covering the plastic tubes with specific UV cut-off foils: (*1*) Control (PAR alone), using *Ultraphan 395* (*Digefra*, Munich, Germany) which cuts off all UV radiation. (*2*) UV-B treatment, using *Ultraphan 295* (*Digefra*, Munich, Germany) which cuts off UV-C radiation.

A UV-A control was not included in the experimental design because we had already shown that there were no clear differences between the PAR and the UV-A controls (Martínez-Abaigar *et al.* 2003), as established also by Niemi *et al.* (2002a) with other bryophytes. To ensure stability in their properties during use, the filters were pre-irradiated and replaced after every 24 h of irradiation. The lamps were pre-burned for 100 h until they reached a stable output (Gehrke 1998). The plastic tubes in the bath systems were moved on a daily basis to prevent possible place-dependent differences in the irradiance received by the plants. PAR lamps gave a PFD of around 98 μmol m^{-2} s^{-1} to the bryophytes with a 10 : 14 h photoperiod (light : dark). UV-B lamps were switched on around noon for 4 h each day (square-wave), and the biologically effective UV-B irradiance ($UV\text{-}B_{BE}$) was estimated using the generalized plant damage action spectrum of Caldwell (1971) normalized to 300 nm. The $UV\text{-}B_{BE}$ applied in our experiment was 0.67 W m^{-2}, equivalent to an exposure to 9.6 kJ m^{-2} d^{-1}. This extra UV-B exposure was required to mimic a 20 % ozone depletion at the latitude of the sampling site as calculated with a computer model for clear sky conditions and aerosol level zero (Björn and Teramura 1993, Björn, personal communication). The maximum UV-B values calculated by the model were confirmed *in situ* by measuring the solar ambient UV-B. The spectral irradiances were measured, and the transmission characteristics of the filters were regularly checked with a spectroradiometer (*Macam SR9910*, *Macam Photometrics*, Livingstone, Scotland), and PFD was measured with a quantum sensor (*LI-190SA*). The bryophytes were cultivated for 82 d.

Physiological variables were measured just before the beginning of the experiment, and the analyses were repeated on days 6, 12, 36, and 82 of the culture period, taking two samples from each tube, for a total of 6 replicates for each physiological variable of each species, radiation regime, and temperature. Before the analysis, it was microscopically confirmed that the specimens had few algal epiphytes. For the analysis of the photosynthetic pigment composition, the fresh mass (FM) of several shoot apices (approximately 25–50 mg) was firstly measured after blotting them with filter paper, and three more samples were weighed to obtain dry mass (DM), exposing the samples at 80 ºC for 24 h. The extraction of pigments was performed on fresh samples with cold 80 % acetone and mortar and pestle. The pigment extract was filtered using *GF/C Whatman* filters, and a 5-cm^3 sample was read in a spectrophotometer (*Perkin-Elmer λ3B UV/Vis*, *Perkin-Elmer*, Wilton, CT, USA). Each extract was then acidified with 0.02 cm^3 1 M HCl to pH 2.5–3.0, stirred, and read again. Chl (*a*+*b*) concentration was calculated on the basis of Ziegler and Egle equations (cf. Šesták 1971) and carotenoid's concentration following Hendry and Price (1993). Both concentrations were calculated per unit DM and also per unit of shoot area (measured with a *LI-COR LI-3000* area meter). Starting from these data, the sclerophylly index, SI [g(DM) m^{-2}] was calculated. The Chl *a/b* ratio was also obtained, as well as the following indices (OD is optical density): OD_{430}/OD_{665}, OD_{430}/OD_{410}, and OD_{665}/OD_{665a} (acidified). The first index represents the ratio of the combined Chls and Cars (both types of pigments absorb radiation at 430 nm) to the Chls, the only pigments which absorb at 665 nm. This index may be indicative of the photoprotective capacity of bryophytes and increases in stress situations (Martínez-Abaigar and Núñez-Olivera 1998). The other two indices decrease when the proportion of phaeophytins increases with respect to Chls, since (*1*) the blue maximum of Chls at 430–435 nm shifts to 410–415 nm in phaeophytins, and (*2*) the red maximum of phaeophytins is lower than that of Chls, and acidification forces the conversion of Chls into phaeophytins. Both phaeopigment indices are often used as vitality indices since they decrease under the influence of adverse factors (Martínez-Abaigar and Núñez-Olivera 1998). P_N and R_D were measured at 10 ºC in a liquid phase oxygen-electrode (*DW2/2*, *Hansatech Instruments*, King's Lynn, Norfolk, UK), using a 0.1 M $NaHCO_3$ solution as carbon source. Irradiation was provided by a red LED source (660 nm *Hansatech LH36*). Samples of *ca*. 40 mg FM were kept in the dark for 20 min and then sealed in the chamber for 5 additional min, also in the dark. R_D values were calculated in the subsequent 3 min of this period. Then, the light was turned on and P_N was calculated by measuring the oxygen evolution for 3 min under 500 μmol m^{-2} s^{-1} (saturating PFD previously determined). Later, the DM of each sample was obtained (24 h, 80 ºC). *In vivo* Chl fluorescence of PS2 was measured with a portable pulse amplitude modulation fluorometer (*MINI-PAM*, *Walz*, Effeltrich, Germany). Minimal and maximal fluorescence (F_0 and F_m) were measured in specimens

dark-adapted for 20 min, using a 600 Hz modulated beam at 0.03 μmol m^{-2} s^{-1} PFD, and "white" saturating flashes of 12 000 μmol m^{-2} s^{-1} PFD and 0.8 s duration. Then a "white actinic light" was switched on (100 μmol m^{-2} s^{-1} PFD), with a 0.8-s saturating pulse every 22 s until F_t and F_m' stabilized at steady values (*ca.* 8 min). The maximum quantum yield of PS2 was given by the ratio F_v/F_m, where $F_v = F_m - F_0$ (Schreiber *et al.* 1995), and the effective quantum yield of photosynthetic energy conversion of PS2 (Φ_{PS2}) by the ratio $(F_m' - F_t)/F_m'$ (Genty *et al.* 1989). Quenching due to non-photochemical dissipation of absorbed photon energy (NPQ) was determined as $(F_m - F_m')/F_m'$ (Schreiber *et al.* 1995). Also, Φ_{PS2} and NPQ were recorded in relation to increasing PFD from 0 to 1 600 μmol m^{-2} s^{-1}. The apparent electron transport rate through PS2 (ETR) was calculated as: $ETR = \Phi_{PS2} \times PFD \times 0.5 \times 0.84$, assuming that two photosystems were involved and that 84 % of the incident quanta were absorbed by the plant (Schreiber *et al.* 1995). The curves ETR *vs.* irradiance and NPQ *vs.* irradiance were fitted to the hyperbolic tangent model equation of Jassby and Platt (Falkowski and Raven 1997) to calculate maximal ETR (ETR_{max}) and maximal NPQ (NPQ_{max}). Protein concentration was determined by the method of Bradford (1976). Methanol-extractable UV-absorbing compounds (MEUVAC) were extracted from *ca.* 50 mg FM of apices. Extraction took place for 15 h at room temperature, in the dark, in 5 cm^3 of a medium containing methanol : water : 7 M HCl, equivalent to 70 : 29 : 1 (v/v/v). The extract was centrifuged for 15 min at 6 000×*g* and an absorbance spectrum was measured between 250 and 400 nm with a spectrophotometer (*Perkin-Elmer λ3B*, UV/Vis). Since a sizeable component of UV-absorbing compounds might be located bound to the cell walls of bryophytes (Searles *et al.* 1999), extraction with NaOH (Schnitzler *et al.* 1996) was also assayed in some cases. However, no significant change was detected between the two methods, as Searles *et al.* (2002) previously found, and the first one was chosen as routine method. The amount of MEUVAC was expressed in arbitrary units as the area under the absorbance curve in the interval 280–315 nm ($AUC_{280-315}$) calculated per unit of both DM and surface area. Growth was measured at the end of the experiment. In *F. antipyretica*, the length of the segments of new growth (both in the main axis and in the branches) was measured to the nearest 0.1 cm in 50 shoots per radiation treatment and temperature. In *J. cordifolia*, ten 4-cm^2 compact turfs were prepared per radiation treatment and temperature, and the number of apices of new growth was counted under a dissection microscope. This variable was highly correlated to the total length growth ($r = 0.83$, $p<0.001$; $n = 25$). In both species, the new tissue was unequivocally identified by its light green colour.

Statistical analysis: The effects of the radiation regime, temperature, and culture period on the physiological responses of each species were tested using a 3-way analysis of variance (ANOVA) if the data met the assumptions of normality and homoscedasticity. If not, a Kruskal-Wallis test was used. Student's *t*-tests were conducted to compare the means of each physiological variable obtained at the end of the culture period for the two radiation regimes (control *vs*. UV-B) and the two temperatures (2 *vs*. 10 ºC); previously, normality and homoscedasticity of the data were confirmed. Two Principal Components Analyses (PCA), one for each species, were conducted to rank the results of the physiological variables obtained on the days 36 and 82 of the culture period for each radiation regime and each temperature, adding also the results obtained at the beginning of the experiment. For PCAs, the concentrations of Chl, Cars, and proteins, together with P_N and R_D, and MEUVAC, were introduced only on a DM basis. All the statistical procedures were performed with *SPSS 9.1 for Windows* (*SPSS*, Chicago, Illinois, USA).

Results

In *F. antipyretica*, most of the physiological variables were significantly affected by the radiation regime, temperature, and, especially, culture period (Table 1). In *J. cordifolia*, the culture period and temperature affected significantly a considerable number of variables, whereas the radiation regime hardly had any significant effect.

In the first 12 d after the imposition of the radiation regimes, both species showed physiological changes that might be related to their acclimation to the culture conditions (data not shown). Thus, only the results obtained at the end of the culture period, together with the initial values of the variables studied, will be mainly shown (Tables 2 and 3). Values at the end of the culture period were probably the results most attributable to UV-B radiation and/or temperature, and the percentage differences between treatments were summarized in Figs. 1 and 2. However, the results obtained on the 36th d began to show the tendency of those gained on the 82nd d, and thus both results were used for the PCAs (see below).

In *F. antipyretica*, after 82 d of culture, the samples under the UV-B treatment showed, against the control, generalized significant decreases in most pigment variables both at 2 and at 10 ºC, as it occurred in three variables related to Chl fluorescence: F_v/F_m, Φ_{PS2}, and ETR_{max} (Fig. 1). The rest of the variables did not show significant changes at any temperature, except for the increase in protein concentration per area at 10 ºC and the strong (49.1 %) decrease in growth at 2 ºC in UV-B-irradiated samples.

In *J. cordifolia*, after 82 d of culture, the values of most variables in the UV-B-irradiated specimens did not show significant changes against the control ones at any

Table 1. Overall effects of the radiation regime, temperature, and culture period on the physiological variables of the two bryophyte species, tested by either a 3-way ANOVA or a Kruskal-Wallis test. Growth was tested by a 2-way ANOVA since it was measured only at the end of the culture period. $^{***}p<0.001$, $^{**}p<0.01$, $^{*}p<0.05$, NS non-significant. For abbreviations see the text.

	Fontinalis			*Jungermannia*		
	Radiation	Temp.	Period	Radiation	Temp.	Period
Chl per DM	***	**	***	NS	*	***
Chl per shoot area	**	***	***	NS	NS	***
Chl *a/b*	**	NS	***	NS	NS	***
Cars per DM	***	NS	***	*	NS	***
Cars per shoot area	**	*	***	NS	***	*
OD_{665}/OD_{665a}	***	NS	***	NS	NS	***
OD_{430}/OD_{410}	***	NS	***	NS	***	***
OD_{430}/OD_{665}	NS	NS	***	NS	NS	***
F_v/F_m	***	***	***	NS	*	NS
Φ_{PS2}	***	*	*	NS	NS	NS
ETR_{max}	***	NS	NS	NS	***	NS
NPQ_{max}	NS	***	***	NS	***	***
P_N per DM	NS	***	***	NS	***	***
P_N per shoot area	NS	***	***	NS	***	*
P_N per Chl	NS	***	*	NS	***	***
R_D per DM	*	***	NS	NS	NS	NS
R_D per shoot area	*	**	**	NS	NS	**
Protein per DM	NS	***	***	NS	NS	***
Protein per shoot area	NS	NS	***	NS	NS	***
MEUVAC ($AUC_{280-315}$) per DM	NS	NS	***	NS	NS	***
MEUVAC ($AUC_{280-315}$) per shoot area	NS	NS	***	NS	NS	***
Sclerophylly index (SI)	NS	***	***	NS	NS	***
Growth	**	***	-	NS	***	-

Table 2. Initial values (mean ± SE) of the physiological variables shown by *Fontinalis antipyretica*, and values recorded at the end of the culture period (82 d) in the control and the UV-B-irradiated samples at 10 and 2 ºC.

Variable	Initial value	10 ºC control	10 ºC UV-B	2 ºC control	2ºC UV-B
Chlorophyll [g kg^{-1}(DM)]	6.38 ± 0.20	5.15 ± 0.23	4.78 ± 0.13	5.14 ± 0.14	4.54 ± 0.14
Chlorophyll [mg m^{-2}]	260.7 ± 7.6	120.8 ± 5.4	114.0 ± 3.1	202.7 ± 5.5	178.4 ± 5.4
Chlorophyll *a/b*	1.83 ± 0.04	1.65 ± 0.06	1.47 ± 0.05	1.64 ± 0.04	1.44 ± 0.06
Carotenoids [g kg^{-1}(DM)]	0.98 ± 0.04	1.03 ± 0.02	0.68 ± 0.02	1.00 ± 0.05	0.80 ± 0.02
Carotenoids [mg m^{-2}]	36.9 ± 1.5	24.3 ± 0.5	16.4 ± 0.5	39.9 ± 2.1	31.8 ± 1.0
OD_{665}/OD_{665a}	1.55 ± 0.00	1.51 ± 0.01	1.36 ± 0.01	1.50 ± 0.01	1.32 ± 0.00
OD_{430}/OD_{410}	1.26 ± 0.00	1.13 ± 0.01	1.00 ± 0.00	1.13 ± 0.02	0.98 ± 0.01
OD_{430}/OD_{665}	1.79 ± 0.02	1.77 ± 0.04	1.63 ± 0.04	1.87 ± 0.05	1.77 ± 0.07
F_v/F_m	0.694 ± 0.006	0.694 ± 0.005	0.663 ± 0.011	0.716 ± 0.001	0.605 ± 0.017
Φ_{PS2}	0.269 ± 0.002	0.323 ± 0.028	0.239 ± 0.006	0.324 ± 0.010	0.233 ± 0.009
ETR_{max}	34.5 ± 3.3	38.9 ± 2.5	27.5 ± 1.7	42.5 ± 1.9	28.2 ± 3.9
NPQ_{max}	3.36 ± 0.21	2.53 ± 0.09	2.71 ± 0.15	3.50 ± 0.23	3.26 ± 0.25
P_N [µmol(O_2) kg^{-1}(DM) s^{-1}]	25.2 ± 1.0	24.8 ± 2.9	19.6 ± 3.0	15.9 ± 1.7	11.4 ± 1.9
P_N [µmol(O_2) m^{-2} s^{-1}]	0.92 ± 0.04	0.58 ± 0.06	0.46 ± 0.06	0.64 ± 0.07	0.45 ± 0.07
P_N [mmol(O_2) kg^{-1}(Chl) s^{-1}]	3.9 ± 0.1	4.8 ± 0.6	4.1 ± 0.6	3.1 ± 0.3	2.5 ± 0.4
R_D [µmol(O_2) kg^{-1}(DM) s^{-1}]	5.4 ± 0.4	15.6 ± 1.7	13.4 ± 3.6	5.0 ± 1.0	5.4 ± 0.3
R_D [µmol(O_2) m^{-2} s^{-1}]	0.20 ± 0.01	0.36 ± 0.03	0.31 ± 0.09	0.20 ± 0.04	0.21 ± 0.02
Protein [g kg^{-1}(DM)]	63.0 ± 1.1	29.5 ± 1.2	33.9 ± 1.6	33.5 ± 3.1	36.3 ± 1.3
Protein [g m^{-2}]	2.592 ± 0.084	0.695 ± 0.028	0.815 ± 0.039	1.330 ± 0.122	1.440 ± 5.1
MEUVAC [$AUC_{280-315}$ mg^{-1}(DM)]	1.53 ± 0.05	0.62 ± 0.03	0.56 ± 0.02	0.64 ± 0.05	0.70 ± 0.04
MEUVAC [$AUC_{280-315}$ cm^{-2}]	6.29 ± 0.44	1.52 ± 0.13	1.41 ± 0.11	2.72 ± 0.32	2.88 ± 0.27
Sclerophylly index [g m^{-2}]	41.1 ± 1.3	23.6 ± 1.1	24.0 ± 1.3	39.7 ± 1.7	39.6 ± 2.6
Growth [cm per shoot]	0	4.64 ± 0.40	3.90 ± 0.34	1.90 ± 0.24	0.97 ± 0.19

Table 3. Initial values (mean ± SE) of the physiological variables shown by *Jungermannia cordifolia*, and values recorded at the end of the culture period (82 d) in the control and the UV-B-irradiated samples at 10 and 2 ºC.

Variable	Initial value	10 ºC control	10 ºC UV-B	2 ºC control	2 ºC UV-B
Chlorophyll [g kg^{-1}(DM)]	7.95 ± 0.09	7.40 ± 0.11	7.16 ± 0.17	6.65 ± 0.21	6.29 ± 0.11
Chlorophyll [mg m^{-2}]	234.7 ± 2.3	176.5 ± 2.8	168.8 ± 3.9	177.0 ± 5.5	167.6 ± 2.9
Chlorophyll *a/b*	2.11 ± 0.01	2.17 ± 0.01	2.15 ± 0.01	2.16 ± 0.02	2.14 ± 0.02
Carotenoids [g kg^{-1}(DM)]	1.24 ± 0.03	1.28 ± 0.03	1.17 ± 0.04	1.32 ± 0.03	1.26 ± 0.03
Carotenoids [mg m^{-2}]	37.0 ± 0.9	28.3 ± 0.4	27.7 ± 1.0	35.3 ± 0.8	33.8 ± 0.8
OD_{665}/OD_{665a}	1.59 ± 0.00	1.60 ± 0.00	1.60 ± 0.00	1.60 ± 0.00	1.61 ± 0.00
OD_{430}/OD_{410}	1.34 ± 0.00	1.33 ± 0.00	1.33 ± 0.00	1.35 ± 0.00	1.33 ± 0.00
OD_{430}/OD_{665}	1.91 ± 0.01	1.81 ± 0.01	1.82 ± 0.02	1.90 ± 0.01	1.93 ± 0.01
F_v/F_m	0.699 ± 0.003	0.689 ± 0.010	0.682 ± 0.008	0.691 ± 0.009	0.688 ± 0.005
Φ_{PS2}	0.271 ± 0.003	0.192 ± 0.005	0.228 ± 0.005	0.226 ± 0.010	0.259 ± 0.015
ETR_{max}	35.7 ± 3.3	23.5 ± 2.1	24.3 ± 2.0	47.7 ± 1.6	49.8 ± 4.4
NPQ_{max}	2.66 ± 0.11	1.67 ± 0.15	1.64 ± 0.12	3.18 ± 0.08	2.94 ± 0.23
P_N [µmol(O_2) kg^{-1}(DM) s^{-1}]	5.2 ± 0.7	8.4 ± 1.9	7.6 ± 2.3	7.4 ± 1.8	6.5 ± 1.3
P_N [µmol(O_2) m^{-2} s^{-1}]	0.19 ± 0.02	0.23 ± 0.05	0.21 ± 0.06	0.22 ± 0.05	0.18 ± 0.03
P_N [mmol(O_2) kg^{-1}(Chl) s^{-1}]	0.66 ± 0.09	1.14 ± 0.27	1.06 ± 0.31	1.10 ± 0.27	1.03 ± 0.20
R_D [µmol(O_2) kg^{-1}(DM) s^{-1}]	5.8 ± 0.2	5.5 ± 1.7	5.2 ± 1.3	7.6 ± 1.5	5.9 ± 1.4
R_D [µmol(O_2) m^{-2} s^{-1}]	0.14 ± 0.01	0.15 ± 0.04	0.15 ± 0.04	0.22 ± 0.04	0.16 ± 0.04
Protein [g kg^{-1}(DM)]	44.9 ± 1.7	22.2 ± 1.2	22.1 ± 0.9	26.1 ± 1.8	26.7 ± 1.2
Protein [g m^{-2}]	1.331 ± 0.043	0.534 ± 0.028	0.516 ± 0.022	0.699 ± 0.047	0.717 ± 0.033
MEUVAC [$AUC_{280\text{-}315}$ mg^{-1}(DM)]	7.6 ± 0.2	11.7 ± 0.4	13.6 ± 0.6	11.9 ± 0.2	16.9 ± 0.2
MEUVAC [$AUC_{280\text{-}315}$ cm^{-2} area]	21.8 ± 0.8	28.9 ± 0.7	31.9 ± 0.9	29.4 ± 0.5	39.0 ± 0.6
Sclerophylly index [g m^{-2}]	29.7 ± 0.9	24.7 ± 1.1	23.7 ± 0.7	26.3 ± 0.6	26.3 ± 1.2
Growth [new apices cm^{-2}(turf area)]	0	13.5 ± 0.8	9.4 ± 0.4	4.3 ± 0.6	3.1 ± 0.3

temperature (Fig. 1). Only a few responses were significant, among which the increases in MEUVAC per DM and shoot area at 2 ºC were remarkable. Growth decreased strongly and significantly (30.7 %) in UV-B-irradiated samples at 10 ºC.

The samples of *F. antipyretica* cultured at 2 ºC showed significantly higher SI and higher concentrations of Chl, Cars, proteins, and MEUVAC per shoot area compared to those cultured at 10 ºC, but significantly lower values of P_N per DM and Chl unit, R_D per DM and shoot area, and growth, generally both in UV-B-irradiated samples and control ones (Fig. 2). OD_{665}/OD_{665a}, OD_{430}/OD_{410}, and F_v/F_m were slightly (1.6–8.7 %) although significantly lower in the 2 ºC samples than in the 10 ºC ones, but only under UV-B radiation.

In *J. cordifolia*, the 2 ºC samples showed, as compared to the 10 ºC ones, significantly lower growth and concentration of Chl per DM, and significantly higher values of Cars and proteins per shoot area, OD_{430}/OD_{665}, ETR_{max}, and NPQ_{max} (Fig. 2). These responses occurred both in UV-B-exposed and control plants. The 2 ºC samples showed also higher values of OD_{430}/OD_{410} and Φ_{PS2}, but only in the control samples. SI, MEUVAC per DM and per shoot area, proteins per DM, and OD_{665}/OD_{665a} showed significantly higher values in 2 ºC samples than in the 10 ºC ones, but only in UV-B-exposed plants.

The first two components of the PCA for *F. antipyretica* accounted for 75 % (47 and 28 %, respectively), and the first three components for 87 %, of the total variance. In the plot generated with the scores of the first two components (Fig. 3, *top*), the initial samples were located at the extreme right part of the diagram, while the 36- and 82-d-samples were intermixed and clearly separated from the initial ones. Control and UV-B-treated specimens were clearly separated along the first axis, with the former closer to the initial state than the latter. For the first axis, the positive loading factors were compact group of physiological variables, while there was no significant negative loading factor. Samples cultured at 2 and 10 ºC were separated along the second axis, with the former placed towards the positive part and the latter towards the negative one. The most significant positive loading factors for this axis were SI and NPQ_{max}, and the negative ones were growth and R_D.

The first two components of the PCA for *J. cordifolia* accounted for 59 % (39 and 20 %, respectively), and the first three components for 75 %, of the total variance. In the plot generated with the scores of the first two components (Fig. 3, *bottom*), the initial state was placed again in the extreme right part of the diagram, whereas the states after 36 and 82 d of culture were displaced to the left due to the progressively stronger physiological changes of the samples. Growth and P_N were the most significant negative loading factors for the first component, and SI, Φ_{PS2}, NPQ_{max}, and OD_{430}/OD_{665} were the most significant positive ones. There was a clear separation

along the second axis between the samples cultured at 10 ºC, which were placed towards the bottom of the diagram, and those cultured at 2 ºC. This could be due to a combination of high values of growth and P_N (negative loading factors for the first axis) in the 10 ºC specimens, since the loading factors for the second axis were less significant. Control and UV-B-treated samples were not separated at all; for each day of culture, both types of samples were close together in the diagram, reflecting a similar physiological state.

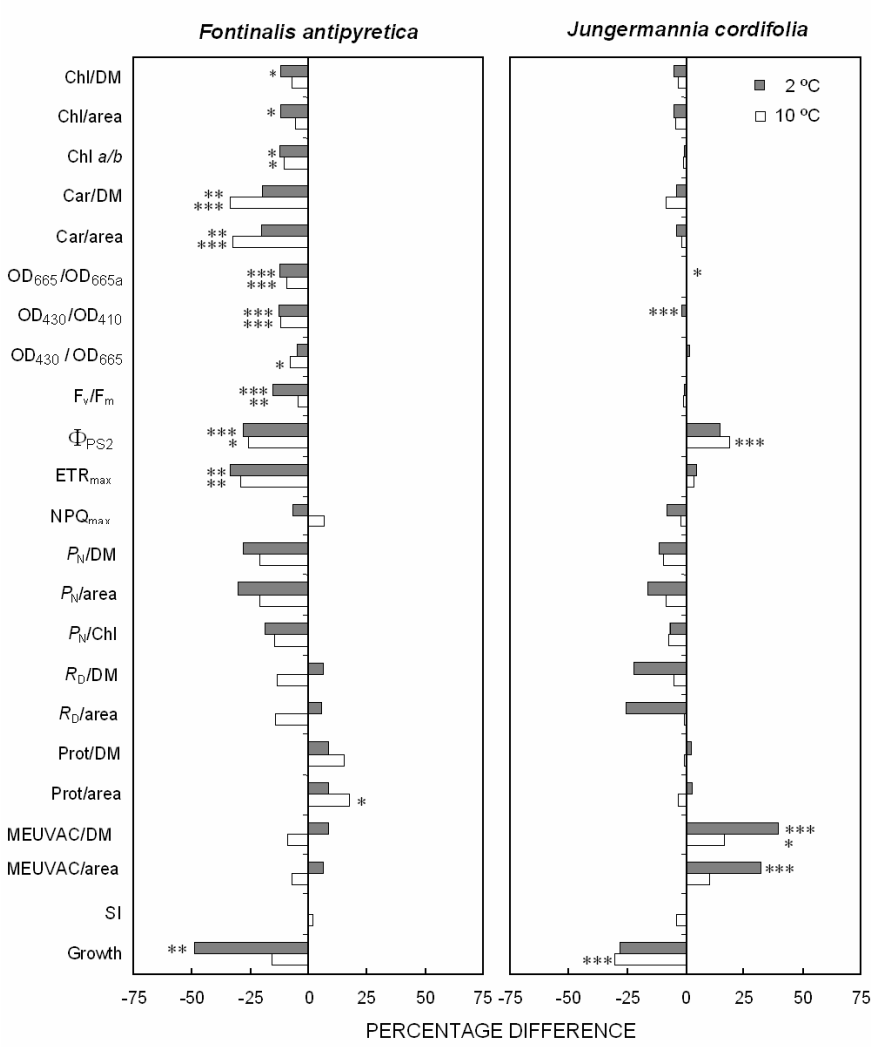

Fig. 1. Percentage differences in different variables between UV-B-treated and control samples at the end of the culture period (82 d) at 10 and 2 °C in *Fontinalis antipyretica* and *Jungermannia cordifolia*. Negative differences indicate that the value of the respective variable in the UV-B-exposed samples was lower than that in the control ones. The significant differences between UV-B-exposed and control samples for each variable at each temperature are shown (***p<0.001, **p<0.01, *p<0.05).

Discussion

The two bryophyte species used in this study responded differently to the experimental conditions of culture period, temperature, and radiation regime. The culture period affected significantly most physiological variables in both species; this was due to both the drastic changes recorded in the first days of culture, probably due to acclimation to the culture conditions, and to subsequent changes which seem to be more attributable to the influence of temperature and radiation regime. This succession of changes was similar to that reported by Martínez-Abaigar *et al.* (2003) when cultivating *F. antipyretica* and *J. cordifolia* for 36 d under enhanced UV-B. Temperature also affected significantly a large number of variables in both species, in line with its key role in plant metabolism. However, the radiation regime influenced differently the physiological state of both species. This is especially clear after analyzing the results obtained at the end of the culture period. UV-B-irradiated samples of *F. antipyreti-*

ca showed, compared to control ones, a clear and global physiological damage, including significant decreases in a number of important variables at both culture temperatures (Fig. 1). In contrast, UV-B-irradiated samples of *J. cordifolia* hardly evidenced significant physiological changes, either at 10 or 2 ºC. The different responses shown by the two species are in line with previous results (Martínez-Abaigar *et al.* 2003), and the PCAs performed for both species with the physiological data obtained after 36 and 82 d of culture (Fig. 3) summarized the above mentioned responses.

The weaker influence of UV-B on *J. cordifolia* compared to *F. antipyretica* might be partly explained by notable and mostly significant MEUVAC increases in the UV-B-treated samples of the liverwort, whereas in the moss the MEUVAC levels were approximately only 5 % of those detected in the liverwort and decreased compared to its initial amount. Thus, the possible protection provided by MEUVAC against enhanced UV-B radiation seems to be negligible in the moss. The increase in UV-absorbing compounds in response to natural or enhanced UV-B radiation has been rarely found in bryophytes (Ihle and Laasch 1996, Newsham *et al.* 2002, Martínez-Abaigar *et al.* 2003), in contrast with its commonness in other photosynthetic organisms (Searles *et al.* 2001, Day and Neale 2002). A possible explanation of this rarity might be a poor methanol extraction of those UV-absorbing compounds located in the cell walls of bryophytes

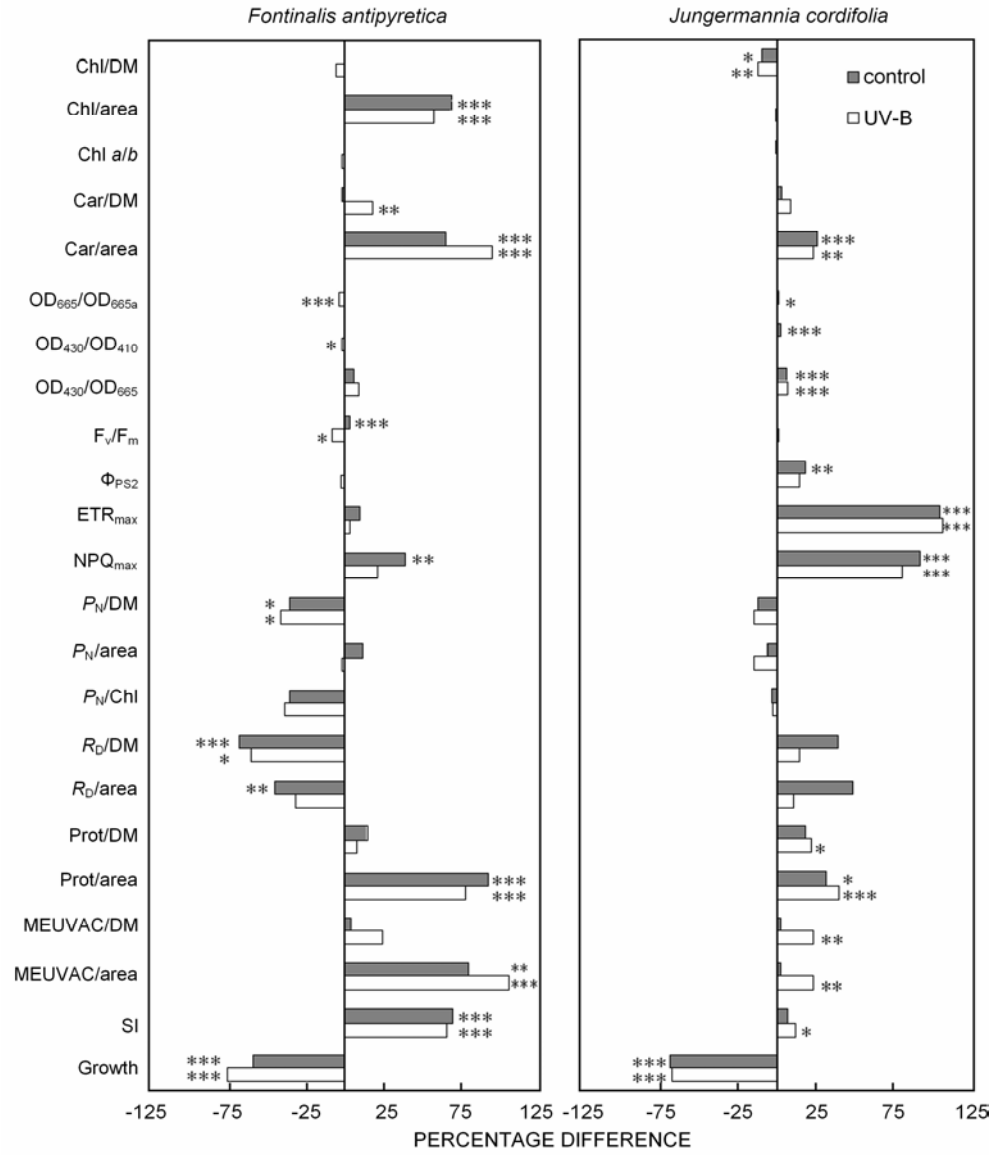

Fig. 2. Percentage differences in different variables between the values recorded at 2 or 10 °C at the end of the culture period (82 d) in control and UV-B-irradiated samples, in *Fontinalis antipyretica* and *Jungermannia cordifolia*. Negative differences indicate that the value of the respective variable in the 2 °C samples was lower than that in the 10 °C ones. The significant differences between 2 and 10 °C samples for each variable in both control and UV-B-exposed plants are shown (***p<0.001, **p<0.01, *p<0.05).

(Niemi *et al.* 2002a, Martínez-Abaigar *et al.* 2003), but further research is needed to confirm this hypothesis.

J. cordifolia seems to be more tolerant to UV-B-radiation than *F. antipyretica* under our experimental conditions, but it should be specified that both species are UV-B-sensitive since their growth decreased under enhanced UV-B radiation. It is somewhat surprising that growth decline was not accompanied by significant reductions in P_N rates in either *J. cordifolia* or *F. antipyretica*. In the moss, this last effect was detected in a previous experiment (Martínez-Abaigar *et al.* 2003), and its absence in this work might be probably due to the great variability of P_N measurements, since other photosynthetic variables that showed a more modest variability (F_v/F_m, Φ_{PS2}, ETR_{max}) were significantly reduced under the UV-B treatment. In the liverwort, not even these variables were affected by UV-B. Thus, its photosynthetic machinery may be somewhat protected by the increase in MEUVAC. In addition, declines in biomass production in the absence of reductions in P_N have been formerly

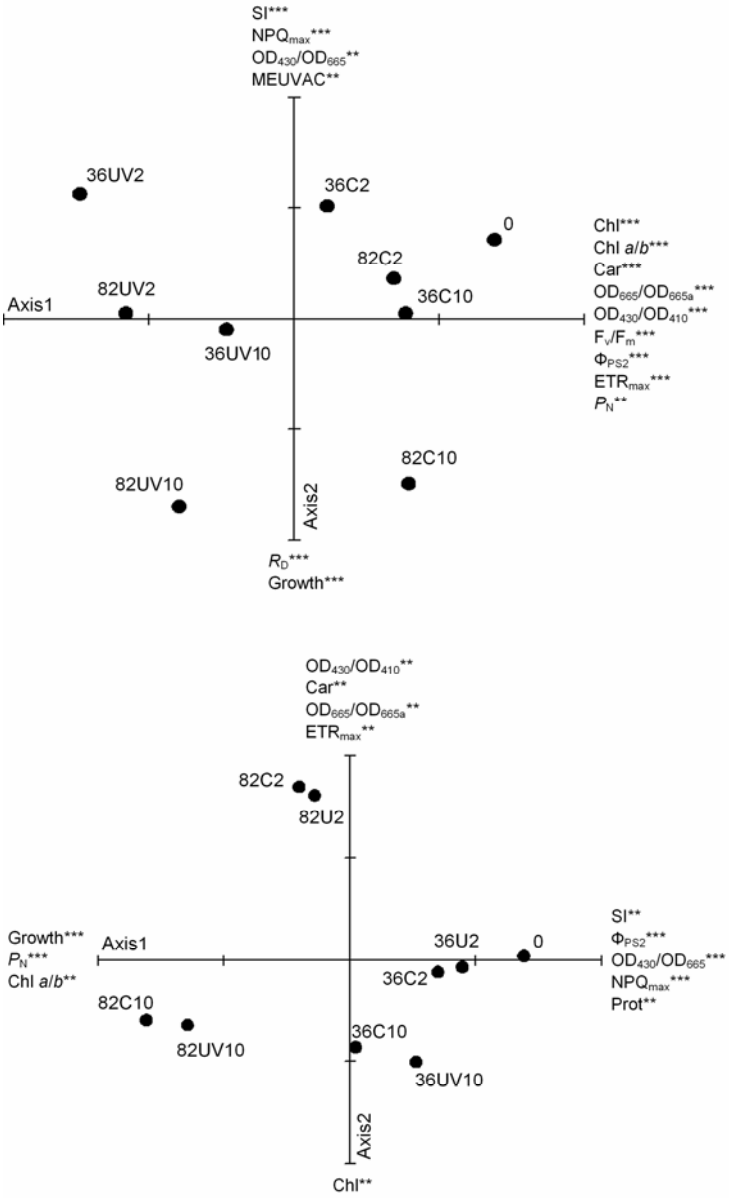

Fig. 3. Principal Components Analyses (PCA), one for each species (*top* = *Fontinalis antipyretica*; *bottom* = *Jungermannia cordifolia*), performed using the physiological variables obtained on the days 36 and 82 of the culture period for each radiation regime (C = control; UV = UV-B) and temperature (2 = 2 °C; 10 = 10 °C). The initial state of the samples of both species (0) is also added. Significant loading factors for the positive and negative parts of each axis are shown when $p<0.001$ (***) or $p<0.01$ (**). The concentrations of chlorophyll (Chl), carotenoids (Car), proteins (Prot), and methanol-extractable UV-absorbing compounds (MEUVAC), together with the rates of net photosynthesis (P_N) and dark respiration (R_D), were introduced in the PCAs only on a dry mass basis. SI = sclerophylly index [g(DM) m^{-2}]. OD = optical density. *Each tic-mark on axes 1 and 2* represents 1 unit.

reported in terrestrial vascular plants exposed to solar UV-B (Day and Neale 2002). Also, in a 5-month laboratory experiment, enhanced UV-B reduced growth in the terrestrial moss *Hylocomium splendens*, but no effect on photosynthesis was detected (Sonesson *et al.* 1996). Growth reductions under UV-B radiation without concomitant photosynthetic alterations could be explained by UV-B-induced damage on DNA and cell division, since DNA damage is closely related to reduction in growth (Mazza *et al.* 1999, Rousseaux *et al.* 1999). This explanation may be compatible with our results, because increased DNA damage can occur under enhanced UV-B without affecting F_v/F_m, photosynthetic gas exchange, or Chl concentration, as it has been demonstrated in the terrestrial moss *Sanionia uncinata* outdoors (Lud *et al.* 2002).

Within a bryological background, almost every physiological response described here has been found separately in bryophytes exposed to enhanced UV-B, either in the field or in the laboratory (for detailed references, see Martínez-Abaigar *et al.* 2003). However, consistent physiological responses affecting several different variables have been rarely found, and artificially enhanced UV-B radiation added to bryophytes can stimulate, depress, or have no effect on their performance (Sonesson *et al.* 2002). These discrepancies, probably due to the different species and experimental conditions used, still hinder the comprehension of the true effects of UV-B radiation on bryophytes. However, it is already clear that the sensitivity of bryophytes to artificially enhanced UV-B depends primarily on the species considered (Gehrke 1999, Csintalan *et al.* 2001, Newsham *et al.* 2002, Niemi *et al.* 2002a,b, Sonesson *et al.* 2002, Martínez-Abaigar *et al.* 2003), and thus bryophytes should not be taken as a homogeneous group regarding their sensitivity to UV-B.

The non-UV-B-irradiated samples of *F. antipyretica* and *J. cordifolia* cultured at 2 ºC showed drastic and comparable growth reductions compared to those cultured at 10 ºC. This was to be expected since growth in aquatic bryophytes varies unimodally depending on temperature with a maximum around 15 ºC (Glime 1987). The changes found for other variables in the moss (SI, contents of Chl, Cars, proteins, and MEUVAC per shoot area, and P_N and R_D per DM), were influenced by the lower sclerophylly of the apices of the 10 ºC samples, which had grown during the culture period, compared to the apices of the 2 ºC samples, which consisted almost completely of "old" tissues. The rest of the changes, both in the moss and the liverwort, were divergent in their physiological meanings (Martínez-Abaigar and Núñez-Olivera 1998, Maxwell and Johnson 2000): the 2 ºC samples of the moss showed low P_N rates per Chl unit but high values of F_v/F_m, and the 2 ºC samples of the liverwort showed low contents of Chl per DM and high values of OD_{430}/OD_{665} (indicative of stress) but high values of OD_{430}/OD_{410}, Φ_{PS2}, and ETR_{max} (indicative of vitality). Hence, the lower temperature used in this study hardly affected itself the integrity of the metabolic machinery (particularly, the photosynthetic apparatus) of the bryophyte species studied.

The interaction of temperature and UV-B in the two bryophytes depended on the species considered, as it is found in other photosynthetic organisms (marine algae: Altamirano *et al.* 2003, Van de Poll *et al.* 2003). Also, it is not surprising that temperature only influences the effects of UV-B radiation on specific variables, since, for instance, F_v/F_m is more sensitive to enhanced UV-B than growth or DNA damage in marine algae (Van de Poll *et al.* 2002). In *F. antipyretica*, the samples cultured at 2 ºC showed lower values of OD_{665}/OD_{665a}, OD_{430}/OD_{410}, and F_v/F_m than those cultured at 10 ºC only when they had been UV-B-irradiated (Fig. 2). These decreases are indicative of stress (Martínez-Abaigar and Núñez-Olivera 1998, Maxwell and Johnson 2000). The changes were certainly slight compared to the control samples (1.6–8.7 % decrease), in accordance with the own nature of these variables, all of which are indices. The three variables mentioned are sclerophylly-independent and thus may represent more reliable effects of UV-B radiation than other variables sclerophylly-dependent. In addition, the decrease in growth (another sclerophylly-independent variable) in the 2 ºC samples with respect to the 10 ºC ones was more pronounced under UV-B radiation conditions (75.1 %) than under PAR alone (58.9 %). Thus, in the moss, the adverse effects of cold and UV-B radiation are apparently additive. The striking MEUVAC per area increase in the samples of *F. antipyretica* cultured at 2 ºC and UV-B-treated did not seem to represent any protection, since absolute MEUVAC levels were very low in comparison with those detected in *J. cordifolia*. In this species (Fig. 2), the differences between the samples cultured at 2 ºC and those cultured at 10 ºC were very similar when exposed either to PAR or to UV-B radiation; in addition, the decrease in growth in the 2 ºC samples with respect to the 10 ºC ones was similar under PAR alone (68.4 %) and under UV-B radiation (67.2 %). Hence, the effects of cold and UV-B radiation may not be additive, and the interaction of both factors is less evident in *J. cordifolia* than in *F. antipyretica*. This lack of interaction in the liverwort could be due to its higher tolerance to enhanced UV-B radiation (Martínez-Abaigar *et al.* 2003), which in turn might be based on the protection given by the accumulation of MEUVAC that occurred in the 2 ºC samples when they were exposed to UV-B.

The different interaction of temperature and UV-B in *F. antipyretica* and *J. cordifolia* was also clear in the two diagrams generated by PCA (Fig. 3). In the moss, at the end of the culture period, the distance between the controls cultured at 10 ºC and the controls cultured at 2 ºC (which may be indicative of the effect of cold) is similar to the distance between the former samples and the UV-B-treated samples cultured at 10 ºC (which may show the effect of UV-B radiation). However, the controls cultured at 10 ºC are considerably further from the UV-B-treated

samples cultured at 2 ºC. In the liverwort, at the end of the culture period, the controls cultured at 10 ºC are placed near the UV-B-treated samples cultured at the same temperature, which may reflect the negligible effect of UV-B radiation. Both types of samples are equally distant from both the controls cultured at 2 ºC and the UV-B-treated samples cultured at 2 ºC. Hence, the effect of the lower temperature in the liverwort was stronger than the effect of UV-B radiation.

The combined effects of temperature and UV-B radiation depend not only on the species and physiological variable considered, but also on the temperatures used (both absolute values and range). This hampers the comparison between different studies, since a temperature range of 8 ºC, like ours, may lead to either significant (Altamirano *et al.* 2003) or negligible effects (Van de Poll *et al.* 2002, 2003). Higher temperatures generally attenuate the adverse effects of UV-B radiation on DNA integrity, biomass production, or other physiological variables in vascular plants (Mark and Tevini 1997), algae (Gómez *et al.* 2001), and lichens (Buffoni Hall *et al.* 2003), as we have found for the UV-B-sensitive bryophyte *F. antipyretica*. In contrast, the combination of a relatively high temperature (16 ºC) and enhanced UV-B radiation may cause the death of germlings in *Fucus* (Altamirano *et al.* 2003).

The effects of enhanced UV-B assessed under laboratory conditions cannot be directly extrapolated to the field. However, it could be speculated that the physiological responses of aquatic bryophytes from mountain streams to enhanced UV-B radiation, both indoors and outdoors, depend on internal factors (the species) and environmental factors, such as temperature. With respect to the species, *F. antipyretica* was more UV-B sensitive than *J. cordifolia* under our experimental conditions, but the moss was able to survive at similar high altitudes and UV-B levels as the liverwort under natural conditions. Thus, the protection mechanisms of the moss against UV-B might be inhibited in our study (perhaps because of the relatively low PFD used) and it may produce UV-absorbing compounds and/or develop other alternative protection mechanisms in the field. Regarding the influence of temperature, the possible increase in UV-B sensitivity at the low temperatures used in this study (2 ºC) would be minimized since this water temperature is typical of the winter (Núñez-Olivera *et al.* 2001), and in this season the predictable UV-B levels are relatively low at northern mid-latitudes. In contrast, in summer, the relatively high water temperatures (summer mean temperature of 13.2 ºC in the Senestillos stream: Núñez-Olivera *et al.* 2001) might facilitate, to some extent, the acclimation of aquatic bryophytes to high levels of UV-B. This might be relevant to predict the consequences of concomitant global warming and increasing UV-B radiation.

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