

# Sensitivity of Laminariales zoospores from Helgoland (North Sea) to ultraviolet and photosynthetically active radiation: implications for depth distribution and seasonal reproduction

M. Y. ROLEDA<sup>1</sup>, C. WIENCKE<sup>2</sup>, D. HANELT<sup>3</sup>, W. H. VAN DE POLL<sup>4</sup> & A. GRUBER<sup>1</sup>

<sup>1</sup>Alfred Wegener Institute for Polar and Marine Research, Biologische Anstalt Helgoland, Postfach 180, D-27483 Helgoland, Germany, <sup>2</sup>Foundation Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany, <sup>3</sup>Biozentrum Klein Flottbek, University of Hamburg, Ohnhorst-Str. 18, D-22609 Hamburg, Germany and <sup>4</sup>Department of Marine Biology, University of Groningen, PO Box 14, NL-9750 AA Haren, The Netherlands

## ABSTRACT

Depth distribution of kelp species in Helgoland (North Sea) is characterized by occurrence of *Laminaria digitata* in the upper sublittoral, whereas *L. saccharina* and *L. hyperborea* dominate the mid and lower sublittoral region. *Laminaria digitata* is fertile in summer whereas both other species are fertile in autumn/winter. To determine the light sensitivity of the propagules, zoospores of *L. digitata*, *L. saccharina* and *L. hyperborea* were exposed in the laboratory to different exposure times of photosynthetically active radiation (PAR; 400–700 nm), PAR + UVA radiation (UVAR; 320–400 nm) and PAR + UVAR + UVB radiation (UVBR; 280–320 nm). Optimum quantum yield of PSII and DNA damage were measured after exposure. Subsequently, recovery of photosynthetic efficiency and DNA damage repair, as well as germination rate were measured after 2 and 3 d cultivation in dim white light. Photosynthetic efficiency of all species was photoinhibited already at 20  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$  PAR, whereas UV radiation (UVR) had a significant additional effect on photo-inhibition. Recovery of the PSII function was observed in all species but not in spores exposed to irradiation longer than 4 h of PAR + UVA + UVB and 8 h of PAR + UVA. The amount of UVB-induced DNA damage measured as cyclobutane–pyrimidine dimers (CPDs) increased with exposure time and highest damage was detected in the spores of lower subtidal *L. hyperborea* relative to the other two species. Significant removal of CPDs indicating repair of DNA damage was observed in all species after 2 d in low white light especially in the spores of upper subtidal *L. digitata*. Therefore, efficient DNA damage repair and recovery of PSII damage contributed to the germination success but not in spores exposed to 16 h of UVBR. UV absorption of zoospore suspension in *L. digitata* is based both on the absorption by the zoospores itself as well as by

exudates in the medium. In contrast, the absorption of the zoospore suspension in *L. saccharina* and *L. hyperborea* is based predominantly on the absorption by the exudates in the medium. This study indicates that UVR sensitivity of zoospores is related to the seasonal zoospore production as well as the vertical distribution pattern of the large sporophytes.

*Key-words:* *Laminaria digitata*; *Laminaria saccharina*; *Laminaria hyperborea*; cyclobutane-pyrimidine dimers; DNA damage and repair;  $F_v/F_m$ ; germination rate; photosynthesis.

## INTRODUCTION

Zoospore production and its subsequent release and recruitment on suitable substrate are important processes in maintaining kelp population in coastal marine environments (Reed, Schroeter & Raimondi 2004). Upon the release of zoospores, these propagules are confined in a viscous physical environment due of their small size (approximately 3.0–5.0  $\mu\text{m}$ ) and relatively slow swimming speeds (Amsler, Reed & Neushul 1992). Swimming competency of kelp zoospores has been recorded up to 72 h (Reed, Amsler & Ebeling 1992) and kelp zoospores have been identified from *in situ* plankton samples (Graham 1999). Spore swimming increases the likelihood of settlement. However, even after cessation of swimming, spores were found to germinate in the water column and retained their capacity to produce viable sporophyte recruits (Reed *et al.* 1992). This transitory planktonic phase, which is capable of photosynthesis, can, however, be exposed to variable environmental stress conditions with respect to light, ultraviolet radiation (UVR) and temperature. Spore dispersal as source of recruits has been extensively studied in Laminariales (e.g. Reed *et al.* 1992, 2004; Fredriksen *et al.* 1995), where resulting colonization has been documented over distances of at least 4000 m (Reed, Laur & Ebeling 1988). Moreover, local hydrodynamic condition can enhance dis-

persal; probability of gamete encounters and ensures high fertilization rates (Yund 2000) developing distinct natural population within the locality.

Surface UV wavelength to PAR ratio in Helgoland varies depending on cloud cover and season. The ratios of 320, 340 and 380 nm to PAR were 15–20% higher on the dull days than on bright days, whereas the 305 nm : PAR ratio did not vary between different cloud cover. On the other hand, seasonal variation is characterized as follows: no seasonal increase in 305 nm : PAR ratio; 50% increase in 320 nm : PAR ratio from winter to summer; constant 340 nm : PAR ratio throughout the year; and slight decrease in 380 nm : PAR ratio during summer months (Dring *et al.* 2001a). Underwater measurements estimated 1% depth for 305-nm radiation at 1 m measurable only during summer and autumn. The 1% depths recorded for the other UV wavelengths were 2.0, 2.6 and 4.6 m for 320, 340 and 380 nm, respectively, compared to 12 m for PAR (Dring *et al.* 2001a). Relative to tidal fluctuation, higher PAR transmittance is also observed during neap tides (low tide at midday) than on spring tides (high tide on midday). This association is suggested to be dependent on stronger tide flows that occur during spring tides contributing to the re-suspension of sediments effectively reducing light penetration (Dring & Lüning 1994).

Tolerance and recovery of photosynthesis of young and adult thalli to inhibiting photosynthetically active radiation (PAR) is reported to influence depth distribution of seaweeds (e.g. Han & Kain 1996; Hanelt, Wiencke & Nultsch 1997a; Hanelt *et al.* 1997b). Recent temporary lowering of stratospheric ozone concentrations enhances the UVB fluxes at the earth's surface (Smith *et al.* 1992; von der Gathen *et al.* 1995; Stähelin *et al.* 2001). Consequently, UVR has also been reported to influence the vertical distribution pattern of seaweeds (e.g. Dring *et al.* 1996a; Bischof, Hanelt & Wiencke 1998a, 2001; Hanelt 1998; Dring, Wagner & Lüning 2001b).

The effect of ultraviolet radiation (UVR) on algal metabolism and physiology is manifold. Early developmental stages of seaweeds have been reported to be more susceptible to UVR when compared to adult stages (reviewed by Coelho, Rijstenbil & Brown 2000). Exposure to increased UVR induces spore mortality and photoinhibition of photosynthesis (Wiencke *et al.* 2000; Wiencke, Clayton & Schoenwaelder 2004; Roleda *et al.* 2004a). The primary targets for the UV-suppression of photosynthetic activity are still under debate (Baker, Noguez & Allen 1997). UVBR appears to degrade the D1 protein and part of the D1/D2 heterodimer; the major structural complex within PSII (Aro *et al.* 1990; Melis, Nemson & Harrison 1992; Jansen *et al.* 1993). Other studies have demonstrated decreases in the pool size of carbon fixation enzymes such as carbonic anhydrase (Dionisio, Tsuzuki & Miyachi 1989) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) (Bischof, Hanelt & Wiencke 2000). Moreover, UVBR exposure causes DNA damage in spores of Laminariales and Gigartinales (Wiencke *et al.* 2000; Roleda *et al.* 2004a). Most UVB-induced lesions involve dimerization of adja-

cent pyrimidine bases which results in cyclobutane-pyrimidine dimers (CPDs). These lesions are cytotoxic because they disrupt cell metabolism and division (van de Poll *et al.* 2001).

To counteract the negative effects of UVBR, ultraviolet sunscreens are used as photoprotective mechanisms in planktonic organisms (Garcia-Pichel 1994). Among 1–< 10 µm size class cells, sunscreens accumulation can afford considerable benefits but only at the expense of relatively heavy energetic investment and with restricted efficiencies (Garcia-Pichel 1994). However, the protective potential of phlorotannin containing physodes in kelp zoospores (3–5 µm) has been described by Wiencke *et al.* (2004). Moreover, kelp phlorotannin exudates along coastal shores from macroalgal sources have been reported to reduce the impact of UVBR, forming UV-refugia for kelp zoospores within the water column (Swanson & Druehl 2002).

Zonation pattern of kelp species in Helgoland (North Sea) is characterized by typical occurrence of *Laminaria digitata* (Hudson) Lamouroux in the uppermost part of the sublittoral region, whereas *Laminaria saccharina* (Linnaeus) Lamouroux and *Laminaria hyperborea* (Gunnerus) Foslie dominate the middle and deeper parts of the kelp zone, respectively (Lüning 1979). Initiation of reproduction in Helgolandic Laminariales is limited to a distinct reproductive season. Reproductive tissues (sori) are observed in *L. digitata* during late spring to summer (May–August), *L. saccharina* during autumn to mid winter (September–February) and *L. hyperborea* during late autumn to winter (November–February). This reproductive strategy is speculated to be associated to the seasonal environmental pattern ensuring higher probability of success in reproduction (De Wreede & Klinger 1988). A recent study on the germination of five species of Laminariales from the Arctic showed that UVR susceptibility of zoospores is also related to the depth distribution of the adult sporophytes (Wiencke *et al.* 2004). In this regard, the present study will re-examine the susceptibility of the early developmental stages of the three Helgolandic *Laminaria* species to varying irradiance in relation to their depth distribution. This relationship was previously sought but not found by Dring *et al.* (1996b). We focus on zoospores and germinating spores, because they can be found to be planktonic for an extended period of time exposed to environmental stress such as high photon fluence rate and UVR. However, settling zoospores under algal canopies experiences different low-light microenvironment which is suitable for germination and growth. This study extends to investigate the impact of increasing exposure time to varying light spectrum on the photosynthetic efficiency and DNA damage of zoospores. The presence of UV-absorbing compounds in zoospore suspension is quantified and this is the first study on the capacity of Laminariales zoospores for DNA repair and its implication on germination capacity. We hypothesize that susceptibility of zoospores of the three *Laminaria* species to PAR and UVR influence the vertical distribution pattern of the adult sporophytes and the eventual reproductive strategies of adult sporophytes.

## MATERIALS AND METHODS

### Zoospore material

Fertile specimens of *L. digitata* were collected by hand in the upper sublittoral (0.5–1 m) during low tide, and *L. saccharina* and *L. hyperborea* were collected by scuba diving in the mid (2–4 m) and lower (5–7 m) sublittoral, respectively, around the island of Helgoland. Thallus parts with sori were blotted with tissue paper and kept for 2 d in a wet chamber in dim light at  $5 \pm 1$  °C. Spores were released from five individual sporophytes per species by flooding Provasoli enriched seawater (Starr & Zeikus 1993) to the thallus in separate Petri-dishes. Spore density released from individual sporophytes was adjusted to  $2.0 \times 10^5$ – $4.0 \times 10^5$  spore ml<sup>-1</sup> using a Neubauer Chamber (Brand GmbH, Wertheim, Germany). Due to the extent of the experimental work, sori for photosynthesis, absorption spectra, DNA damage and recovery, and germination experiments were separately collected four times during the peak fertile season of *L. digitata* (May–July), *L. saccharina* (September–November) and *L. hyperborea* (December–February).

### Irradiation treatments

Photosynthetically active radiation (PAR) was provided by white fluorescent tubes (L65 Watt/25S, Osram, Munich, Germany) and ultraviolet radiation (UVR) was generated by UVA-340 fluorescent tubes (Q-Panel, Cleveland, OH, USA), emitting a spectrum similar to solar radiation in the range 295–340 nm. Three kinds of filter foils were used to cut off different wavelength ranges from the spectrum emitted by the fluorescent tubes. Experimental units were covered with the following filters: Ultraphan transparent (Digefra GmbH, Munich, Germany); Folanorm (Folex GmbH, Dreieich, Germany) or Ultraphan URUV Farblos corresponding to the PAR + UVA + UVB (PAB), PAR + UVA (PA) and PAR (P) treatments, respectively. Irradiation was measured using a cosine sensor connected to a UV-VIS Spectrometer (Marcel Kruse, Bremerhaven, Germany) below the cut-off filters. The biologically effective doses (BED) between 280 and 320 nm applied were calculated using two action spectra for well-known biological responses: the generalized plant damage (280–312 nm,

Caldwell 1971) and DNA damage for *Escherichia coli* (280–320 nm, Setlow 1974). Minimal erythral dose (MED) below the cut-off filters was also measured using an ELUV-14 UV-Data logger (El Naggar *et al.* 1995). Both unweighted and weighted irradiances for each treatment are compiled in Table 1. The 1.26 UV (total UVR) : PAR ratio in this study is within the highly variable ratio of UV (305, 320, 340, and 380) : PAR ratio measured in Helgoland which ranges between 0.002 and 1.4 depending on cloud cover and season (Dring *et al.* 2001a).

### Chlorophyll fluorescence measurements

Photosynthetic efficiency measured as variable fluorescence of photosystem II (PSII), was determined using a xenon pulse amplitude modulation fluorometer (XE-PAM) connected to a PC with WINCONTROL software (Heinz Walz GmbH, Effeltrich, Germany). Immediately after adjustment of spore density (approximately 1 h after spore release), spore suspension was filled into 5 mL quartz cuvettes and the maximum quantum yield ( $F_v/F_m$ ) was measured to determine initial photosynthetic efficiency at time zero ( $T_0$ ,  $n = 5$ ) as described by Hanelt (1998). Photosynthesis (in terms of relative electron transport rate,  $ETR = PFR \times \Delta F/F_m'$ ) versus irradiance curves (P–I curve) were also measured in the  $T_0$  samples ( $n = 3$ , chosen at random from the five replicates) as described by Bischof *et al.* (1998b). Saturating irradiance level ( $I_k$ ) and  $ETR_{max}$  were estimated. Spore suspensions used for  $T_0$  measurements were filled into corresponding Petri dishes. To evaluate the effect of different radiation and exposure time treatments, 5 mL of fresh spore suspension were filled into each 35 mm × 10 mm cell culture dish and exposed to the three radiation conditions in a series of time treatments (1, 2, 4, 8 and 16 h;  $n = 5$  per treatment combination) at  $10 \pm 1$  °C. After treatments,  $F_v/F_m$  was measured and spore suspension was returned to the same culture dish and cultivated under dim white light ( $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for recovery. Spore suspension measured at  $T_0$  was also maintained at the same condition. Measurements of photosynthetic efficiency were repeated after 2 d to determine recovery and handling effect on untreated  $T_0$  samples (now designated as  $T_2$  measurement), which were eventually used as control. Settled and germinating spores

**Table 1.** Experimental treatments applied with the corresponding weighted irradiances using the biologically effective weighing function for general plant damage (Caldwell 1971), DNA damage of *E. coli* (Setlow 1974), and minimal erythral dose (MED, El Naggar *et al.* 1995)

Treatment	Experimental irradiance (W m <sup>-2</sup> )			Weighted irradiance (W m <sup>-2</sup> )		
	PAR (400–700 nm)	UVA (320–400 nm)	UVB (280–320 nm)	General plant damage (Caldwell 1971)	DNA damage (Setlow 1974)	MED (El Naggar <i>et al.</i> 1995)
Ultraphan transparent (PAR + UVA + UVB)	4.74	5.86	0.36	$1.8 \times 10^{-2}$	$4.7 \times 10^{-3}$	$8.7 \times 10^{-2}$
Folanorm 320 (PAR + UVA)	4.44	4.95	0.03	0	$9.5 \times 10^{-5}$	$4.2 \times 10^{-3}$
Ultraphan URUV farblos (PAR)	4.59	0.04	0.00	0	0	$1.2 \times 10^{-3}$

were slowly re-suspended by sucking and jetting the medium against the bottom of the culture dish using Eppendorf pipettes.  $F_v/F_m$  after exposure and after recovery was expressed as percentages of  $T_0$  and  $T_2$  control, respectively.

### Absorbance spectrum

To determine the presence of UV-absorbing compounds in the zoospore suspension, untreated samples were filled into quartz cuvettes and scanned in the 250–700 nm waveband using Shimadzu photometer (UV 2401PC; Shimadzu, Tokyo, Japan) equipped with an integrating sphere. Absorbance spectrum of the zoospore suspension, the medium (filtrate) and zoospores were measured from: (1) zoospore suspension with seawater as reference, (2) filtrate with seawater as reference, and (3) zoospore suspension and filtrate as reference, respectively. The filtrate was obtained by filtering the zoospores out of the suspension through 44-mm-diameter, 1.0  $\mu\text{m}$  pore size Nuclepore<sup>®</sup> polycarbonate membrane (Whatman, Maidstone, Kent, UK) using a vacuum pump at 400–600 millibars to minimize damage to the cells.

### DNA damage and repair

DNA damage and its subsequent repair were determined after exposure to the same treatments. From the spore suspension, 40 mL was used for each experimental unit. For each treatment, six experimental units were prepared. After the irradiation treatment, three experimental units (as replicates) were processed immediately while the other three were allowed to recover for 2 d in low white light before processing. Settled and germinating spores were re-suspended from the bottom of the Petri dishes by jetting pressurized seawater from a wash bottle. The spore samples were filtered through 44-mm-diameter, 1.0  $\mu\text{m}$  pore size Nuclepore<sup>®</sup> polycarbonate membrane (Whatman). Filters were individually filled into 2 mL Eppendorf tubes and frozen at  $-80^\circ\text{C}$  for further DNA extraction and analysis of CPDs.

### Spore germination

Cover slips were put inside the 85 mm  $\times$  15 mm culture dishes and filled with 40 mL Provasoli-enriched seawater. To ensure that the density of spores per unit area is similar throughout the experiments, 2–4 drops of the working spore suspension were put into each dish. The dishes were then exposed to the same experimental treatments, in triplicates. After treatment, spores were allowed to germinate in low white light (10  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) for 3 d. Triplicate of untreated samples (control) were also allowed to grow at the same low light condition. Spores settled on the cover slip were scored as germinated or not germinated by counting 300 cells per replicate using a light microscope (Olympus CH-2; Olympus, Tokyo, Japan) equipped with a 20 $\times$  seawater immersion objective. A spore was classified as germinated when at least a germ-tube was formed. Dead and

living cells were not differentiated. Since non-germinated cells were also observed under control, germination rate under P, PA and PAB treatments was expressed as percentage of control.

### DNA extraction

Frozen spores on polycarbonate filters were treated with extraction buffer (CTAB) and DNA was isolated as described by van de Poll *et al.* (2001) and modified by Roleda *et al.* (2004a). After DNA extraction, the pellet was dissolved in 0.2 mL TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid, pH 8.0), treated with RNAase (5  $\mu\text{L}$  10 mg  $\text{mL}^{-1}$ , 30 min, 37  $^\circ\text{C}$ ; Sigma, St Louis, MO, USA) and stored at  $-20^\circ\text{C}$ . The DNA concentration was quantified fluorometrically using the PicoGreen assay (Molecular Probes, Eugene, OR, USA) and a Cary Eclipse Fluorescence Spectrophotometer (Variance Scientific Instrument, Palo Alto, CA, USA). A dilution series with a known amount of DNA (Serva, Heidelberg, Germany) was included for calibration purposes.

### Assay for CPDs detection

The immunoassay for CPDs was modified after Vink *et al.* (1994) and van de Poll *et al.* (2001). Heat-denatured samples containing 50 ng DNA were transferred to a nitrocellulose membrane (Protran BA 79, pore size 0.1  $\mu\text{m}$ ; Schleicher & Schuell, Keene, NH, USA) with a Minifold I SRC96 dot blot apparatus (Schleicher & Schuell). After a two-step antibody assay, the membrane was treated with ECL Western blotting detection reagent (Amersham, Little Chalfont, Bucks., UK) and sealed in a transparent vinyl plastic folder (Leitz, Stuttgart, Germany). This was subsequently exposed to photosensitive ECL films (Amersham) at different exposure times. The films were developed using X-ray film developer. Developed films were scanned using Bio-Rad imaging densitometer (Model GS-700; Bio-Rad Laboratories, Hercules, CA, USA) and grey scale values were quantified using MULTI-ANALYST (Macintosh Software for Bio-Rad Image Analysis Systems). A calibration series of UV-irradiated calf thymus DNA (Serva, Heidelberg, Germany) supplemented with unexposed DNA was included giving 1  $\mu\text{g mL}^{-1}$  DNA for each calibration point. The UV-irradiated DNA was previously calibrated against UV-irradiated Hela DNA with known amounts of CPDs (kindly provided by A. Vink). CPDs were quantified by comparing the grey scales within the linear range of the film.

### Statistical analysis

Data were tested for homogeneity of variances (Levene Statistics) and normality (Kolmogorov–Smirnov test). Corresponding transformations were done to heteroskedastic and non-normal data. The response of the dependent factors were tested using multiple analyses of variance (MANOVA,  $P < 0.05$ ) with interaction effect between spe-

cies, irradiance and exposure time. When two-way and three-way interactions were observed, significantly different subgroups were determined by plotting the means of each dependent factor against the levels of each independent (main) factor (Underwood 1981). Groupings were based on *post hoc* multiple comparisons test. Statistical analyses were done using SPSS program (SPSS, Chicago, IL, USA).

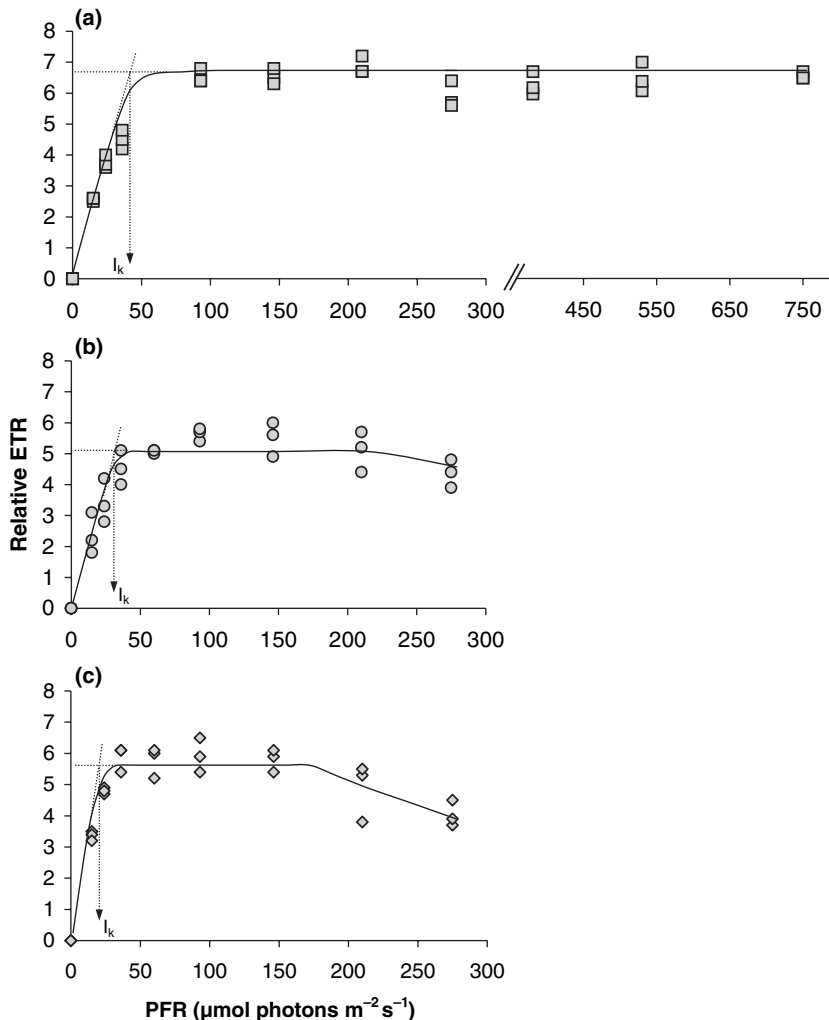
## RESULTS

### Photosynthetic efficiency

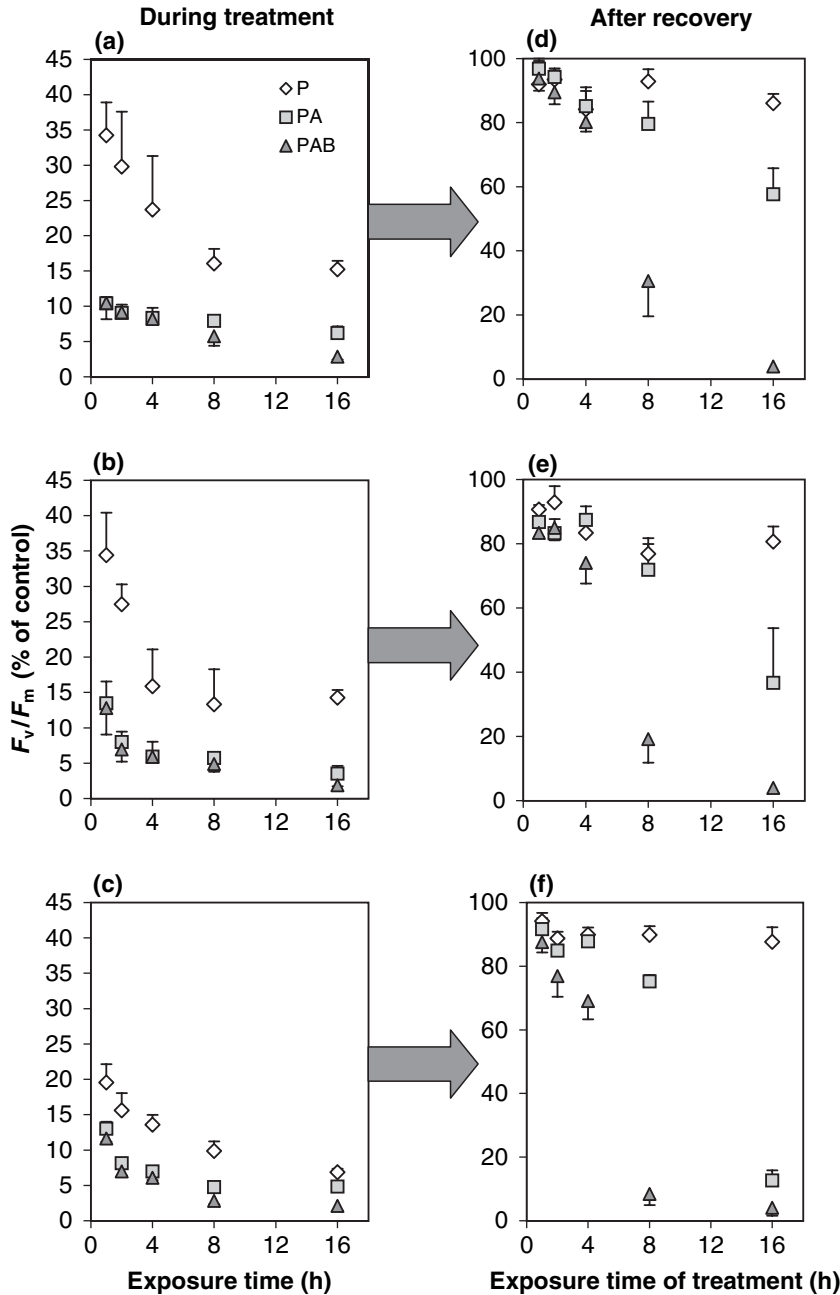
Measurements of initial photosynthetic efficiency of the controls showed highest maximum quantum yield ( $F_v/F_m$ ) in zoospores of *L. hyperborea* ( $0.533 \pm 0.005$ ), followed by *L. saccharina* ( $0.476 \pm 0.003$ ) and lowest in *L. digitata* ( $0.466 \pm 0.010$ ). During the short irradiation period (3 min) for the measurement of the P-I curve,  $ETR_{max}$  of the controls in *L. digitata* did not decrease until the highest actinic light level of  $750 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  was reached. A slight decrease in  $ETR_{max}$  was observed in *L. saccharina* at

$275 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  whereas the  $ETR_{max}$  of *L. hyperborea* decreases already above  $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Visual estimate of the P-I curve showed higher saturating irradiance ( $I_k$ ) in *L. digitata* (approximately  $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; Fig. 1a) compared to *L. saccharina* and *L. hyperborea* (approximately 30 and  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ , respectively; Fig. 1b & c). After 1 h of higher light pretreatment at  $\pm 20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  photosynthetically active radiation (PAR = P), the optimum quantum yield ( $F_v/F_m$ , expressed as a percentage of control) was already reduced to 34% in *L. digitata* (Fig. 2a) and *L. saccharina* (Fig. 2b) and to 20% in *L. hyperborea* (Fig. 2c). Increasing exposure time further decreased the  $F_v/F_m$ , indicating that zoospores are quite low light adapted. Light supplemented with UV-radiation further decreased zoospore photosynthetic efficiency. After 1 h exposure to PAR + UVA (PA) and PAR + UVA + UVB (PAB), an additional reduction in the  $F_v/F_m$  of UVR exposed samples (approximately 7–20%) was observed compared to the PAR only. Increasing exposure time further exacerbate the effect of UVR.

After 2 d in dim white light, photosynthetic efficiency of the untreated (control) germinating spores was reduced by



**Figure 1.** Photosynthetic performance (P-I curve) of zoospores from (a) *Laminaria digitata* (b) *L. saccharina* and (c) *L. hyperborea* ( $n = 3$ ) immediately after release from the sori. PFR is the respective photon fluence rate of actinic white light and ETR is the electron transport rate. Saturating irradiance ( $I_k$ ) is estimated as the point at which the extrapolated initial slope crosses maximum photosynthesis ( $ETR_{max}$ ). Maximum effective quantum yields are  $0.424 \pm 0.01$ ,  $0.318 \pm 0.09$  and  $0.453 \pm 0.02$  for *L. digitata*, *L. saccharina* and *L. hyperborea*, respectively.



**Figure 2.** Mean optimum quantum yield ( $F_v/F_m$ ) of zoospores during treatment to photosynthetically active radiation (PAR = P), PAR + UVA (PA) and PAR + UVA + UVB (PAB) at different exposure times in (a) *Laminaria digitata*; (b) *L. saccharina*; and (c) *L. hyperborea* expressed as percentage of control. Absolute means at  $T_0$  are  $0.466 \pm 0.010$ ,  $0.476 \pm 0.003$  and  $0.533 \pm 0.005$ , respectively. Corresponding photosynthetic recovery (d, e and f, respectively) after 48 h post-culture in dim white light ( $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ). Vertical bars are standard deviations (SD,  $n = 5$ ). Absolute means at  $T_2$  are  $0.387 \pm 0.005$ ,  $0.431 \pm 0.008$  and  $0.513 \pm 0.011$ , respectively.

4–17% in comparison with the freshly released zoospores of all controls.  $F_v/F_m$  was measured in decreasing order in *L. hyperborea* ( $0.513 \pm 0.011$ ); *L. saccharina* ( $0.431 \pm 0.008$ ); and *L. digitata* ( $0.387 \pm 0.005$ ). Photosynthetic efficiency of all species treated with P was able to recover in dim light to 80–95% of the control (Fig. 2d–f). An efficient recovery of photosynthetic efficiency (70–90% of control) was also observed in spores of all species treated to a maximum of 8 h PA and 4 h PAB (Fig. 2d–f). All data (absolute values) are compiled in Table 2.

Multiple analysis of variance (MANOVA,  $P = 0.05$ ) showed a significant effect of the main factors, two-way and three-way interactions (Table 3). *Post hoc* multiple compar-

isons test showed several significantly different subgroups. Ranking the subgroups from the lowest to higher photosynthesis level, photosynthetic efficiency was lowest in the subgroup consisting of 16 h PAB in all species, 16 h PA in the mid and lower sublittoral species *L. saccharina* and *L. hyperborea* and 8 h PAB in *L. hyperborea*. The second from lowest subgroup showed minimal photosynthetic efficiency among 4 h PAB and 8 h PA in *L. saccharina* and *L. hyperborea*, 8 h PAB in *L. digitata* and *L. saccharina*, 16 h PA in *L. digitata* and 16 h P in *L. hyperborea*. After 2 d in dim white light, no recovery of photosynthetic efficiency was observed in the subgroup consisting of 16 h PAB treatment in all species and minimal recovery was observed in the

**Table 2.** Mean absolute values ( $\pm$  SD) of zoospore photosynthetic efficiency (optimum quantum yield,  $F_v/F_m$ ) and germination after treatment to photosynthetically active radiation (PAR = P); PAR + UVA radiation (PA); PAR + UVAR + UVB radiation (PAB)

		<i>Laminaria digitata</i>				<i>Laminaria saccharina</i>				<i>Laminaria hyperborea</i>			
		PA	PAB	P	PA	PAB	P	PA	PAB	P	PA	PAB	
Optimum quantum yield ( $F_v/F_m$ )													
Control ( $T_0$ )		0.466 $\pm$ 0.010						0.476 $\pm$ 0.003					0.533 $\pm$ 0.005
Control ( $T_2$ )		0.387 $\pm$ 0.005						0.431 $\pm$ 0.008					0.513 $\pm$ 0.011
Treatment (h)													
1		0.160 $\pm$ 0.022	0.049 $\pm$ 0.004	0.164 $\pm$ 0.029	0.064 $\pm$ 0.015	0.061 $\pm$ 0.018	0.104 $\pm$ 0.014	0.069 $\pm$ 0.005	0.062 $\pm$ 0.002				
2		0.139 $\pm$ 0.036	0.042 $\pm$ 0.005	0.131 $\pm$ 0.013	0.038 $\pm$ 0.007	0.033 $\pm$ 0.008	0.083 $\pm$ 0.013	0.043 $\pm$ 0.004	0.037 $\pm$ 0.003				
4		0.110 $\pm$ 0.036	0.039 $\pm$ 0.007	0.076 $\pm$ 0.025	0.028 $\pm$ 0.010	0.028 $\pm$ 0.003	0.072 $\pm$ 0.007	0.037 $\pm$ 0.003	0.032 $\pm$ 0.002				
8		0.075 $\pm$ 0.010	0.037 $\pm$ 0.003	0.063 $\pm$ 0.024	0.027 $\pm$ 0.003	0.023 $\pm$ 0.005	0.053 $\pm$ 0.007	0.025 $\pm$ 0.004	0.015 $\pm$ 0.003				
16		0.071 $\pm$ 0.006	0.029 $\pm$ 0.004	0.068 $\pm$ 0.005	0.017 $\pm$ 0.005	0.009 $\pm$ 0.001	0.037 $\pm$ 0.003	0.026 $\pm$ 0.002	0.011 $\pm$ 0.003				
48 h recovery after exposure [Treatment exposure(h)]													
1		0.356 $\pm$ 0.028	0.375 $\pm$ 0.012	0.391 $\pm$ 0.006	0.374 $\pm$ 0.008	0.359 $\pm$ 0.005	0.483 $\pm$ 0.013	0.470 $\pm$ 0.017	0.449 $\pm$ 0.017				
2		0.362 $\pm$ 0.011	0.365 $\pm$ 0.010	0.400 $\pm$ 0.022	0.359 $\pm$ 0.019	0.366 $\pm$ 0.017	0.455 $\pm$ 0.010	0.435 $\pm$ 0.010	0.395 $\pm$ 0.033				
4		0.326 $\pm$ 0.022	0.330 $\pm$ 0.023	0.359 $\pm$ 0.017	0.377 $\pm$ 0.018	0.319 $\pm$ 0.028	0.461 $\pm$ 0.012	0.451 $\pm$ 0.013	0.354 $\pm$ 0.030				
8		0.359 $\pm$ 0.015	0.308 $\pm$ 0.027	0.331 $\pm$ 0.021	0.310 $\pm$ 0.034	0.083 $\pm$ 0.032	0.461 $\pm$ 0.014	0.386 $\pm$ 0.010	0.043 $\pm$ 0.018				
16		0.333 $\pm$ 0.011	0.223 $\pm$ 0.031	0.348 $\pm$ 0.020	0.158 $\pm$ 0.074	0.017 $\pm$ 0.006	0.450 $\pm$ 0.023	0.065 $\pm$ 0.016	0.021 $\pm$ 0.013				
Germination (%)													
Control		93 $\pm$ 2.5				90 $\pm$ 3.2				95 $\pm$ 3.2			
Treatment (h)													
1		83 $\pm$ 0.3	69 $\pm$ 0.7	75 $\pm$ 2.2	69 $\pm$ 3.5	61 $\pm$ 0.6	80 $\pm$ 0.9	63 $\pm$ 1.2	61 $\pm$ 7.6				
2		75 $\pm$ 1.8	61 $\pm$ 3.0	63 $\pm$ 4.6	67 $\pm$ 4.4	57 $\pm$ 1.5	71 $\pm$ 1.0	55 $\pm$ 5.3	54 $\pm$ 1.6				
4		69 $\pm$ 0.8	51 $\pm$ 4.4	61 $\pm$ 4.0	61 $\pm$ 3.7	50 $\pm$ 4.5	69 $\pm$ 1.0	55 $\pm$ 0.9	50 $\pm$ 1.5				
8		66 $\pm$ 5.1	37 $\pm$ 7.9	59 $\pm$ 2.2	58 $\pm$ 1.3	26 $\pm$ 1.3	63 $\pm$ 2.8	53 $\pm$ 1.4	25 $\pm$ 3.7				
16		59 $\pm$ 2.4	26 $\pm$ 0.9	56 $\pm$ 3.6	57 $\pm$ 2.0	23 $\pm$ 0.6	62 $\pm$ 6.8	45 $\pm$ 15.5	19 $\pm$ 1.9				

Photon flux density is 20–24  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  ( $\pm 4.5 \text{ W m}^{-2}$ ). Photosynthetic recovery was initiated in dim white light of 10  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  after treatment. Control at time zero ( $T_0$ ) was measured after spore release while control at day 2 ( $T_2$ ) was measured after post-cultivation at the same dim light condition with that of the treated samples. Germination controls were untreated samples simultaneously cultivated at 10  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  together with the treated samples after treatment.

**Table 3.** Multiple analysis of variance (MANOVA) and significance values for the main effects and interactions of species, irradiance and exposure time on the photosynthesis, DNA damage and repair and germination of zoospores from three species of Laminariales in Helgoland

Experiment	Variable	Source of variation	d.f.	F-value	P-value
Photosynthesis	$F_v/F_m$ (after treatment)	Species (A)	2	51.286	< 0.001*
		Irradiance (B)	2	533.834	< 0.001*
		Exposure time (C)	4	133.264	< 0.001*
		A × B	4	31.238	< 0.001*
		A × C	8	3.376	0.001*
		B × C	8	14.561	< 0.001*
		A × B × C	16	2.502	0.002*
	$F_v/F_m$ (recovery)	Species (A)	2	47.502	< 0.001*
		Irradiance (B)	2	825.265	< 0.001*
		Exposure time (C)	4	729.542	< 0.001*
		A × B	4	11.663	< 0.001*
		A × C	8	5.532	< 0.001*
		B × C	8	209.925	< 0.001*
		A × B × C	16	9.352	< 0.001*
DNA damage and repair	CPDs induction	Species (A)	2	49.608	< 0.001*
		Exposure time (B)	4	40.684	< 0.001*
		A × B	8	3.769	0.004*
	Remaining CPDs	Species (A)	2	12.053	< 0.001*
		Exposure time (B)	4	5.243	0.003*
		A × B	8	1.872	0.102 <sup>ns</sup>
Germination		Species (A)	2	58.614	< 0.001*
		Irradiance (B)	2	390.725	< 0.001*
		Exposure time (C)	4	185.816	< 0.001*
		A × B	4	16.497	< 0.001*
		A × C	8	1.265	0.272 <sup>ns</sup>
		B × C	8	25.338	< 0.001*
		A × B × C	16	0.741	0.745 <sup>ns</sup>

\*Significant; <sup>ns</sup> not significant.

subgroup of 16 h PA and 8 h PAB treatments in the lower sublittoral species *L. hyperborea*. This corresponds to the zonation pattern of the respective species at the coast.

### Absorbance spectrum

Spectral analysis of the zoospore suspension (Fig. 3a), filtrate (Fig. 3b) and zoospores (Fig. 3c) showed strong absorbance below 360 nm. The absorbance maxima in the 260–280 nm range are characteristic of phlorotannins. Higher absorbance was measured in all *L. digitata* samples in comparison with the two other species. The filtrate containing exudates strongly absorbed UVBR in the medium of *L. digitata* more than the zoospores itself. UV absorbance of the filtrate was always higher than that of the zoospores. Relatively similar absorbance was measured in the zoospores of *L. saccharina* and *L. hyperborea* (Fig. 3c).

### DNA damage and repair

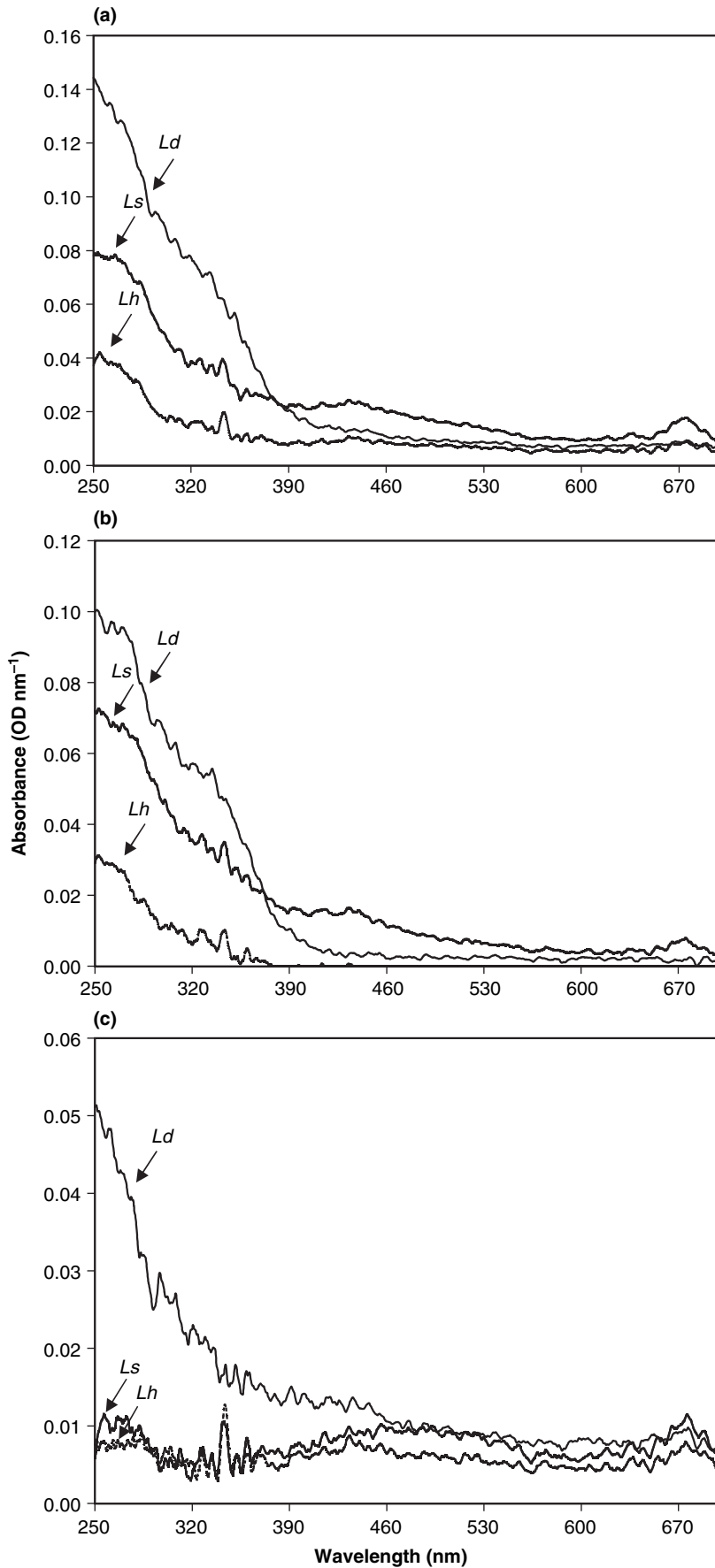
Differential cyclobutane-pyrimidine dimers (CPDs) induction was observed in the spores of the three species examined. CPD induction significantly increases with UV exposure time and higher CPD induction was observed in *L. hyperborea* (Fig. 4a). However, significant two-way interaction was observed in CPD induction (Table 2),

implying that DNA damage may either be significantly different among the three species, but not for all exposure times or significantly different among all exposure times but not for all species. After 2 d post-culture in dim white light, all species were observed to repair DNA damage. No detectable CPDs were observed in *L. digitata* spores exposed to 1–8 h of PAB (Fig. 4b). Repair of DNA damage was observed to be significantly different between species ( $P < 0.001$ ) and exposure time ( $P = 0.003$ ). *Post hoc* test showed that the difference between repair of *L. saccharina* and *L. hyperborea* DNA damage was not significantly different.

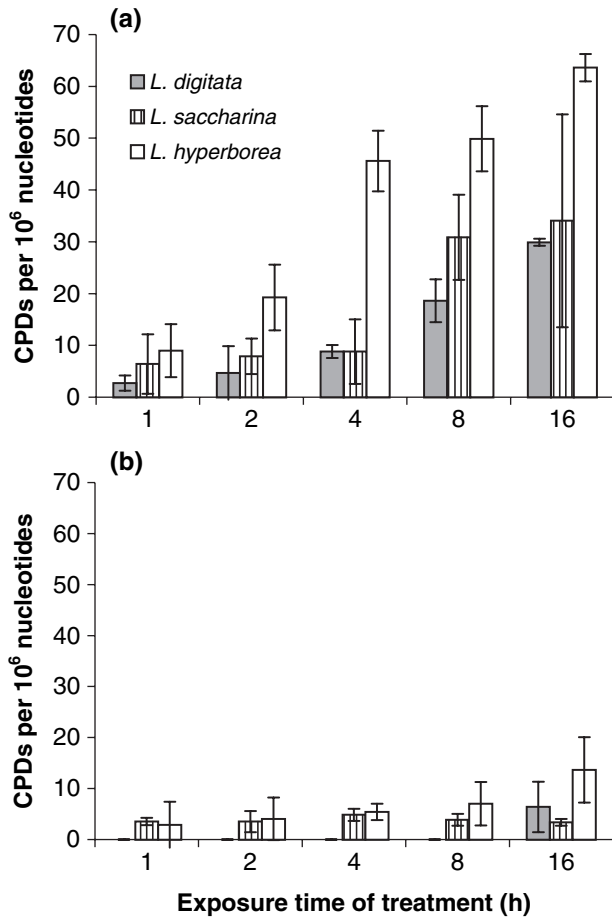
### Germination

After 3 d in dim white light, spore germination of untreated samples were  $93 \pm 2.5$ ,  $90 \pm 3.2$  and  $95 \pm 3.2\%$  in *L. digitata*, *L. saccharina* and *L. hyperborea*, respectively (all non-standardized data are presented in Table 2). Among treated samples, germination rate decreased with increasing exposure treatment in all species (Fig. 5a–c). Germination was 85–90% after 1 h PAR and 60–65% after 16 h PAR exposure. Germination capacity was effectively reduced in spores exposed to light supplemented with UVR. UVA had no effect on the germination capacity of *L. digitata* (Fig. 5a) and *L. saccharina* (Fig. 5b). In contrast, germination rate in





**Figure 3.** Absorbance spectrum of *Laminaria digitata* (*Ld*), *L. saccharina* (*Ls*) and *L. hyperborea* (*Lh*) (a) zoospore suspension (b) filtrate = seawater medium and (c) zoospores.



**Figure 4.** UVB-induced DNA damage (induced CPD concentrations per million nucleotides) in zoospores after (a) exposure to increasing time of PAR + UVA + UVB and (b) corresponding CPD repair after 2 d recovery in  $10 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ . Vertical bars are standard deviations (SD,  $n = 3$ ).

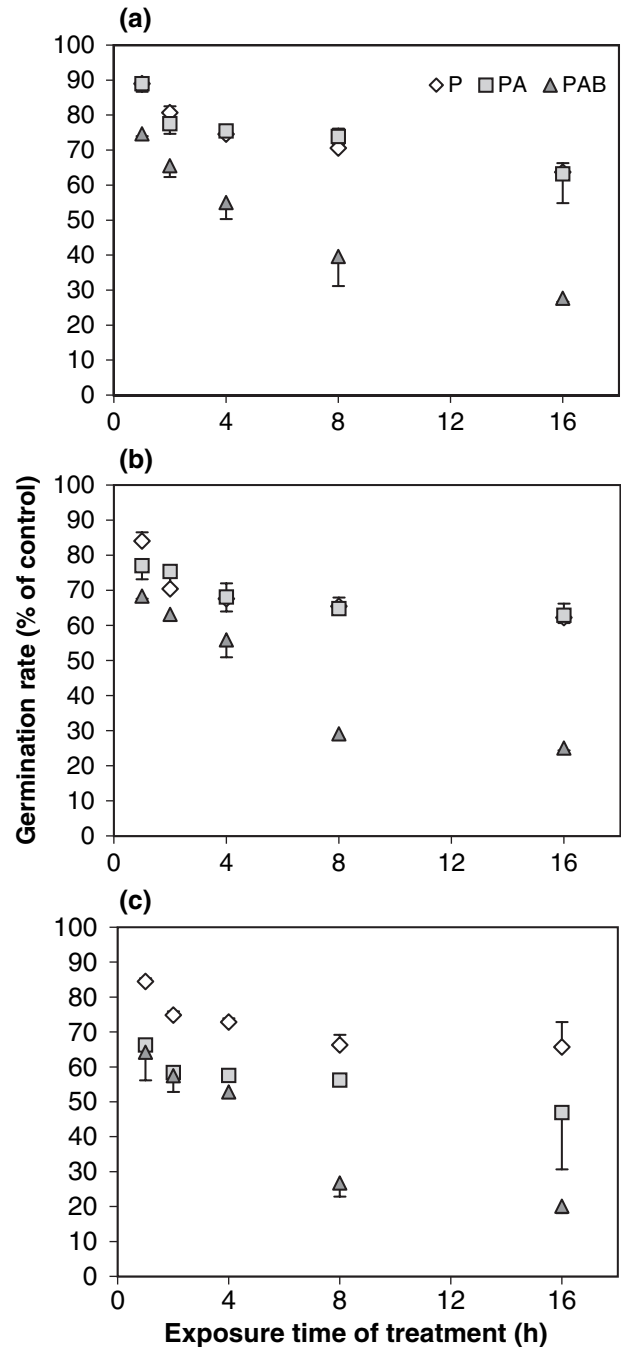
*L. hyperborea* decreased to 50% after 16 h of exposure to PA (Fig. 5c). Additional UVB reduced germination rate to less than 30% in all species exposed to 16 h PAB and to *L. saccharina* and *L. hyperborea* exposed to 8 h PAB (Fig. 5b & c). MANOVA ( $P = 0.05$ ) showed significant effect of the main factors and two-way interaction between species and irradiance, as well as between irradiance and exposure time (Table 3).

## DISCUSSION

This study indicates that zoospore photosynthesis is adapted to low light conditions and UVR causes significant photoinhibition of photosynthesis. Moreover, it is the first report of the capability of spores to repair UVB-induced DNA damage which enhanced spore viability and germination capacity of UVR treated samples. Absorbance of UVR in the zoospore suspensions was found to be based on the absorbance capacity of the spores as well as of the exudates in the medium. Tolerance of spores to PAR and UVR was

found to be related to the depth distribution of the adult sporophytes.

The initial differences in  $I_k$  values of the three *Laminaria* species investigated are related to the natural depth distribution of the algae. The saturating irradiance showed a



**Figure 5.** Spore germination, expressed as percentage of control in (a) *Laminaria digitata* (b) *L. saccharina* and (c) *L. hyperborea*, 3 d after exposure to photosynthetically active radiation (PAR = P), PAR + UVA (PA) and PAR + UVA + UVB (PAB) at different exposure time. Post-cultivation is at  $10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Vertical bars are standard deviations (SD,  $n = 3$ ).

correlation with habitat whereby zoospores photosynthetic performance of the upper sublittoral *L. digitata* saturated at approximately  $40 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  whereas the performance of the lower sublittoral *L. hyperborea* saturated at approximately  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ . The decrease in  $\text{ETR}_{\text{max}}$  with increasing actinic light level during P–I curve measurement further established the association between light sensitivity and depth distribution of adult plants. Zoospores from *L. saccharina* and *L. hyperborea* which inhabit the deeper kelp zone are clearly more sensitive to higher photon fluence rates. This differential light adaptation is also reported in young sporophytes of *L. hyperborea*, which are able to survive in low light, requiring only  $1 \mu\text{mol photon m}^{-2} \text{s}^{-1}$ , in contrast to *L. digitata* (Han & Kain 1996). Another *Laminaria* species, *Laminaria farlowii* Setchell showed an  $I_k$  ( $41 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ; Amsler & Neushul 1991) comparable to *L. digitata* in our study. Thus, light requirement and tolerance to high light is thought to be genetically fixed. Adult *L. digitata* sporophytes can be photosynthetically characterized as ‘sun plants’, which are not able to produce sufficient photosynthate for growth in the ‘shade’ regions of the deeper kelp zones (Lüning 1979).

The sensitivity of zoospore photosynthesis is already shown in the depression of the maximum quantum yield ( $F_v/F_m$ ) of PSII at the low fluence rate of  $20 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of PAR. This is attributed to the occurrence of photoinhibition even below the saturating light intensity (Hanelt, Huppertz & Nultsch 1992; Osmond 1994). Ögren & Sjöström (1990) also reported that the rate of net photosynthesis can be depressed by photoinhibition over the whole natural range of the photosynthetic photon fluence rates. The primary site of photoinhibition has been suggested to be located in the PSII reaction centre. Key stages of photoinhibition are as follows: functional PSII  $\leftrightarrow$  inactivated PSII  $\rightarrow$  non functional PSII  $\rightarrow$  damage D1 protein removed  $\rightarrow$  D1 protein resynthesized  $\rightarrow$  recovery of functional PSII (Long, Humphries & Falkowski 1994). If low light adaptation is a general feature of brown algal zoospores, light may exert a significant effect on survival of all zoospores in the water column. Differences in P, PA and PAB sensitivity of maximum quantum yield are presumably caused by the degree of damage to PSII components versus the xanthophyll cycle mediated down regulation of PSII (Gevaert *et al.* 2003). Although a similar reduction in maximum quantum yield was also observed in PA and PAB treatments, recovery after prolonged exposure to PAB treatments was only minimal compared with the first, indicating that UVBR causes more damage to PSII function than UVAR. When D1 protein of the PSII is impaired, the effect is only reversible over a longer time scale (hours) because synthesis of new D1 protein is required (Mattoo *et al.* 1984; Long *et al.* 1994; Hanelt, Wiencke & Bischof 2003). This usually occurs in species growing in the lower subtidal zone exposed to high irradiances (Hanelt 1998). Due to the activity of the xanthophyll cycle, seaweeds are able to recover rapidly (within minutes) after the offset of light stress (Long *et al.* 1994; Osmond 1994; Franklin & Forster 1997). Gametophytes exposed to

unnaturally high UVR ( $2.36 \times 10^5 \text{ J m}^{-2}$ ) showed no full recovery in *L. digitata*, *L. saccharina* and *L. hyperborea* (Dring *et al.* 1996a). Slow photosynthetic recovery can therefore reduce the accumulation of photosynthetic products disabling cellular division and delaying initiation of germination in zoospore. Modelling studies on photoinhibition indicate a clear cost in terms of potential carbon acquisition, whereby stress-induced photoinhibition decreases the efficiency in the conversion of intercepted light into dry matter (Long *et al.* 1994).

Harmful effects of UVR on the cell is suggested to operate in a more direct way, through its absorption by aromatic and sulphhydryl-containing biomolecules causing direct molecular damage (Vass 1997) and, by proteins and DNA forming CPDs (Setlow 1974). These photoproducts inhibit transcription and replication of DNA and consequently disrupt cell metabolism and division (Buma *et al.* 1995, 2000), directly constraining cell viability and growth. Hence, UVB-induced DNA damage further compromised germination capacity where adverse effect was elicited already after 1 h of exposure, especially for zoospores of *L. saccharina* and *L. hyperborea*. Damage to microtubules causing inhibition of nuclear division in the zoospore nucleus of *Macrocystis pyrifera* (Huovinen *et al.* 2000) and cell division in *Fucus* spp. (Schoenwaelder *et al.* 2003) were also responsible for mortality and failure of germination in spores exposed to UVR. In young sporophytes, chronic exposures to UVR causes lower growth rate, tissue damage and morphological deformations in *Laminaria ochroleuca* Bachelot de la Pylaie (Roleda *et al.* 2004b).

The impact of UVR on the germination capacity of brown algal zoospores inhabiting different water depth has been demonstrated for Laminariales from Spitsbergen and from southern Spain (Wiencke *et al.* 2000, 2004). Conversely, in a previous study on UVR sensitivity of zoospore germination and gametophyte photosynthesis showed no differences among the three species of Laminariales around Helgoland (Dring *et al.* 1996b). This may be attributed to the two- to four-fold higher levels of UVA and UVB that was supplemented to about  $35\text{--}50 \mu\text{mol photons m}^{-2} \text{s}^{-1}$  of PAR (UV : PAR ratio = 1.525). A higher order of magnitude in UVR : PAR ratio has been reported to intensify the UV effect on plants (Caldwell *et al.* 1995; Rozema *et al.* 1997), which may magnify UVR effect on one species and at the same time obscure its effects between species. Comparison between experimental treatments showed that the 6 h UVR applied by Dring *et al.* (1996b) is equivalent to  $3.54 \times 10^5 \text{ J m}^{-2}$ , a dose that is higher in comparison with our 16 h PAR + UVA + UVB treatment ( $3.32 \times 10^5 \text{ J m}^{-2}$ ). Consequently, the study of Dring and coworkers observed lower germination rates in *L. digitata* (approximately 20%) and *L. hyperborea* (>10%) compared with our study (UVR : PAR ratio = 1.257) on the same species with germination rate of 27.6 and 20.1%, respectively. Higher UVR dose ( $5.46 \times 10^5 \text{ J m}^{-2}$ , UVR : PAR ratio = 1.523) was also used by Wiencke *et al.* (2004), which resulted in >1% germination in Arctic *L. digitata* and *L. saccharina*. However, it may be possible that the Arctic population of these two

Laminariales is more susceptible to UVR in comparison with their cold temperate counterparts.

In this experiment, we supplemented lower UVA and UVB levels to the predetermined saturating light intensity ( $I_k$ ) of the deep kelp species. We used a more realistic UV:PAR ratio compared to the study by Dring *et al.* (1996b). We observed that the physiological responses of *L. hyperborea* were more impaired under longer exposure time of PA and PAB treatments than those of *L. digitata* and *L. saccharina*. Species-specific difference in DNA damage accumulation became obvious after 4 h and longer exposures, where the degree of DNA damage was also higher in *L. hyperborea*. This response was observed to be related to the amount of UV absorbing compounds present in the zoospore and in the medium. In brown algae, phlorotannin containing vesicles (called physodes) which strongly absorb in the UVC region of the spectrum were invoked to play a role in chemical UV defence because their synthesis is inducible by UVBR (Pavia *et al.* 1997; Schoenwaelder 2002). Exudation of this compound from macroalgae into seawater can at low concentrations reduce the impact of UVB exposure to UV-sensitive kelp meiospores (Swanson & Druhl 2002). In Arctic Laminariales zoospores, an increase in number and size of phlorotannin-containing physodes was observed after UV exposure which contributed UVR protection against cellular damage (Wiencke *et al.* 2004). Although we observed strong absorbance of UVBR in the medium where zoospores were released, the source of these phlorotannin exudates (either released from the sori or from zoospores itself) is yet to be determined. All species were able to repair DNA damage, which could either be mediated by light-dependent photolyases or light-independent nucleotide excision repair (Pakker, Beekman & Breeman 2000; van de Poll *et al.* 2002). However, higher remaining DNA damage was also observed in *L. hyperborea* previously exposed to 8 and 16 h PAB. Although no direct correlation was observed between germination and DNA repair rates among species, DNA repair mechanism certainly had contributed to the germination success in *L. digitata* relative to the other two investigated deep kelp species.

Although zoospores of all species were able to recover in dim white light, 2 d after exposure to 4 h of different UV-spectra, future study should measure time series regeneration to determine the rate of photosynthetic recovery. Field experiments are also necessary because UVB radiation applied in our experiment could be accompanied by a 10- to 20-fold higher PAR. Studies on the acclimation potential of zoospores to high PAR are also necessary because under these conditions UVB tolerance may be enhanced by increasing activity of photorepair enzymes (Warner & Caldwell 1983). To estimate the ecological impact of enhanced UVR, seasonal variation in solar radiation has to be related to the reproductive seasonality of kelps as well as the diel periodicity in zoospore release (Reed *et al.* 1988; Amsler & Neushul 1989). Zoospore production in perennial kelp is either continuous (Chapman 1984; Joska & Bolton 1987) or seasonal (tom Dieck 1991; Reed *et al.* 1996;

Graham 1999). The distinct reproductive seasons of Helgolandic *Laminaria* spp. is remarkable compared to other population of the same species. *Laminaria digitata* plants from Nova Scotia are found to be fertile throughout the year (Chapman 1984). To ensure reproductive success, propagules production should be synchronized with the onset of favourable environmental conditions (e.g. light, photoperiod or temperature) (Lüning 1980; Santelices 1990; Kinlan *et al.* 2003). Inferring from the result of our study, the summer reproductive season of the upper sublittoral *L. digitata* would suggest that sporogenic tissues as well as zoospores of this species could tolerate or possess effective protective mechanism against high solar radiation. On the other hand, winter reproduction in the lower sublittoral *L. hyperborea* is thought to be a strategy to avoid reproductive failure due to the relative sensitivity of their zoospores to high PAR and UVR. Samples collected at specific sites around Helgoland belong most likely to the same population because water motion disperses spores and fertilize gametes between different individual sporophytes around the small island (approximately 35 km<sup>2</sup> of rocky shore area). The population of *Laminaria* around Helgoland is exposed to the same environmental factors and therefore, collection site (geographic) is unlikely to play a role in the species response to the experimental treatments. However, the difference in the abiotic factors occurring at different water depths may cause a different adaptation status of the samples. In conclusion, zoospores susceptibility to UVR could therefore determine spore viability and germination success of kelp propagules. Susceptibility of spores indicates a relation to the observed zonation pattern of kelp community around Helgoland.

## ACKNOWLEDGMENTS

The first author is supported by a scholarship from the German Academic Exchange Service (DAAD). We thank R. Dizon for valuable statistical input. This is publication awi-n14900

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Received 25 August 2004; received in revised form 18 October 2004; accepted for publication 19 October 2004