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Effects of UVB radiation and salt stress on growth, pigments and antioxidative defence of the marine diatom *Cylindrotheca closterium*

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ABSTRACT: During emersion, diatoms are exposed to sunlight (with UVB) on tidal flats, where the salinity may increase as a result of evaporation. In order to investigate whether a combination of UV radiation and desiccation would cause oxidative stress under such conditions, an experiment has been carried out with the diatom *Cylindrotheca closterium*. Cell division rates, photosynthetic efficiencies, pigment contents, and activities of antioxidant enzymes (SOD, APX, MDHAR, GR) were analysed in exponential-phase batch cultures grown at 35 (normal salinity) and 70 PSU (hyperosmotic) in a 12 h:12 h light:dark cycle (268 µmol photons m^{-2} s^{-1} photosynthetically active radiation; PAR) at 15°C. Within the 12 h photoperiod, UVA (2.80 W m^{-2} unweighted) or UVA+UVB (4.23 and 0.22 W m^{-2}, respectively) were supplemented during 4 h. Separately as well as in combination, UVB (3.45 kJ m^{-2} d^{-1}) and salt stress (70 PSU) caused a decrease in division rates. This UVB dose caused a decrease in photosynthetic efficiency, whereas salt stress did not. Cell volumes of UVB-exposed *C. closterium* increased only at 70 PSU. UVB radiation and salt stress caused significant decreases in chl *a*, chl *c* and fucoxanthin contents, but the UVB effect was stronger than the salt effect. The relatively high β-carotene:chl *a* ratios in UVB-exposed and salt-stressed cells might indicate that β-carotene was used in scavenging singlet oxygen. In high light with UVA (at 35 PSU), SOD, APX, MDHAR and GR activities were not higher than in low light (27 µmol photons m^{-2} s^{-1}). Separately and in combination, UVB and salt stress enhanced SOD activity in *C. closterium*, whereas APX was stimulated by UVB only. MDHAR was stimulated under UVB and salt stress, but there were no effects on GR activity. Under ambient salt and UV conditions during emersion, oxidative stress may contribute to an inhibition of growth of *C. closterium*.

KEY WORDS: Antioxidants · *Cylindrotheca closterium* · Diatom · Pigments · Salt stress · Ultraviolet

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INTRODUCTION

Benthic diatoms, usually the major group of phototrophic microorganisms on tidal flats, may contribute up to ~50% of total primary production per unit surface area in coastal waters, and achieve the highest production rates during emersion (Underwood & Kromkamp 1999). During emersion, benthic diatoms have to be protected against photodamage and desiccation caused by high sunlight intensities and evaporation. Intertidal benthic diatom communities are mainly composed of multi-layered biofilms of epipelic species, which may perform some vertical migration that limits the UV exposure time of the uppermost cells and offers photoprotection by self-shading (Underwood & Kromkamp 1999). An effective photoprotection by UV-absorbing and xanthophyll-cycle pigments (Demers et al. 1991, Roy 2000) was not observed in benthic diatoms (Underwood et al. 1999, Wulff et al. 1999). Exposure to ambient UVB does not inhibit the primary production of most benthic diatoms (Peletier et al. 1996, Underwood et al. 1999, Wulff et al. 1999). However, the group of semi-planktonic benthic diatoms, which are partially resuspended during high tides and deposited on tidal

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flats during low tides, may be more UV-sensitive; such examples include the benthic diatoms *Nitzschia* and *Synedra* (Francoeur & Lowe 1998, Wulff et al. 2000). The experiment in this paper will focus on a representative of these relatively UV-sensitive diatoms, *Cylindrotheca closterium*, in particular as regards its defence against UV- and salt-induced production of active oxygen species. UV-induced production of active oxygen species via photochemical reactions in the aqueous environment (Zhou & Mopper 1990, Scully et al. 1996), which increases the antioxidative defence in intertidal animals (Buchner et al. 1996, Abele-Oeschger et al. 1997), suggests that such photoreactions may also be a cause of oxidative stress in benthic diatoms during tidal emersion. UV radiation also stimulates the intracellular active oxygen production in plants and algae (Foyer et al. 1994, Mallick & Mohn 2000, Vincent & Neale 2000). Hyperosmotic conditions (salt and drought stress) also stimulate intracellular active oxygen production, triggering antioxidative defences in plants (Navari-Izzo et al. 1997, Hoekstra et al. 2001, Rout & Shaw 2001). So far, these oxidative desiccation effects have not been studied in diatoms. Photosynthesis and growth in emersed benthic diatom communities may be inhibited if the salinity of the adhering water exceeds 50 PSU as a result of wind-induced evaporation (Admiraal 1977, Visscher & Van Gemerden 1991, Van Bergeijk 2000), although benthic diatoms may be protected against desiccation by the polymeric substances (EPS) they excrete (Decho 1990).

As regards benthic diatoms, no studies have been reported on (1) combined effects of UV and high salinity on benthic diatoms, and (2) their enzymatic antioxidative defence in general. To get a first insight into the effect of emersion conditions, an experiment was carried out with a relatively sensitive semi-planktonic benthic species, *Cylindrotheca closterium*. This species has been grown at a high photosynthetically active radiation (PAR) intensity, optionally excluding and including UVB, and at salinities of 35 and 70 PSU (4 combinations). Under low-light conditions the production rate of active oxygen species, and thus activities of antioxidative defence enzymes, are low. Therefore, in order to measure 'control values' of antioxidative enzyme activities, a fifth series was included in which *C. closterium* was grown in dim light (low PAR). Cell division rates, photosynthetic efficiencies, pigment contents, and activities of the antioxidative key enzymes, superoxide dismutase (SOD), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR), were measured under the 5 experimental conditions. The hypothesis to be tested was that realistic (ambient) levels of UVB radiation and hypersalinity inhibit cell growth and stimulate the antioxidative defence in *C. closterium*.

MATERIALS AND METHODS

Culture conditions and irradiance. Cells were grown in batch cultures, at low light intensity and a salinity of 35 PSU (encoded L35), and at high light (H) at 35 PSU with a 4 h supplement of UVA (encoded A35) or a supplement of UVA+UVB (encoded B35), or at 70 PSU with a 4 h supplement of UVA (encoded A70) or a 4 h supplement of UVA+UVB (encoded B70). Batch cultures of *Cylindrotheca closterium* (Ehrenberg) were grown axenically at 15°C in glass Petri dishes (18 cm diameter) with 500 ml artificial medium. The composition and the preparation of the marine diatom medium (35 PSU) are described in De Brouwer et al. (2002). In the 70 PSU medium, concentrations of the major salts (NaCl, Na_2SO_4, $MgCl_2$, KCl, $CaCl_2$) were doubled.

Cylindrotheca closterium was grown in a 12 h:12 h light:dark cycle (LDC; photoperiod 08:00 to 20:00 h) at low (L) and high (H) light intensities. Spectra from 280 to 700 nm were scanned with a MACAM SR-9910 spectroradiometer at 1 nm intervals. PAR intensities (400 to 700 nm) are given as photon irradiance (Fig. 1a). Under low-light the cultures were illumi-

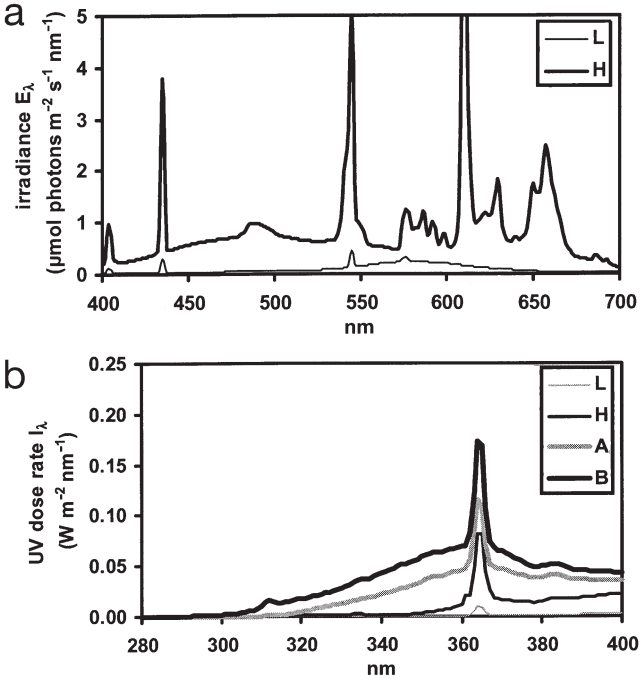

Fig. 1. Spectra of artificial light supplied to *Cylindrotheca closterium*, scanned with a MACAM SR-9910 spectroradiometer at 1 nm wavelength (λ) intervals: (a) PAR (400 to 700 nm), as photon irradiance (E_λ) in µmol photons m^{-2} s^{-1} nm^{-1} vs λ, under low (L) and high (H) light conditions (08:00 to 20:00 h); (b) dose rates of UV (280 to 400 nm) in W m^{-2} nm^{-1} vs λ, for low-light spectrum L (08:00 to 20:00 h) and high-light spectra H (08:00 to 10:00 h and 14:00 to 20:00 h), A (10:00 to 14:00 h) and B (10:00 to 14:00 h); see Table 1 for details

nated at 27 µmol photons m^{-2} s^{-1} (400 to 700 nm) by Philips TL18W33 white fluorescent lamps. To simulate an 'emersion' light spectrum with UV and PAR (with sufficient UVA, blue and red-orange light), illumination was provided by 36 W fluorescent lamps (Activa 172 and Grolux, Sylvania) at 268 µmol photons m^{-2} s^{-1} PAR (08:00 to 20:00 h). In this high-light mode, UV was supplemented during 4 h (10:00 to 14:00 h) by 40 W fluorescent lamps (4 × UVA Sylvania Black Light and 1 × UVB Philips TL12) at 2 levels: (a) basic Emersion Condition A, where UVB was blocked by Mylar® sheet on the glass covers of the dishes; (b) Emersion Condition B with UVB, where the glass covers blocked UVC (and UVB below 290 nm). The duration of the UV exposure was limited to 4 h by turning off the UV lamps at 14:00 h until the next morning at 10:00 h in order to allow a repair of UV damage. Fig. 1b shows the UV spectra (MACAM SR-9910) under low light (L; 08:00 to 20:00 h) and high light (H; 08:00 to 10:00 h and 14:00 to 20:00 h), respectively, as well as those applied during the 4 h UV exposure under conditions A and B (10:00 to 14:00 h). Table 1 gives the unweighted dose rates of UVB and UVA, and Biologically Effective UV-Dose Rates (BEDRs) derived from different biological weighting functions (BWF); these assign a relative weight (W_λ) to the UV radiation (I_λ) at each wavelength (λ), assuming that $W_{\lambda=300} = 1$. Effective UV-dose rates were calculated as $BEDR = {}^{\lambda=280-400}\Sigma(W_\lambda \times I_\lambda)$, in W m^{-2}. $BEDR_{PLA}$ uses a BWF designed for 'whole-plant' damage that attributes effects to UVB only (Caldwell 1971). $BEDR_{DNA}$ uses a BWF that weighs the UVB-damage to DNA (Setlow 1974). $BEDR_{DIA}$ uses a BWF derived from models for UV-induced inhibition of photosynthesis in the diatom *Phaeodactylum* (Cullen et al. 1992, Neale 2000). $BEDR_{CHL}$ uses a BWF weighing the inhibition of photosynthesis in chloroplasts, that attributes more relative weight to UVA (Jones & Kok 1966). Table 1 also gives daily unweighted UVB doses, as well as weighted UV doses (kJ m^{-2} d^{-1}) for each BWF.

Division rates, cell volume, photosynthetic efficiency and pigments. Cells of *Cylindrotheca closterium* were resuspended from the bottom of the cul-

Table 1. Experimental UV spectra. (a) unweighted UVB (280 to 320 nm) and UVA (321 to 400 nm), and biologically effective dose rates (BEDR) using UV-weighting functions (BWF) of: [a]Caldwell (1971); [b]Setlow (1974); [c]Cullen et al. (1992); and [d]Jones & Kok (1966); (b) cumulative daily doses of unweighted UVB, and weighted biologically effective doses (BED) under experimental conditions L, H, A and B: conditions L and H were in low and high light intensities respectively; in condition A UVB was blocked; and in condition B UVC was blocked (and UVB below 290 nm)

(a) UV dose rates per spectrum (see Fig. 1b)

Light spectrum	L	H	A	B
Unweighted UV	W m^{-2}			
UVB	0.00	0.01	0.03	0.22
UVA	0.10	1.04	2.80	4.23
Total UV	0.10	1.05	2.83	4.45
Weighted UV	W m^{-2} (280–400 nm)			
$BEDR_{PLA}$[a]	0.00	0.00	0.00	0.06
$BEDR_{DNA}$[b]	0.00	0.00	0.03	0.09
$BEDR_{DIA}$[c]	0.00	0.03	0.11	0.27
$BEDR_{CHL}$[d]	0.02	0.22	0.67	1.26

(b) Daily UV doses

Experimental condition	L	A	B
Time of exposure	12 h spectrum L (08:00–20:00 h)	8 h spectrum H (08:00–10:00 h and 14:00–20:00 h); 4 h spectrum A (10:00–14:00 h)	8 h spectrum H (08:00–10:00 h and 14:00–20:00 h); 4 h spectrum B (10:00–14:00 h)
Unweighted UVB dose	kJ m^{-2} d^{-1} (280–320 nm)		
	0.10	0.66	3.45
Weighted UV dose	kJ m^{-2} d^{-1} (280–400 nm)		
BED_{PLA}[a]	0.01	0.06	0.84
BED_{DNA}[b]	0.00	0.05	1.27
BED_{DIA}[c]	0.12	2.42	4.75
BED_{CHL}[d]	0.86	16.0	24.3

ture dishes with a stirring bar before counting, and cells were counted at regular intervals during exponential growth (Coulter Counter® Multisizer II; 100 µm pore tube; 500 µl sample size). Division rates (div. d^{-1}) were calculated as: $1/\Delta t \times {}^2\log(N_t/N_{t-\Delta t})$, where Δt is the time interval (d) at which cells were counted, N_t is the cell number at time t (cells ml^{-1}), and $N_{t-\Delta t}$ is the number of the previous count (at $t-\Delta t$). Lengths (l) and widths (w) of 50 cells $sample^{-1}$ were measured with a microscope (Olympus A031, 40× objective), a video camera (JVC) attached to a computer with a frame grabber, and the image analysis package Qwin (Leica). As, by approximation, the shape of *C. closterium* is a double cone, cell volumes (μm^3) were calculated as: $l/3 \times \pi \times (w/2)^2$. Photosynthetic efficiencies (Fv/Fm)[1], i.e. maximum PSII quantum yields (Schreiber et al. 1986), were measured with a MiniPAM Photosynthesis Yield Analyzer (H. Walz) in 30 min dark-adapted cell cultures, previously exposed to the different spectra. For pigment analysis, cultures were sampled (20 ml), collected onto cellulose nitrate filters (pore size 0.22 µm), flash-frozen in liquid nitrogen, and stored at –80°C. Pigment extracts (in 90%-methanol/ 0.5 M ammonium acetate) were separated on a reversed-phase column (Nova-pak C_{18}, 4 µm, 15 cm; Waters Milennium HPLC system). Gradient-mixing pumps delivered 3 mobile-phase solvents: methanol/ammonium acetate, 90% acetonitril and 100% ethyl acetate (Wright et al. 1991, Kraay et al. 1992). Peaks were identified on the basis of retention times and absorption characteristics (Waters 996 photodiode array detector; Milennium 2.15 software) using commercially available standards (VKI Hørsholm) and absorption data (Jeffrey et al. 1997). Using the values of the standards, peak areas of chl *a*, chl *c*, fucoxanthin, diadinoxanthin and β-carotene were converted to pigment contents normalised to biovolume (mg m^{-3} cell volume), and to chl *a* (mg pigment mg^{-1} chl *a*).

Determination of activities of SOD, APX, MDHAR and GR. Cell suspensions (50 ml) of *Cylindrotheca closterium* grown under experimental conditions (L35, A35, A70, B35, B70) were sampled, filtered onto 0.45 µm cellulose acetate filters, and frozen and stored in liquid nitrogen until analysis. Cells were scraped off the filter after thawing, transferred into an extraction buffer, and sonicated (Soniprep 150 MSE; 3 min; amplitude 14 µm; on ice). Homogenates were centrifuged (20 min; $7000 \times g$; 4°C) and the supernatants (extracts) were kept on ice, prior to activity measurements. A subsample of extract (50 µl) was stored (–20°C) for protein analysis, as activities of SOD, APX, MDHAR and GR were normalised to protein. Protein concentrations (Pr) were measured spectrophotometrically (Bradford 1976, Rijstenbil 2002). SOD activity measurements were based on the principle that superoxide anions ($O_2^{\bullet -}$) generated by the xanthine-xanthine oxidase system reduce cytochrome c (cyt *c*), and that SOD inhibits cyt *c* reduction (McCord & Fridovich 1969). Specific SOD activities were measured in enzyme units (U mg $protein^{-1}$), as described in Rijstenbil (2002). APX activity measurements were based on the decrease in the absorption of ascorbate at 290 nm (Mittler & Zilinskas 1991, Rao et al. 1996, Weckx & Clijsters 1996). GR activity measurements were based on the spectrophotometrically determined oxidation rate of NADPH (Rao et al. 1996, Weckx & Clijsters 1996). The details of the assays for SOD, APX and GR activities are described in Rijstenbil (2002). MDHAR activity measurements were based on the conversion of ascorbate with ascorbate oxidase that renders the MDHA radical, and reduction of MDHA to ascorbate catalysed by MDHAR, whereby the conversion from NADPH to $NADP^+$ is monitored spectrophotometically (Jahnke et al. 1991). The NADPH absorption decrease rates were read during 3 min, at 25°C and λ = 340 nm (specific NADPH absorbance, ε = 6.22 mM^{-1} cm^{-1}) in a 1 cm quartz cuvette (µmol NADPH [ml extract]$^{-1}$ min^{-1}). Specific activities of APX, MDHAR and GR, normalised to protein (Rijstenbil 2002), were presented as enzyme units, U mg $protein^{-1}$ defined, respectively, as µmol ascorbate mg $protein^{-1}$ min^{-1} (APX) or as µmol NADPH mg $protein^{-1}$ min^{-1} (MDHAR, GR).

Sampling scheme. Analyses were performed on exponential-phase *Cylindrotheca closterium* grown under 5 conditions: L35, A35, A70, B35, and B70, in replicate batch cultures. Under each condition, division rates (n = 4) and cell volumes (n = 6) were measured. Cultures were sampled when the actively dividing, exponential-phase cells reached densities that were sufficiently high for reproducible measurements of Fv/Fm, pigments and enzyme activities (~5×10^5 cells ml^{-1}). Fv/Fm was measured in 30 min dark-adapted cultures sampled from series L35, A35, B35, A70 and B70, at 10:00 h (0 min exposure) and in samples pre-exposed during 30, 60, 120, 180 and 240 min to the corresponding light spectra (L, A, B). Fv/Fm data were averages of 4 PAM-readings (per series, per sampling time), in duplicate cultures (n = 2). Samples were taken at 14:00 h from replicate batch cultures grown under the experimental conditions (L35, A35, B35, A70, B70) for pigment analysis (n = 2), and measurements of the activities of SOD, APX, MDHAR, and GR (n = 5). For all measured parameters, effects of the experimental light and salinity treatments were analysed with Statistica 6.0® (StatSoft). Effects of high light vs low light (A35 vs L35) were tested with 1-factorial ANOVAs. Combined

[1]Fv/Fm = (Fm – Fo)/Fm, where Fm is the maximum cell fluorescence after a saturating light pulse, and Fo is the basal fluorescence of the dark-adapted cells

UV and salt effects (comparing series A35, B35, A70, B70) were analysed with 2-factorial ANOVAs, followed by post-hoc Tukey tests; p-values of <0.05 were accepted as representing significant differences.

RESULTS

Growth, photosynthetic effciency and pigments

Compared to *in situ* emersion conditions, where maximum light intensities may exceed 1000 µmol photons m^{-2} s^{-1}, PAR provided by the fluorescent lamps in Series A and B was rather low. On sunny days, solar UVB radiation is ~0.2 to 0.4% of PAR (µmol photons m^{-2} s^{-1}). In Series B, a UVB:UVA:PAR ratio of 0.2:4.7:100 was obtained through the glass covers on the dishes, at a dose of 3.45 kJ m^{-2} d^{-1} (Table 1). The diatoms did not grow when UVB was provided during the entire photoperiod, in either a 4 h:20 h LDC or a 12 h:12 h LDC. UVB exposure was therefore limited to 4 h Spectrum B and 8 h Spectrum H in a 12 h:12 h LDC (Fig. 1, Table 1). Growth of *Cylindrotheca closterium* was observed from the day of inoculation until the final day of sampling in the exponential phase (~2 wk). At 35 PSU (L35, A35, B35) the duration of the lag phase was 3 d, whereas at 70 PSU (A70, B70) its duration was 5 d. Under Condition A35 (Fig. 2a) division rates were not different from the rates of 0.48 (±0.04) div. d^{-1} that were additionally measured in cultures grown without UV lamps (Spectrum H: Fig. 1b) from 08:00 to 20:00 h (ANOVA: n = 4; p > 0.05 not significant [ns]). Thus, since UVA had no adverse effect on growth, Series A35 served as the 'reference' for Series A70, B35, and B70. A 2-factorial ANOVA (n = 4; factors UVB and salinity) on division rates in Series A35, B35, A70 and B70 showed a negative effect of UVB (p < 0.001) and high salinity (p < 0.0001); as revealed by the post-hoc test, this was only due to differences between the high division rates in Series A35 compared to those in B35, A70 and B70 (Fig. 2a). Cell volumes (Fig. 2b) did not differ between conditions L35, A35, B35, and A70 (p > 0.05 ns), but volumes of UVB-exposed cells grown at 70 PSU (B70) were 43% larger compared to cells grown at 35 PSU (p < 0.05).

At the start of the photoperiod, photosynthetic efficiency of 30 min dark-adapted cells (Fv/Fm) was 0.70 in low light (L35), and decreased to 0.67 after 4 h (Fig. 3). UVA caused a minor decrease of Fv/Fm (~0.02) after 4 h (A35, A70), and the next morning at 10:00 h,

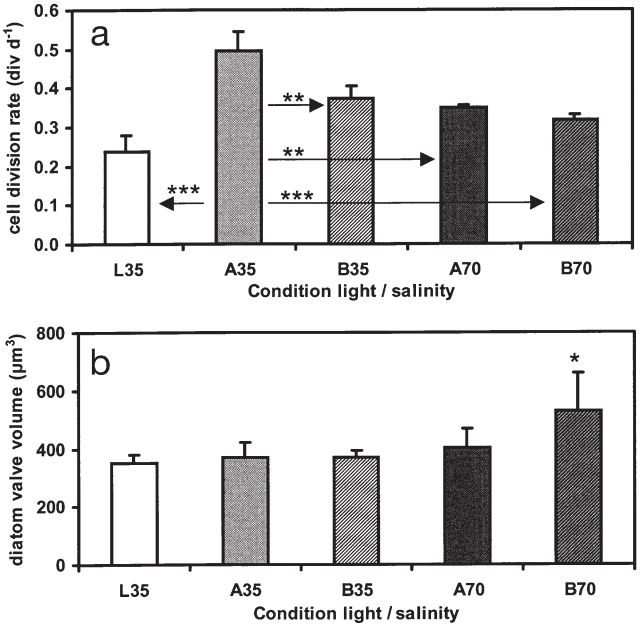

Fig. 2. *Cylindrotheca closterium*. Growth and cell size vs experimental light and salinity conditions: (a) division rates (div. d^{-1}) of exponential-phase cells (means + SD; n = 4) grown at 35 PSU in low light (L35) and in high light with 4 h UVA (A35), or 4 h UVA+UVB (B35), and at 70 PSU in high light with 4 h UVA (A70), and 4 h UVA+UVB (B70); (b) volumes of exponential-phase cells (means + SD; n = 6) grown under conditions L35, A35, B35, A70 and B70. Levels of significant differences are indicated in the graphs as: *p < 0.05; **p < 0.01; ***p < 0.001

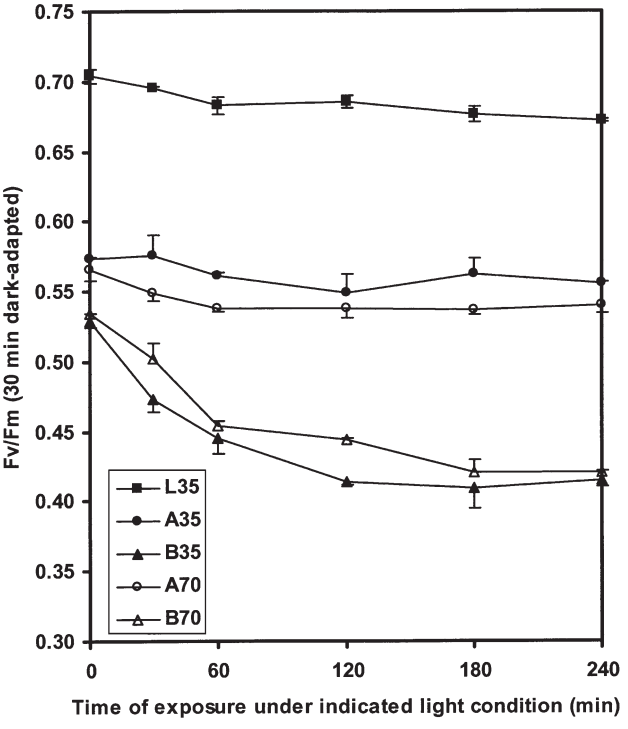

Fig. 3. *Cylindrotheca closterium* grown at a salinity of 35 and 70 PSU: change of the maximum PSII efficiency (Fv/Fm) of 30 min dark-adapted cells with the time of pre-exposure (min) to the spectral conditions (L, A, B; see Table 1) under which the exponential phase cultures have been grown. For explanations of experimental conditions, see Fig. 2

Fv/Fm had recovered to its initial value (0.58; not shown). UVB (B35, B70) caused a large decrease of Fv/Fm from 0.54 at 10:00 h, to 0.42 at 14:00 h ($p < 0.00001$); recovery of Fv/Fm to its initial value (0.55) the next morning at 10:00 h (not shown) suggests that UVB-damage had been repaired overnight. The 'high-salinity' effects on Fv/Fm were negligible under conditions A and B ($p > 0.05$ ns).

Data of pigment contents and pigment:chl *a* ratios were presented in Table 2, with the significant differences between series indicated. As expected, chl *a* contents of shade-adapted, low-light grown cells (L35) were higher than those of high-light grown cells (A35), as was also the case with chl *c*, fucoxanthin and β-carotene contents. A 2-factorial ANOVA (n = 2) on Series A35, B35, A70 and B70 indicated that the factors 'UVB' ($p < 0.0001$) and 'high salinity' ($p < 0.01$) caused a loss of chl *a*. A similar loss was found in chl *c* contents, with an effect of both UVB ($p < 0.001$) and high salinity ($p < 0.01$). A minor decrease of 'chl *c*:chl *a*' was caused by UVB ($p < 0.05$). Both UVB ($p < 0.01$) and high salinity ($p < 0.05$) caused a decrease of fucoxanthin contents, but effects on 'fucoxanthin:chl *a*' were not significant ($p > 0.05$ ns). The diadinoxanthin-diatoxanthin (xanthophyll) cycle in diatoms protects the photosystems against high light, via heat dissipation of photon energy that downregulates the photosynthetic electron flow. Compared to low-light condition L35, diadinoxanthin (DD) contents were far higher under 'UVA' conditions A35 and A70. However, since diatoxanthin (DT) could not be quantified, the effects of high light, UV and salinity on the DD-DT cycle could not be measured. A 2-factorial ANOVA indicated that diadinoxanthin contents were lower in UVB-exposed cells ($p < 0.05$), whereas salinity had no effect. 'DD:chl *a*' was significanty higher under high light (A35) compared to low light (L35). Neither UVB nor high salinity had any effect on 'DD:chl *a*' ($p > 0.05$ ns). Two-factorial ANOVAs (A35, A70, B35, B70) indicated that only UVB caused a relative increase of 'β-carotene:chl *a*' ($p < 0.05$); however absolute β-carotene contents were lower in UVB-exposed diatoms ($p < 0.01$). There was no effect of high salinity ($p > 0.05$ ns) on either β-carotene contents or 'β-carotene:chl *a*'.

Table 2. Pigments in duplicate samples (±SD) of *Cylindrotheca closterium* (exponential phase) under conditions L35, A35, B35, A70 and B70 (see 'Culture conditions and irradiance' for details): (a) contents per cm^3 biovolume; (b) pigment:chl *a* ratios. Fuc; fucoxanthin; DD: diadinoxanthin; β-car: β-carotene. Significant differences between parameter values in the different series have been indicated: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$

Condition	L35	A35	B35	A70	B70	Remarks
(a) Content			mg pigment per cm^3 cell volume			
Chl *a*	2.71 (±0.12)	1.64 (±0.02)	0.92 (±0.03)	1.29 (±0.01)	0.61 (±0.07)	L35>A35*; A35>B35***; A35>A70**; A35>B70****; B35>B70**; A70>B70***
Chl *c*	0.269 (±0.009)	0.182 (±0.006)	0.093 (±0.004)	0.137 (±0.010)	0.047 (±0.004)	L35>A35*; A35>B35**; A35>A70**; B35>B70*; A70>B70**; A35>B70**
Fuc	1.06 (±0.06)	0.65 (±0.01)	0.34 (±0.01)	0.53 (±0.06)	0.22 (±0.02)	L35>A35*; A35>B35**; A35>B70**; A70>B70**
DD	0.106 (±0.002)	0.240 (±0.014)	0.095 (±0.004)	0.224 (±0.060)	0.119 (±0.009)	L35<A35*
β-car	0.0185 (±0.0011)	0.0116 (±0.0016)	0.0080 (±0.0005)	0.0100 (±0.0009)	0.0063 (±0.0005)	L35>A35*; A35>B35*; A35>B70*; B35>B70*
(b) Ratio			mg pigment per mg chl *a*			
Chl *c*:chl *a*	0.111 (±0.001)	0.110 (±0.005)	0.102 (±0.009)	0.106 (±0.008)	0.078 (±0.002)	A35>B70*; A70>B70*
Fuc:chl *a*	0.437 (±0.012)	0.396 (±0.010)	0.373 (±0.004)	0.409 (±0.047)	0.357 (±0.010)	
DD:chl *a*	0.046 (±0.001)	0.146 (±0.011)	0.104 (±0.002)	0.172 (±0.047)	0.195 (±0.006)	L35<A35*
β-car:chl *a*	0.0076 (±0.0002)	0.0071 (±0.0002)	0.0087 (±0.0003)	0.0077 (±0.0007)	0.0104 (±0.0003)	A35>B70*; A70>B70*

Responses of the antioxidative defence

Activities of the antioxidative defence enzymes, and significant differences between series, are shown in Fig. 4. Differences in SOD activities (Fig. 4a) between low-light (control) condition L35 and high-light condition A35 (reference) were not significant (ANOVA, n = 5; $p > 0.05$ ns). A 2-factorial ANOVA on SOD activities (n = 5; A35, B35, A70, B70) showed a stimulation caused by UVB and high salinity, where the salinity effect was stronger ($p < 0.00001$) than the UVB effect ($p < 0.001$). APX activity (Fig. 4b) in the high-light reference series A35 was not different ($p > 0.05$ ns) from the control, grown under low light (L35). A 2-factorial ANOVA (n = 5; A35, A70, B35, B70) on APX activities showed no effect of salinity ($p > 0.05$ ns), but a stimulation by UVB ($p < 0.01$). Using the data from the replicated enzyme measurements (n = 5) under all conditions (L35, A35, B35, A70, B70), a positive correlation was found between APX vs SOD (n = 25; $R^2 = 0.40$; $p < 0.01$): APX activity was enhanced by the SOD-catalysed production of H_2O_2. A positive correlation also existed between MDHAR vs APX (n = 25; $R^2 = 0.58$; $p < 0.00001$). As APX activity increases, more ascorbate will be converted to monodehydroascorbate (MDHA), the substrate of MDHAR (Fig. 4c). In Series L35, A35, B35 and A70, MDHAR activities of *Cylindrotheca closterium* were not different from each other ($p > 0.05$ ns). A 2-factorial ANOVA (n = 5) on MDHAR activities in series A35, B35, A70 and B70 indicated a strong effect of both UVB exposure ($p < 0.00001$) and high salinity ($p < 0.000001$). However, this was merely caused by the high MDHAR activities in the UVB-exposed cells at 70 PSU (B70). MDHA radicals disproportionate non-enzymatically to DHA. During reduction of DHA to ascorbate (Halliwell-Asada cycle) glutathione (GSH) is oxidised to GSSG, and the reduction of GSSG to GSH is catalysed by GR. However, a correlation APX vs GR did not exist, and effects of UVB or high salinity on GR activities (Fig. 4d) were not found ($p > 0.05$ ns). Although UVB and salt stress may inhibit growth through a wide variety of mechanisms, cell energy will be allocated for enzymatic antioxidative defence; this energy investment may account for some reduction of division rates. On the basis of the data of series A35, B35, A70 and B70 (pairing the same 4 replicates in which enzyme activities and division rates were determined), a negative correlation between division rates and SOD activities could be found: (division rate) = 0.481 – 0.0005 × (SOD activity) (n = 16; $R^2 = 0.42$; $p < 0.01$). Antioxidative defence only has an indirect link with growth, since inhibition by oxidative stress may also be a result of oxidative damage caused by active oxygen production, induced either by UVB or salt stress.

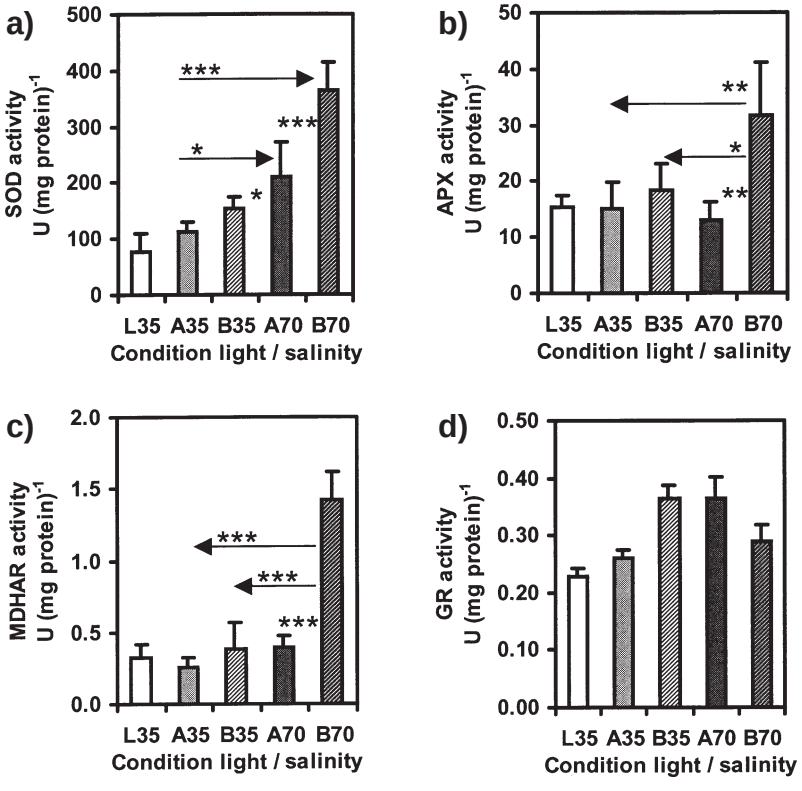

Fig. 4. *Cylindrotheca closterium*. Means (+SD; n = 5) of specific activities of antioxidative enzymes in exponential-phase cells, grown under the conditions L35, A35, B35, A70 and B70, in U mg protein^{-1}: (a) superoxide dismutase (SOD); (b) ascorbate peroxidase (APX), (c) monodehydroascorbate reductase (MDHAR) and (d) glutathione reductase (GR). Levels of significant differences are indicated in the graphs as: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. For explanations of experimental conditions, see Fig. 2

DISCUSSION

In studies addressing the impact of UV on photosynthesis, UV dose rates must be weighted and quantified (Neale 2000), UV:PAR ratios should be realistic, and ideally, artificial PAR spectra should resemble the solar spectrum. PAR is usually provided as cool-white light by fluorescent growth lamps (Lesser et al. 1994, Peletier et al. 1996). As such, PAR spectra deviate from the solar spectrum that diatoms experience during emersion; cool-white lamps were combined in this experiment with lamps that added UVA, blue (315 to

450 nm, UVA lamps), and orange-red light (625 to 675 nm, Grolux). Although the gaps in the UVA-blue and orange-red regions were partly filled up, the experimental spectrum was still irregular (compared with the smooth solar spectrum), which is a characteristic of fluorescent lamps. It should be mentioned that this may have resulted in photosynthetic (and other) responses that would deviate from those obtained under natural solar radiation. In this experiment, effective UV-dose rates and doses were determined (Table 1). Different responses were the result of higher UV:PAR ratios (B35, B70) compared to reference condition A35. An extrapolation of these responses to natural situations should be made with caution, since solar light intensities may be higher during emersion, and the solar spectrum is different from the artificial spectrum. This experiment was conducted under controlled artificial conditions, with the intention to give a first indication of the combined UV and salt effects on antioxidative defence.

Maximum quantum efficiencies of PSII photochemistry (Fv/Fm) of *Cylindrotheca closterium* (Fig. 3) reached a value of ~0.7 in low light (L35). This is about the maximum Fv/Fm for diatoms, and indicates that cells are in optimum physiological condition. Under Condition A, Fv/Fm was ~0.1 lower, which might have been caused by a limited supply of inorganic carbon, since pH values in Series A35 and A70 (8.9 to 9.2) were higher than in Series L35 (8.3 to 8.4). As a result of this, initial (morning) values of Fv/Fm in the separate batch cultures of A35, B35, A70, and B70 were somewhat lower compared to L35. However, initial Fv/Fm values from 0.55 to 0.58 indicate that cells were hardly carbon-limited during exponential growth. The minor decrease of Fv/Fm (0.02 to 0.03) in Series A35 and A70 showed that the effect of UVA was negligible. A steady decrease of Fv/Fm in the time-course measurements (Fig. 3) would indicate reciprocity between UV-dose rate and duration of exposure (Neale 2000), but this did not apply to *C. closterium*. Also in Series B35 and B70, the Fv/Fm values appeared to stabilise within 4 h: a balance between photodamage and repair was established, so that the UV-inhibition was UV-dose rate, rather than UV dose-dependent. A similar loss of reciprocity in UV dose-effect relationships has also been found in the planktonic diatom *Thalassiosira pseudonana* (Cullen & Lesser 1991, Rijstenbil 2002). Cellular responses (including antioxidative defence) measured after 4 h exposure may therefore be considered as an effect of the UV dose rates at the 2 different UV:PAR ratios that were applied. With the UV-inhibition model for photosynthesis (Cullen et al. 1992, Neale 2000), an inhibition factor of E^*_{inh} = 0.12 was derived for spectrum B (Fig. 1b), predicting a value of $(100/[(1 + E^*_{inh})])$, which is 89% of the photosynthetic rate that would have been achieved without UV. The 4 h decrease of Fv/Fm to 78% of its initial value (Fig. 3) suggests that the effect of spectrum B on photosynthesis may have been greater than that predicted from E^*_{inh}. The daily UVB dose of 3.45 kJ m^{-2} d^{-1} (Condition B) fell within the range of ambient doses (2.6 to 11.6 kJ m^{-2} d^{-1}) that inhibited growth of periphytic freshwater *Synedra* spp. (Francoeur & Lowe 1998). At a weighted dose of 1.27 kJ m^{-2} d^{-1} (Table 1, after Setlow 1974), division rates of *C. closterium* in Series B35 were 75% of those in Series A35, and in Series B70 were 81% of those in Series A70 (Fig. 2a). At a dose of ~1.5 kJ m^{-2} d^{-1} (Setlow 1974), growth rates of *Nitzschia* (*C.*) *closterium* started to decrease, and were 0 to 25% of their maximum from 2.3 to 6.8 kJ m^{-2} d^{-1} (Buma et al. 1996, Peletier et al. 1996). Persistently high Fv/Fm values (0.68 to 0.75) in a diatom mat (*Gyrosigma balticum*) exposed to 4–10 kJ m^{-2} d^{-1} unweighted UVB were attributed to photoprotection provided by migration of diatoms into sediment pores (Underwood et al. 1999). During low tides, semi-planktonic benthic diatoms (e.g. *Bacillaria*, *Nitzschia*) are deposited on tidal flats, and although their vertical motility may provide some self-shading, such species (including *C. closterium*) are often exposed to full sunlight. An unweighted dose of 12.6 kJ m^{-2} d^{-1} UVB inhibited the motility of *Nitzschia lineariz* on 'overcast' days (UVB:PAR = 0.45:100, in kJ m^{-2} d^{-1}), but on sunny days (UVB:PAR = 0.11:100) this dose had no effect (Moroz et al. 1999). Hence, it is unlikely that the motility of *C. closterium* was inhibited by a UVB dose of 3.45 kJ m^{-2} d^{-1} (Table 1) at a UVB:PAR ratio of 0.13:100 (kJ m^{-2} d^{-1}).

Combined effects of salinity and UVB on *Cylindrotheca closterium* (*Nitzschia closterium*) were not examined earlier. Compared to some other benthic diatoms, *N. closterium* showed a high UV sensitivity: occurring partly in the water column, this species may not be well adapted to solar radiation when deposited on tidal flats during emersion (Peletier et al. 1996). As a result of wind-induced evaporation, salinities on tidal flats may reach 100 PSU (Visscher & Van Gemerden 1991). At 70 PSU, division rates of *C. closterium* were 70% (Condition A) and 86% (Condition B) of the rates measured at 35 PSU (Fig. 2a). At 55 PSU, growth rates of *C. closterium* were ~75% of the optimum at 22 PSU (Van Bergeijk 2000). Maximum photosynthetic rates (mg O_2 mg chl a^{-1} h^{-1}) of *N. closterium* were at 70 PSU, ~75%, and at 100 PSU, ~50% of those found at ~20 PSU (100%) (Admiraal 1977). UVB exposure may disturb division processes, and thereby cause cell swelling, as in *Phaeodactylum tricornutum* (Behrenfeld et al. 1992). At 35 PSU, volumes of UVB-exposed *C. closterium* were similar to those grown in low light and in high light with UVA, and only at 70 PSU was an increase in volumes (more specifically, in diameters)

of UVB-exposed cells observed (Fig. 2b). Buma et al. (1996) observed an increase in cell volume in UVB-exposed *Cyclotella*, but not in *N. closterium*. It has been reported that cell swelling may be a response to oxidative challenge (Schliess & Haussinger 2002), but at this stage it is not clear whether this 'high-salinity' effect on cell diameters was related to active oxygen production.

Chl *a*, chl *c*, fucoxanthin, diadinoxanthin and β-carotene contents were lower in UVB-exposed than in UVA-exposed cells (Table 2). A decrease in chl *a*, chl *c*, fucoxanthin and diadinoxanthin has been found in diatoms (including *Nitzschia*) after 2 h UVB exposure (Döhler 1998). UVB-induced H_2O_2 production caused a decrease in the transcript of a gene (*Lhcb*) encoding for the light-harvesting complex binding proteins (Mackerness et al. 2001). In UVB-exposed *Cylindrotheca closterium* this may have contributed to an inhibition of pigment synthesis, resulting in the decrease of Fv/Fm. β-carotene contents were lower, but 'β-carotene:chl *a*' ratios were higher in UVB-exposed than in UVA-exposed *C. closterium*. Zudaire & Roy (2001) found a decrease in β,β-carotene contents in the planktonic diatom *Thalassiosira weissflogii* grown in a 10:14 h LDC with an additional daily 4 h UV-dose of 3.45 kJ m^{-2} d^{-1} (BWF of Cullen et al. 1992). Underwood et al. (1999) found an increase in the 'β-carotene:chl *a*' ratio in benthic diatoms (*Gyrosigma balticum*) after a 5 d exposure to 'ambient + 15%' UVB. Since β-carotene is a scavenger of singlet oxygen ($^1O_2$) in light-stressed cells (Malanga et al. 1997, Mallick & Mohn 2000), it may have contributed to the antioxidative defence in *C. closterium*. In cells grown in high light (+UVA), the diadinoxanthin:chl *a* ratio and diadinoxanthin contents were higher than in low-light grown cells. In order to provide high-light protection via the de-epoxidation of diadinoxanthin to diatoxanthin, diatoms increase their pools of diadinoxanthin (Foyer et al. 1994, Zudaire & Roy 2001). Diadinoxanthin contents were much lower in UVB-exposed than in UVA-exposed *C. closterium*; the results were contradictory, in that in UVB-exposed cells diadinoxanthin contents and the diadinoxanthin:chl *a* ratio were higher at 70 PSU than at 35 PSU. Xanthophylls may serve as antioxidants (Mallick & Mohn 2000), whereby active oxygen species cause the epoxidation of diatoxanthin to diadinoxanthin (Lichtenthaler 1998). However, since diatoxanthin was not quantified, there is no evidence that a salt-induced active oxygen production has reversed the xanthophyll cycle in *C. closterium*, and caused a high diadinoxanthin:chl a ratio (B70). An overall 'high salinity' effect on pigment contents, pigment:chl *a* ratios, and Fv/Fm was not found in *C. closterium*.

The 4 h decrease of Fv/Fm at the dose rates indicated in Table 1 (Condition B) has most likely been caused by UV-induced oxidative- or photodamage to chloroplasts, including the destruction of thylakoid membranes, pigments, and other sensitive PSII constituents such as the D1 proteins (Foyer et al. 1994, Hazzard et al. 1997, Landgraf et al. 1997). This type of photodamage is mainly caused in peroxidation reactions by hydroxyl radicals ($^{\bullet}OH$) that are formed in a non-enzymatic reaction of $O_2^{\bullet -}$ with H_2O_2 (Haber-Weiss reaction: e.g. Foyer et al. 1994). A relative increase of the activities of SOD and APX under conditions B35 and B70 (Figs. 4a,b) indicates that UVB stimulated the production as well as the elimination of active oxygen species. A recovery of Fv/Fm to its initial value (0.55) the next morning at 10:00 h indicates that the UVB-induced inhibition of PSII disappeared in the course of the light–dark cycle. For repair of UVB damage to PSII, in particular for new synthesis of D1 proteins, several hours of PAR are usually required (Foyer et al. 1994). The decrease in division rates (Fig. 2a) may be a result of UVB-induced oxidative damage to proteins, DNA and membranes, as well as an investment in energy (ATP) and reductive capacity for enzymatic repair and antioxidative defence (Buma et al. 1995, Ishida et al. 1997, Dawar et al. 1998, Mallick & Mohn 2000). Both in low light and PAR + UVA (A35), SOD activities had low values of ~100 U mg protein^{-1}, but attained values of ~360 U mg protein^{-1} in UVB-exposed cells at high salinity (Fig. 4a). SOD activities in *Cylindrotheca closterium* fell within the range of activities found in *Peridinium gatunense* (Butow et al. 1996: 0 to 500 U mg protein^{-1}), but were higher compared to SOD activities in the UVB-exposed dinoflagellates *Symbiodinium bermudense* and *Prorocentrum micans* (Lesser 1996a,b: 7 to 24 U mg protein^{-1}). APX activities (Fig. 4b) were far above the values found in the species above (Lesser 1996a,b, Butow et al. 1997: 0.2 to 11 U mg protein^{-1}). An increase in activities of APX and MDHAR (Fig. 4b,c) was found only in UVB-exposed cells grown at 70 PSU. MDHAR activities in *C. closterium* (Fig. 4c) were far higher than in *P. gatunense* (Butow et al. 1997: 0 to 0.05 U mg protein^{-1}). GR did not clearly respond to UVB or salinity (Fig. 4d); GR activities were similar to those in *P. gatunense* (Butow et al. 1997).

SOD activity, in particular, was enhanced by high salinity, which is an indication of oxidative stress in the (partially) dehydrated *Cylindrotheca closterium* cells. Cell dehydration causes a loss of ATP synthase and a decrease of ATP, which inhibits ribulose biphosphate synthesis and, thus, also CO_2 assimilation. As in dehydrating plants, CO_2 assimilation rates decrease faster than photosynthetic electron transport rates (Tezara et al. 1999, Nogués & Baker 2000), a smaller amount of electrons are utilised in carbon reduction and an increasing amount of electrons enhance the active

oxygen production at PSI (Mehler reaction: Foyer et al. 1994). Moreover, dehydrating cells may accumulate cellular iron that catalyses active oxygen production in the Fenton reaction, which stimulates the antioxidative defence (Iturbe-Ormaexte et al. 1998). NADPH oxidase and peroxidases, activated by UVB radiation and pathogen infections (causing cell injury), have recently been identified as the major sources of enzyme-mediated $O_2^{\bullet -}$ production in plant cells (Mackerness et al. 2001). Navari-Izzo et al. (1997) also mentioned that mechanical cell injury caused by dehydration may be a trigger for active oxygen production, activating the antioxidative defence. Salt-induced active oxygen species (in particular hydroxyl radicals) damage cell membranes, thereby disturbing osmoregulation (Dionisio-Sese & Tobita 1998); disturbed cellular ion balances in salt-stressed plants (increase of K^+, Mg^{2+} and Ca^{2+}) may stimulate the transcription of antioxidative enzymes, in particular SOD (Rout & Shaw 2001). Some of the above mechanisms may have contributed to an activation of the antioxidative defence in salt-stressed *C. closterium*.

The moderate UVB exposure had a negative effect on photosynthetic efficiency and division rates of *Cylindrotheca closterium*. Pigment contents were lower in UVB-exposed cells, but β-carotene (scavenger of $^1O_2$) increased in proportion to chl *a*. Activities of SOD and APX were stimulated by UVB. Salt stress (hypersaline conditions) had a negative influence on division rates. The highest activities of SOD, APX and MDHAR were found under combined UVB and salt stress. The antioxidative defence of *C. closterium* was stimulated at values of salinity and irradiance, as may be found during emersion.

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