

OXIDATIVE STRESS RESPONSES IN THE MARINE ANTARCTIC DIATOM *CHAETOCEROS BREVIS* (BACILLARIOPHYCEAE) DURING PHOTOACCLIMATION¹

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The enzyme superoxide dismutase (SOD) holds a key position in the microalgal antioxidant network. The present research focused on oxidative stress responses in the Antarctic diatom *Chaetoceros brevis* F. Schütt during transition to excess (including ultraviolet radiation [UVR]) and limiting irradiance conditions. Over a 4 d period, cellular responses of thiobarbituric acid reactive substances (TBARS, a general oxidative stress indicator), SOD activity, photosynthetic and xanthophyll cycle pigments, PSII efficiency, and growth were determined. In addition, oxidative responses were measured during a daily cycle. Changing irradiance conditions significantly affected growth rates of *C. brevis*. PSII efficiency decreased significantly during periodic excess irradiance and increased under low irradiance conditions. Transition to excess irradiance increased the ratio of xanthophyll to light-harvesting pigments, whereas the opposite was observed for cultures transferred to low irradiance. This acclimation process was completed after 2 d in the new irradiance environment. SOD activity increased significantly after the first day regardless of the new irradiance environment but returned to preexposure values on the fourth day. We hypothesize that SOD activity may be temporarily elevated in *C. brevis* after irradiance shifts, thereby reducing oxidative stress when photoacclimation is in progress.

Key index words: Antarctic microalgae; malondialdehyde; oxidative stress; photoacclimation; photosystem II efficiency; superoxide dismutase; UV radiation; xanthophyll cycle

Abbreviations: DD, diadinoxanthin; DES, de-epoxidation state = $DT/(DT + DD)$; DT, diatoxanthin; ETC, electron transport chains; Fuco, fucoxanthin;

H₂O₂, hydrogen peroxide; LHC, light-harvesting complex; O₂, molecular oxygen; O₂^{-•}, superoxide radical; OH[•], hydroxyl radical; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; UVA, ultraviolet A radiation (315–400 nm); UVB, ultraviolet B radiation (280–315 nm); UVR, ultraviolet radiation

Reactive oxygen species (ROS) are produced as by-products of photosynthetic electron transport and metabolism in photosynthetic organisms (Fridovich 1975, Cadenas 1989, Alscher et al. 1997). The chloroplasts with their high concentrations of O₂ and a constant flow of electrons through the photosystems are a principal site for ROS production (Pinto et al. 2003). When the consumption of the reduced equivalents cannot match their production, electron transport chains (ETCs) become overreduced, and electrons leak onto O₂, by which ROS formation is initiated (Mehler 1951, Asada et al. 1974, Gechev et al. 2006). Environmental conditions, such as the presence of UVR (280–400 nm), stimulate radical formation (Hideg and Vass 1996). Reduction of O₂ occurs through a series of univalent electron transfers (Taube 1965). The first one-electron reduction generates the superoxide radical (O₂^{-•}). Addition of a second electron generates H₂O₂, either by a spontaneous or an enzymatic reaction catalyzed by SOD (Gregory and Fridovich 1973a,b, Halliwell and Gutteridge 1984). Both ROSs are moderately harmful, but in the presence of unchelated bivalent cations (i.e., Fe^{2+/3+}, Cu⁺²⁺), O₂^{-•} is able to reduce H₂O₂, thereby generating the highly reactive hydroxyl radical (HO[•]; Halliwell and Gutteridge 1984, Cadenas 1989, Kehler 2000). If the production of HO[•] is not limited, adverse effects can be expected, ranging from the temporary impairment of photosynthesis to viability loss, possibly

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related with membrane damage due to lipid peroxidation (Halliwell 2006, van de Poll et al. 2006).

Marine microalgae experience excess irradiance and oxidative stress conditions particularly near the water surface (Llabrés and Agustí 2006). In addition, they experience large fluctuations in quantity and quality of incoming irradiance due to changes in their position in the water column (Helbling et al. 1994, Neale et al. 1998, 2003). Exposure to UVR causes additional stress and is responsible for significant decreases in the photosynthetic activity of Antarctic microalgae (Smith et al. 1992, Holm-Hansen et al. 1993, Helbling et al. 1994). In Antarctic regions, the diurnal rhythm and the movement through the upper mixed layer are responsible for large variations in irradiance conditions. The presence of melting sea ice in spring and summer may further contribute to these large fluctuations: microalgae may be advected from the low-irradiance under-ice environment to stratified surface waters in the Marginal Ice Zone within a very short period of time (Lancelot et al. 1991).

To counteract or prevent ROS formation during excess in situ irradiance conditions, Antarctic microalgae need flexible antioxidative mechanisms. For example, similar to plants, algae have the ability to tune the energy flow toward the photosystems by adjusting the composition of the light-harvesting and photoprotective pigments (Falkowski and LaRoche 1991, Harris et al. 2005, van de Poll et al. 2005). Additionally, excess excitation energy can be quenched as heat by activating the xanthophyll cycle (Olaizola et al. 1994, Lavaud et al. 2002, van de Poll et al. 2006). When these defense systems are not sufficient, cells can counteract ROS with a sophisticated network of antioxidant enzymes, which scavenge the various ROS intermediates (Foyer et al. 1994, Asada 1999). The enzyme SOD holds a key position in this network because it is able to scavenge the superoxide radical by which proliferation of ROS is prevented. Although photosynthetic responses during irradiance transitions are well investigated in Antarctic microalgae, virtually nothing is known about their antioxidant responses (Schriek 2000, van de Poll et al. 2006). In temperate marine microalgae, SOD activity and pigment composition were determined to vary over the diurnal cycle as well as during prolonged growth in batch culture (Sigaud-Kutner et al. 2002, 2005). Furthermore, SOD activities in temperate microalgae and zooxanthellae fluctuated with season (Butow et al. 1997), salinity (Jahnke and White 2003, Rijstenbil 2003), irradiance (Lesser and Shick 1989a), and UVR (Lesser 1996a, Litchman et al. 2002, Rijstenbil 2002).

The aim of the present work was to investigate antioxidant responses during irradiance transitions in the Antarctic marine diatom *Chaetoceros brevis*. *C. brevis* was precultured at $180 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and

transferred to low and periodic excess irradiance (including UVR). Growth, PSII efficiency, pigment composition, and SOD activity were followed for 4 d after the transition. In addition, the possible variability in these responses during the diurnal cycle was investigated.

MATERIALS AND METHODS

Experimental design. *Chaetoceros brevis* (Center for Culture of Marine Phytoplankton isolate 163) was cultured for 3 weeks in duplicate UV transparent 2.2 L polymethylmethacrylate boxes containing modified f/2 enriched seawater (Veldhuis and Admiraal 1987) of 34.5 PSU at 4°C. The precultures received $180 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR (initial condition) during a 16:8 light:dark (L:D) cycle. To avoid nutrient limitation, medium was replenished every 10 d. The duplicate cultures were split into four UV transparent boxes (making eight boxes in total) and supplied with 2 L fresh medium. After an additional 3 d of growth, duplicate boxes, each originating from one preculture, were transferred to low irradiance (LL, $20 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high irradiance conditions (HL, $1,350 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), or high irradiance with UV (HL + UV, $1,350 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR, $24.2 \text{ W} \cdot \text{m}^{-2}$ ultraviolet A [UVA: 315–400 nm], and $2.1 \text{ W} \cdot \text{m}^{-2}$ ultraviolet B [UVB: 280–315 nm]). Two cultures remained at the original conditions (CTRL). High irradiance exposures (HL and HL + UV) were given for 4 h per day, after which the cultures were transferred back to the CTRL conditions. This treatment was repeated for 4 d. Samples for SOD activity, TBARS, PSII efficiency, growth, and pigment composition were taken from all cultures, directly after the HL and HL + UV exposures. On the fourth day, samples were obtained during the diurnal cycle: before the light period started, before transfer to HL + UV, after 4 h HL or HL + UV, 2 h after termination of the HL or HL + UV exposures, before the dark period, and before the beginning of the next light period. The entire experiment was repeated a few weeks later, giving a total of four replicates for each irradiance condition.

Irradiance. PAR was provided by Biolux fluorescence tubes (Osram, GmbH, Munich, Germany), whereas UVR was provided by four UVA 340 fluorescent tubes (Q-panel, Cleveland, OH, USA). PAR irradiance was measured with a spherical irradiance meter (QSL-100; Biospherical Instruments, San Diego, CA, USA) in air. UVR was measured using a MACAM SR9910 double monochromator scanning spectroradiometer (Macam Photometrics, Livingston, UK) in air. Cultures exposed to excess PAR irradiance (HL) were shielded from the UVR sources by a wooden cover.

Growth. On each experimental day a 2 mL sample from each replicate was fixed with 20 μL 37% formaldehyde (Merck, Darmstadt, Germany). Cell counts were performed with a Coulter XL-MCL flow cytometer (Beckman-Coulter, Miami, FL, USA) as described by van de Poll et al. (2005). Growth rate was determined by measuring the slope of the natural log (Ln) of cell number as a function of time during 4 d of the experiment. Growth rate of the initial culture was determined over four successive days before the start of the experiment.

SOD activity. Samples for SOD (70 mL) activity and TBARS (70 mL) concentration were filtered over 2.0 μm pore-size polycarbonate filters (\varnothing 47 mm; Osmonics Inc., Livermore, CA, USA) and stored at -80°C for 1 month. SOD analyses were done using the Riboflavin/NitroBlue Tetrazolium (RF/NBT) method modified for algal material, as described in Janknegt et al. (2007). Filters were thawed and resuspended in 0.75 mL buffer X containing 50 mM KH_2PO_4 , pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100 (Aldrich, Steinheim, Germany), 2% PVP (Acros, Morris Plains, NJ, USA), and Complete protease inhibitor cocktail (Roche, Mannheim, Germany). Subsequently,

cells were disrupted by sonication (2×30 s, at $A = 30$) at 4°C , after which the extracts were centrifuged for 25 min ($20,000g$) at 4°C . Supernatants were transferred to a clean 1.5 mL Eppendorf tube, after which 150 μL was taken for duplicate protein quantification according to Bradford (1976). SOD activity was then determined using the RF/NBT method (Beauchamp and Fridovich 1971, Fryer et al. 1998). This assay is based on the competition between SOD and NBT for O_2^- generated by the illuminated riboflavin. SOD activity was defined as the amount of sample required to achieve 50% inhibition of the NBT reduction (V_{50}). NBT reduction was measured (in duplicate) in four reaction mixtures, containing 0 (blank, no inhibition), 12.5, 25, and 50 μL extract. The 1.5 mL reaction mixtures contained SOD buffer (50 mM KH_2PO_4 pH 7.8, 0.1 mM EDTA, and 0.25% Triton X-100 [Sigma, Steinheim, Germany]), 2 mM riboflavin (Sigma), and 57 μM NBT (Sigma). Reaction mixtures were incubated in the dark for 30 min at 4°C and subsequently illuminated from above for 15 min by four fluorescence tubes (Philips TLD/18W, 30 cm distance; Philips, Frankfurt, Germany), giving 199 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Afterward, absorbance was measured at 560 nm on a Cary 3E UV/VIS double beam spectrophotometer (Varian, Middelburg, the Netherlands). Fifty percent inhibition (V_{50}) was calculated by regression using the linear part of a natural semi-log curve. The specific activity of SOD (SA) was calculated according to $1/(V_{50} \cdot [\text{protein}])$.

Pigments. Samples for pigment analysis were filtered over GF/F filters (Ø 25 mm) and stored at -80°C for 1 month. Pigments were analyzed as described by Van Leeuwe et al. (2006). In short, filters were handled under dim light and freeze-dried (48 h), after which pigments were extracted in 4 mL 90% cold acetone (v/v, 48 h, 5°C). Pigments were resolved using HPLC (Waters 2690 separation module; 996 photodiode array detector; Milford, MA, USA) with a C_{18} 5 μm DeltaPak reversed-phase column (Milford, MA, USA) and identified by retention time and diode array spectroscopy. Quantification was performed using standard dilutions of chl a , e_2 , fucoxanthin, diadinoxanthin, diatoxanthin, and β carotene. Cell counts were made using a Coulter XL-MCL flow cytometer (Beckman Coulter, Miami, FL, USA) as described by van de Poll et al. (2005) for calculation of cellular pigment concentrations.

Determination of TBARS. TBARS comprise a rough estimate of the presence of aldehydes, yet most reactivity originates from malondialdehyde (MDA), a product of ROS-induced lipid peroxidation. TBARS analyses were performed after Heath and Parker (1968): filters were resuspended in 0.8 mL MilliQ water and 0.8 mL thiobarbituric acid (0.5% w/v, Sigma) in 20% trichloroacetic acid (TCA). The mixture was heated for 30 min at 90°C and immediately put on ice. After filter removal, the supernatant was centrifuged (10 min, $20,000g$). TBARS were detected by subtracting the nonspecific turbidity measured at 600 nm from the TBARS absorption maximum at 532 nm using a Cary 3E double beam UV/VIS spectrophotometer (Varian, Middelburg, the Netherlands). Extraction solvent was used as the blank.

PSII efficiency. PSII efficiency was measured each day after HL and HL + UV exposures and on the last day during the diurnal cycle. Samples for PSII efficiency were filtered over GF/F filters (Ø 11.3 mm; Whatman, Maidstone, UK). Filtration was stopped before the filter dried, after which the filter was transferred to a temperature-controlled (4°C) seawater-filled cuvette. Chl fluorescence was measured with a PAM 2000 pulse amplitude modulated fluorometer (Walz, Effeltrich, Germany). Maximum quantum yield (F_v/F_m) was determined after 5 min of dark acclimation.

Statistical analyses. Differences in growth rates were analyzed using one-way analysis of variance (ANOVA). Other differences were tested for significance with a repeated measures ANOVA (RM ANOVA). Significant differences were

further analyzed using an LSD post hoc test. Tests were performed using STATISTICA 7 for Windows (Tulsa, OK, USA).

RESULTS

Growth. Under the preexperimental conditions, where irradiance was the same as the experimental control, *C. brevis* grew exponentially at an average rate of $0.45 \text{ div} \cdot \text{d}^{-1}$ (Fig. 1). After transfer to the experimental irradiance conditions, all cultures continued to grow exponentially. The control (CTRL) cultures grew fastest ($0.42 \text{ div} \cdot \text{d}^{-1}$), followed by HL ($0.34 \text{ div} \cdot \text{d}^{-1}$), HL + UV ($0.28 \text{ div} \cdot \text{d}^{-1}$), and LL ($0.12 \text{ div} \cdot \text{d}^{-1}$). The growth rates of the control and preexperimental cultures were not significantly different, while the growth rates of all experimental treatments were significantly different from each other (one-way ANOVA, $P < 0.0005$).

PSII efficiency. At the start of the experiment ($T = 0$), the maximum quantum yield (F_v/F_m) of the initial cultures was on average $0.411 (\pm 0.012 \text{ SD})$; Fig. 2a). During the following 4 d, the F_v/F_m of the CTRL cultures remained on the same level (slope -0.0007 ; RM ANOVA, $P > 0.07$). The diurnal rhythm of the CTRL condition showed a slight but significant decrease during the light period (RM ANOVA, $P < 0.0005$), followed by a significant increase during the dark period (RM ANOVA, $P < 0.0005$; Fig. 2b). The highest maximum quantum yield was measured under the LL condition. The F_v/F_m of LL increased significantly (RM ANOVA, $P < 0.0005$) during the course of the experiment and leveled off at ~ 0.7 (Fig. 2a). No significant changes were observed for LL during the

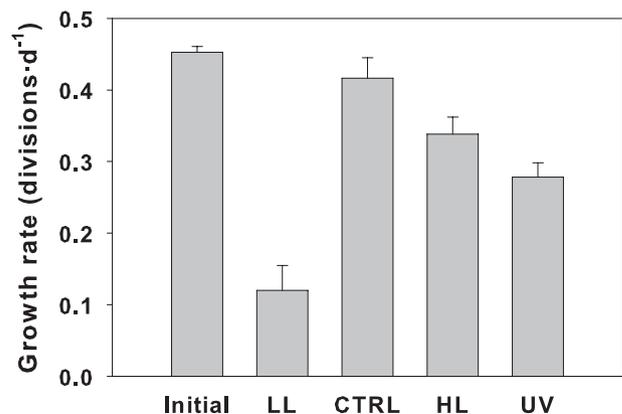


FIG. 1. Growth rates of *Chaetoceros brevis* cultured at 180 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR (initial) before and after transition to low irradiance PAR (LL, 20 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (HL, 1,350 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance (UV, 1,350 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ + 24 $\text{W} \cdot \text{m}^{-2}$ UVA and 2.1 $\text{W} \cdot \text{m}^{-2}$ UVB). The control cultures (CTRL) were kept at the same irradiance condition as the initial culture. Bars show mean growth rates of four replicates; error bars indicate standard deviations. a: significant differences between all other irradiance conditions; b: differs significantly from LL, HL, and UV ($P < 0.0005$).

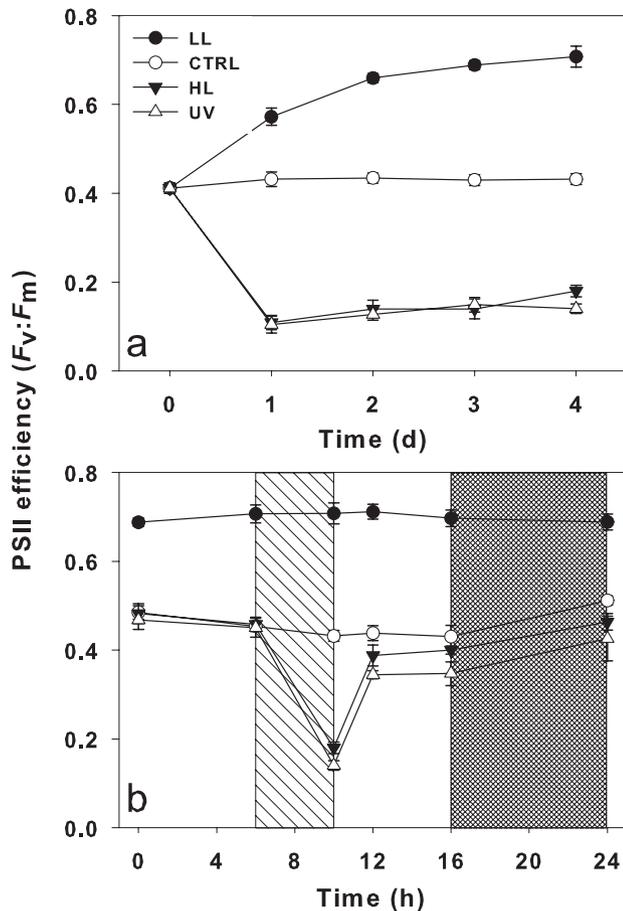


FIG. 2. PSII efficiency before (initial, $180 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) and after a shift to low PAR irradiance (LL, $20 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (HL, $1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance ($1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} + 24 \text{ W} \cdot \text{m}^{-2}$ UVA and $2.1 \text{ W} \cdot \text{m}^{-2}$ UVB). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. (a) During 4 d of photoacclimation. (b) During the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to the new irradiance environment (light dashed area) at $t = 6$ h, and returned to CTRL conditions at $t = 10$ h. The dark dashed area ($t = 16$ until $t = 24$) represents the night period. The daily data points of all cultures were measured at $t = 10$ h. Error bars represent standard deviations.

diurnal cycle. Lowest values were measured directly after HL and HL + UV exposure, resulting in a significant 60%–70% decrease (RM ANOVA, $P < 0.0001$) of F_v/F_m . Only on the fourth day was the F_v/F_m of HL + UV significantly lower than HL (RM ANOVA, $P < 0.01$). The diurnal cycles on the fourth day showed significant recovery (RM ANOVA, $P < 0.0005$) 2 h after the HL or HL + UV exposure, with F_v/F_m increasing to 89% and 79% of the CTRL values, respectively. The following 12 h caused an additional increase of ~2% and 5%, respectively. The F_v/F_m of HL + UV remained significantly lower (RM ANOVA, $P < 0.05$) than that of the HL-exposed cultures until after the dark period.

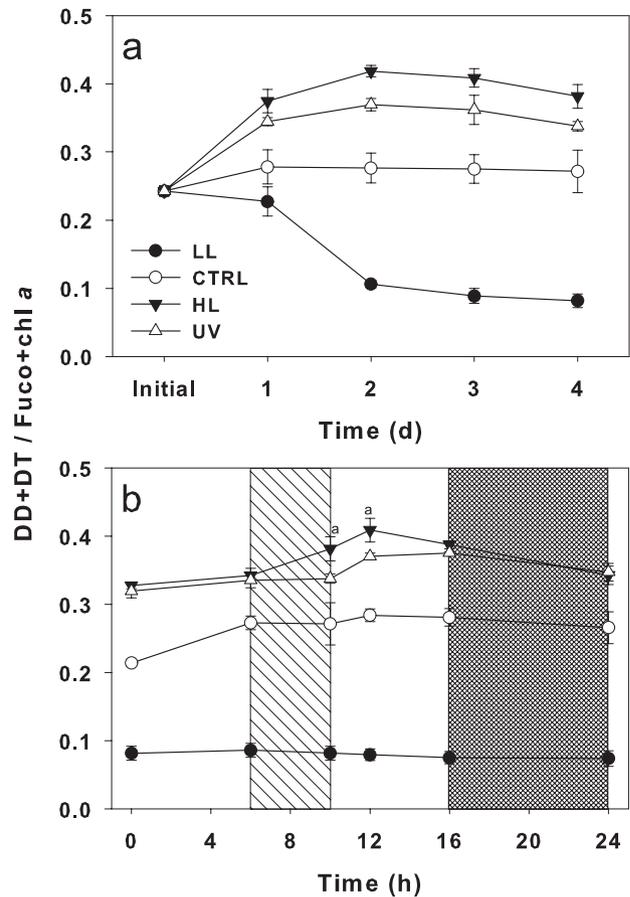


FIG. 3. Light protection pigments (DD + DT) relative to light-harvesting pigments (Fuco + chl *a*) before (initial, $180 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) and after a shift to low PAR irradiance (LL, $20 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (HL, $1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance ($1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} + 24 \text{ W} \cdot \text{m}^{-2}$ UVA and $2.1 \text{ W} \cdot \text{m}^{-2}$ UVB). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. (a) During 4 d of photoacclimation. (b) During the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to a new irradiance environment (light dashed area) at $t = 6$ h, and returned to CTRL conditions at $t = 10$ h. The dark dashed area ($t = 16$ until $t = 24$) represents the night period. The daily data points of all cultures were measured at $t = 10$ h. Error bars represent standard deviations. a: significant UVR effect ($P < 0.05$).

Yet, the recovery rates of both cultures (1.14 h^{-1}) were not significantly different.

Pigment analyses. After 3 weeks of preculturing at CTRL conditions, the ratio of photoprotective xanthophyll pigments to photosynthetic pigments ($DD + DT / \text{Fuco} + \text{chl } a$) was 0.25 and remained on that level during the experiment (Fig. 3a). When transferred to LL, this ratio decreased significantly (RM ANOVA, $P < 0.0005$), eventually dropping to 0.08. The diurnal cycle of this ratio did not show any variability for the LL condition (Fig. 3b). In contrast, when placed under HL and HL + UV, the ratio increased significantly (RM ANOVA, $P < 0.0005$) and reached values as high as 0.42. This

TABLE 1. (A) Cellular pigment content of *Chaetoceros brevis* before (initial, 180 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) and after a shift to low PAR irradiance (low light, 20 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (high light, 1,350 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance (high light + UVR, 1,350 $\mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ + 24 $\text{W} \cdot \text{m}^{-2}$ UVA and 2.1 $\text{W} \cdot \text{m}^{-2}$ UVB). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. (B) Cellular pigment content during their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to the new irradiance environment at $t = 6$ h, and returned to CTRL conditions at $t = 10$ h. The night period was from $t = 16$ until $t = 24$ h. The daily data points of all cultures were measured at $t = 10$ h. (a) Chl a + fucoxanthin; (b) diatoxanthin + diadinoxanthin. LL cultures were kept at low irradiance for the entire photoperiod.

Irradiance condition	Initial	$T = 1$	$T = 2$	$T = 3$	$T = 4$	
(A) Concentration of chl a + fucoxanthin per cell ($\times 10^2$ pg \cdot cell $^{-1}$).						
Low light	1.78 \pm 0.30	1.37 \pm 0.18	1.41 \pm 0.22 ^a	1.79 \pm 0.19	2.18 \pm 0.43	
CTRL	1.78 \pm 0.30	1.30 \pm 0.12	1.25 \pm 0.11	1.44 \pm 0.18	1.41 \pm 0.08	
High light	1.78 \pm 0.30	1.16 \pm 0.16	1.03 \pm 0.15	1.21 \pm 0.18	1.03 \pm 0.08	
High light + UVR	1.78 \pm 0.30	1.10 \pm 0.23	1.10 \pm 0.11	1.15 \pm 0.07	1.27 \pm 0.07	
	$t = 0$	$t = 6$	$t = 10$	$t = 12$	$t = 16$	$t = 24$
Low light	1.96 \pm 0.34	2.10 \pm 0.46	2.18 \pm 0.43	2.16 \pm 0.53	2.36 \pm 0.61	2.59 \pm 0.46
CTRL	1.23 \pm 0.08	1.32 \pm 0.15	1.41 \pm 0.08	1.35 \pm 0.09	1.52 \pm 0.09	1.56 \pm 0.15
High light	0.96 \pm 0.08	1.09 \pm 0.05	1.03 \pm 0.08	1.08 \pm 0.08	1.19 \pm 0.20	1.29 \pm 0.20
High light + UVR	1.09 \pm 0.10	1.19 \pm 0.10	1.27 \pm 0.07	1.34 \pm 0.14	1.19 \pm 0.18 ^b	1.41 \pm 0.18
Irradiance condition	Initial	$T = 1$	$T = 2$	$T = 3$	$T = 4$	
(B) Concentration diatoxanthin + diadinoxanthin ($\times 10$ pg \cdot cell $^{-1}$).						
Low light	4.33 \pm 0.85	3.08 \pm 0.22	1.52 \pm 0.15 ^a	1.57 \pm 0.67	1.75 \pm 0.20	
CTRL	4.33 \pm 0.85	3.61 \pm 0.53	3.47 \pm 0.48	3.99 \pm 0.78	3.82 \pm 0.25	
High light	4.33 \pm 0.85	4.35 \pm 0.75	4.31 \pm 0.70	4.95 \pm 0.78	3.91 \pm 0.21	
High light + UVR	4.33 \pm 0.85	3.79 \pm 0.79	4.06 \pm 0.49	4.17 \pm 0.47	4.27 \pm 0.20	
	$t = 0$	$t = 6$	$t = 10$	$t = 12$	$t = 16$	$t = 24$
Low light	1.58 \pm 0.19	1.78 \pm 0.32	1.75 \pm 0.20	1.69 \pm 0.26	1.74 \pm 0.25	1.88 \pm 0.15
CTRL	2.64 \pm 0.11	3.49 \pm 0.17	3.82 \pm 0.25	3.83 \pm 0.38	4.15 \pm 0.16	4.12 \pm 0.33
High light	3.16 \pm 0.27	3.74 \pm 0.22	3.91 \pm 0.21	4.44 \pm 0.51	4.62 \pm 0.80	4.41 \pm 0.54
High light + UVR	3.46 \pm 0.25	3.99 \pm 0.27	4.27 \pm 0.20	4.96 \pm 0.48	4.47 \pm 0.67 ^b	4.93 \pm 0.64

Values of pigments are means \cdot cell $^{-1}$ \pm SD shown for four replicates. T = time in days; t = time in hours. ^aTriPLICATE; ^bDUPLICATE.

increase was due to a significant decrease (RM ANOVA, $P < 0.05$) in light-harvesting pigments (Table 1). In the absence of UVR, the protective capacity of cells was found to be significantly higher (RM ANOVA, $P < 0.005$) relative to their light absorption capacity. The diurnal cycles of HL and HL + UV cultures showed significantly higher pigment ratios (RM ANOVA, $P < 0.0005$) at the beginning of the light period compared with the CTRL condition. Furthermore, the protection over harvesting ratio of the HL culture increased immediately after HL exposure, while the HL + UV culture did not show an increased ratio until 2 h later. During the exposure period, the ratio of the HL culture increased mainly due to significant reduction in light-harvesting pigments (RM ANOVA, $P < 0.05$). This response was postponed in the HL + UV culture until after the exposure period. UVR significantly affected de-epoxidation of diadinoxanthin (DD) to its energy dissipating form diatoxanthin (DT; RM ANOVA, $P < 0.0005$); the de-epoxidation status [DES = DT/(DT + DD)] of the HL culture was on average 73% (SD = 2.7), while the DES after HL + UV was on average 59% (SD = 6.8). After 2 h of recovery, the DES of both cultures returned to preexposure levels (Fig. 4b). Cultures growing at LL contained no detectable level of DT (Fig. 4, a and b).

SOD activity. During the experiment, the SOD activity of the CTRL culture was on average 135 $\text{U} \cdot \text{mg}^{-1}$ protein and did not change significantly over time (Fig. 5). Four hours after the irradiance transitions, LL, HL, and HL + UV cultures showed significantly increased SOD activities (RM ANOVA, $P < 0.0005$), with values of 255, 350, and 368 $\text{U} \cdot \text{mg}^{-1}$ protein, respectively. Although SOD activities decreased considerably on the second day, their values were still significantly higher (RM ANOVA, $P < 0.005$) than those of the CTRL cultures. After the third exposure period, HL, HL + UV, and CTRL contained similar SOD activity levels, in contrast to the LL culture, which still showed significantly (RM ANOVA, $P < 0.05$) higher values. On the final day, all four cultures showed similar SOD activities, not significantly differing from the CTRL culture. Yet, during the diurnal cycle, the LL culture contained significantly more SOD activity (average = 167 $\text{U} \cdot \text{mg}^{-1}$ protein, SD = 29) than CTRL (average = 106 $\text{U} \cdot \text{mg}^{-1}$ protein, SD = 15), HL (average = 110 $\text{U} \cdot \text{mg}^{-1}$ protein, SD = 13), and HL + UV (average = 116 $\text{U} \cdot \text{mg}^{-1}$ protein, SD = 18) cultures (RM ANOVA, $P < 0.05$), which were all similar to each other throughout the 24 h.

TBARS content. Four hours after the irradiance transitions, the cellular TBARS content of all four

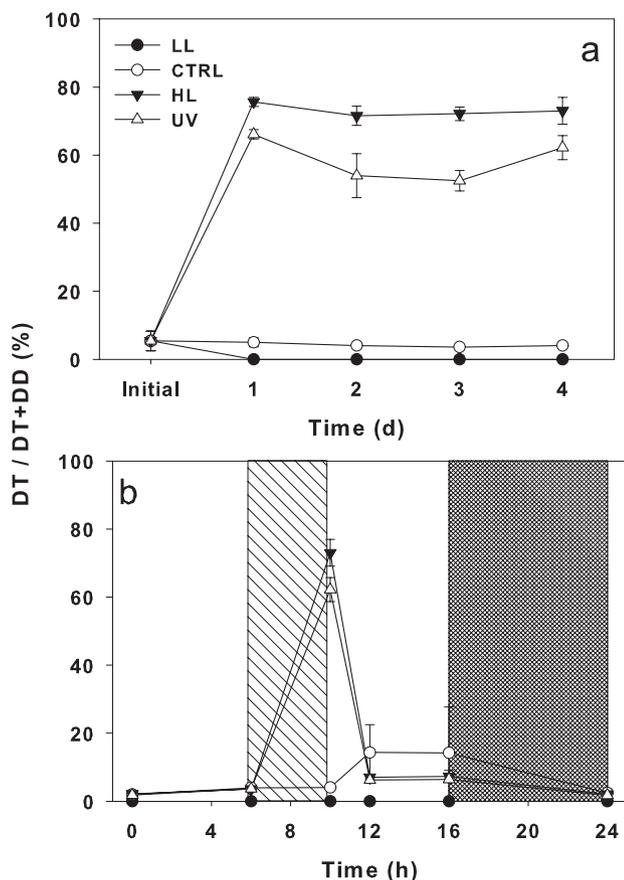


FIG. 4. De-epoxidation state (DES = DT/DT + DD) of the photoprotective xanthophyll pigments before (initial, $180 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) and after a shift to low PAR irradiance (LL, $20 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (HL, $1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance ($1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} + 24 \text{ W} \cdot \text{m}^{-2}$ UVA and $2.1 \text{ W} \cdot \text{m}^{-2}$ UVB). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. (a) During 4 d of photoacclimation. (b) During the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to the new irradiance environment (light dashed area) at $t = 6$ h, and returned to CTRL conditions at $t = 10$ h. The dark dashed area ($t = 16$ until $t = 24$ h) represents the night period. The daily data points of all cultures were measured at $t = 10$ h. Error bars represent standard deviations.

cultures was similar to the initial situation ($T = 0$; Fig. 6a). During the following days, the cell-specific amount of TBARS present in the CTRL, HL, and HL + UV cultures remained at this concentration, while TBARS in LL-cultured cells increased. As a result, the LL cultures contained significantly more TBARS per cell on the fourth day than the other three cultures (RM ANOVA, $P < 0.05$). This trend persisted during the diurnal cycle on day 4 (Fig. 6b).

DISCUSSION

In this study, we investigated oxidative stress responses in the marine diatom *C. brevis* during

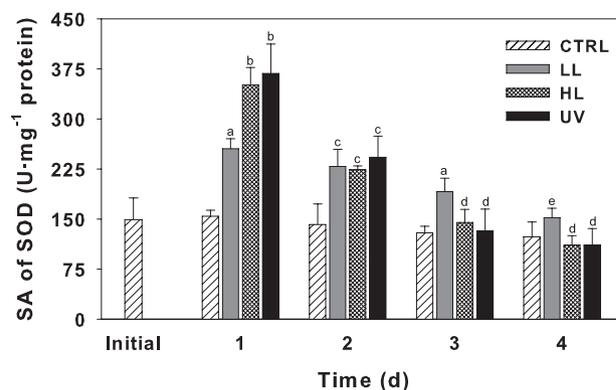


FIG. 5. Specific activity (SA) of superoxide dismutase (SOD) before (initial, $180 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) and after a shift to low PAR irradiance (LL, $20 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (HL, $1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance ($1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} + 24 \text{ W} \cdot \text{m}^{-2}$ UVA and $2.1 \text{ W} \cdot \text{m}^{-2}$ UVB). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. Error bars represent standard deviations. a: significant differences from all other irradiance conditions ($P < 0.05$) on the same day; b: significant differences from CTRL and LL ($P < 0.0005$) on the same day; c: significant differences from CTRL ($P < 0.005$) on the same day; d: significant differences from LL ($P < 0.05$) on the same day; e: significant differences from HL and UV ($P < 0.05$) on the same day.

photoacclimation. Prior to the experiment, cells grew exponentially under saturating irradiance conditions ($180 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$, 16:8 L:D, and 4°C) at rates consistent with literature values (van de Poll et al. 2005). Cultures transferred to the HL + UV regime were exposed to excess irradiance including UVA (320–400 nm) and UVB (280–320 nm) with a spectral ratio of 128:11.3:1 $\text{W} \cdot \text{m}^{-2}$ (PAR:UVA:UVB). This approximates natural Antarctic irradiance ratios measured at Melchior station (80:13.6:1; Helbling et al. 1996) and Palmer station (450:12.5:1; Bouchard et al. 2005b). In this study, UVR resulted in a significant growth reduction of 15%, relative to excess PAR only (HL treatment), probably due to its stimulating effect on ROS production. After transition to excess irradiance, light-harvesting activity of HL and HL + UV cultures probably exceeded light-processing activity, which overreduced photosynthetic ETCs and subsequently enhanced ROS formation (Karentz et al. 1991, Nishiyama et al. 2001).

The relatively low maximal quantum yield of CTRL cultures ($F_v/F_m = 0.4$) indicated suboptimal photosynthetic performance. Yet, the growth rate of the CTRL cultures was the highest of all treatments, suggesting that the low F_v/F_m was not caused by photoinhibitory damage. The highest PSII efficiency was measured in LL cultures (average $F_v/F_m = 0.7$), consistent with values obtained previously (van de Poll et al. 2005, 2006). The PSII efficiency increase was stoichiometrically inverse to the decrease in the DD + DT/Fuco + chl *a* ratio, which resulted from strong cellular decrease in xanthophyll pigments

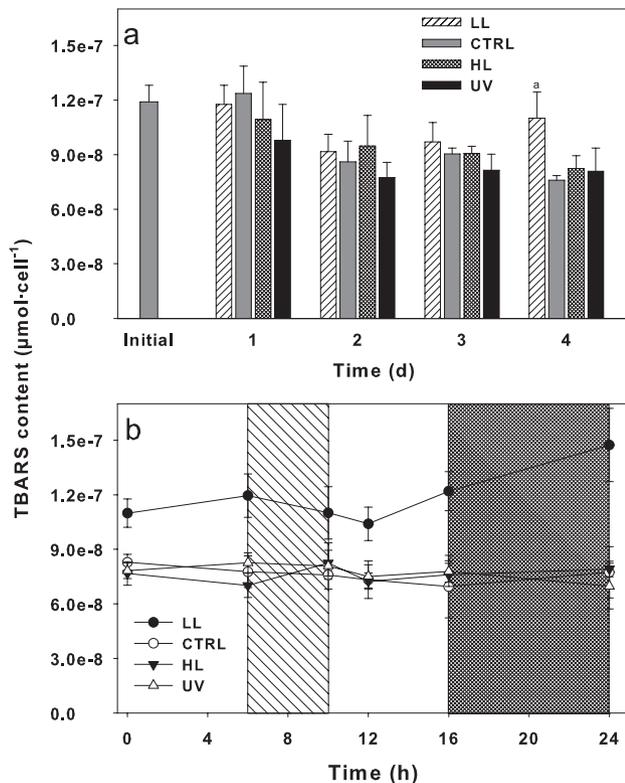


FIG. 6. Cellular TBARS content before (initial, $180 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ PAR) and after a shift to low PAR irradiance (LL, $20 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), high PAR irradiance (HL, $1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and high PAR + UV irradiance ($1,350 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1} + 24 \text{ W} \cdot \text{m}^{-2}$ UVA and $2.1 \text{ W} \cdot \text{m}^{-2}$ UVB). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. (a) During 4 d of photoacclimation. (b) During the diurnal cycle on day 4. LL cultures were kept at low irradiance for the entire photoperiod. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to new irradiance environment (light dashed area) at $t = 6$ h, and returned to CTRL conditions at $t = 10$ h. The dark dashed area ($t = 16$ until $t = 24$) represents the night period. The daily data points of all cultures were measured at $t = 10$ h. Error bars represent standard deviations. a: differs significantly from all other irradiance conditions ($P < 0.005$) on the same day. TBARS, thiobarbituric acid reactive substances.

and a simultaneous increase in light-harvesting pigments. In accordance with Post et al. (1985), these changes could not compensate for the decreased irradiance and resulted in lower growth rates.

The inverse relationship between DD + DT/Fuco + chl *a* ratio and PSII efficiency also applied for cultures subjected to the HL treatment. This finding was also reported in other studies on responses of diatoms to irradiance shifts (Willemoës and Monas 1991, Olaizola and Yamamoto 1994, Mewes and Richter 2002). Furthermore, field studies confirmed that transition from low to high irradiance resulted in an increased DD + DT/Fuco + chl *a* ratio (Moline 1998). In accordance with Claustre et al. (1994), we determined that the increased protection/harvesting ratio of HL cultures resulted

almost entirely from a decrease in light-harvesting pigments. Yet, the increased protection/harvesting ratios and de-epoxidation status could not prevent growth reduction in the HL and HL + UV cultures.

No apparent effect on PSII efficiency was observed directly after the UVR exposures, as reported previously (Vassiliev et al. 1994, Lesser et al. 1996, van de Poll et al. 2006). Furthermore, recovery rates of PSII efficiency over time were similar for HL and HL + UV cultures. However, the HL cultures showed 90% recovery, while PSII efficiency of HL + UV cultures remained 10% lower. These changes could indicate partial UVR-induced photo-damage to the PSII reaction center D1 protein, as found elsewhere (Nishiyama et al. 2001, Bouchard et al. 2005a,b). In addition, the DD + DT/Fuco + chl *a* ratio of HL + UVR-exposed cells was significantly lower than that of the HL culture. This finding is contrary to other studies where UVR did not affect pigment ratios or even cause enhancement of xanthophyll pools and pigment ratios (Goss et al. 1999, Buma et al. 2000, Mewes and Richter 2002). The DES of the HL + UV culture was on average 10% lower than that of HL-exposed cells, similar to values reported by Bischof et al. (2002a), Pfündel et al. (1992), and Mewes and Richter (2002). This reduction in xanthophyll-cycle activity has been linked to the increased production of ROS (Bischof et al. 2002b). More specifically, Rijstenbil (2003) and Mallick and Mohn (2000) suggested that diatoxanthin can act as an antioxidant by which the xanthophyll pigment is reepoxidized to diadinoxanthin. Mewes and Richter (2002) proposed that UVR enhanced thylakoid membrane permeability, resulting in a lower ΔpH gradient, which increases the activity of diatoxanthin epoxidase.

The SOD activity of the CTRL culture remained unchanged throughout the experiment. This observation not only indicated that physiological stress conditions did not change but also that the cultures remained in the same growth phase during the experimental period (Malanga and Puntarulo 1995, Sigaud-Kutner et al. 2002). In contrast, the LL, HL, and HL + UV cultures showed elevated SOD levels right after the irradiance shifts. Evidently, the change in irradiance environment stimulated production of superoxide radicals and subsequently their scavenging counterparts (Bowler et al. 1992, Malanga and Puntarulo 1995, Rijstenbil 2002), regardless of the new irradiance regime. After the first day, SOD activities of all three cultures decreased until they had returned to the CTRL value on the fourth day, while no significant additional UVR effect on SOD activity was seen.

To the best of our knowledge, no other study has reported an acclimation response in SOD activity to irradiance changes per se. Previous studies described SOD responses of temperate microalgae to elevated irradiance conditions after one exposure (Malanga et al. 1999, Rijstenbil 2002, van de Poll

et al. 2006), an acclimation period (Lesser and Shick 1989a, Lesser 1996a,b, Rijstenbil 2001, 2003), or a shift in growth-phase (Malanga and Puntarulo 1995, Sigaud-Kutner et al. 2002). Other studies described a UVR-induced increase in SOD activity, although no effect or even decreased activity was reported (Malanga et al. 1999, Zhang et al. 2005, van de Poll et al. 2006). All these studies suggest that there is no uniform response of SOD to excess irradiance including UVR. Genetic studies on plants and algae reveal that the expression levels of SOD-encoding genes are regulated by the redox status of the cell (Bowler et al. 1992, Kohen and Nyska 2002, Mittler et al. 2004) and the intracellular location of $O_2^{\cdot-}$ production. Because the cellular redox status depends on the amount of pro- and antioxidants, all genes involved in ROS scavenging influence each other's expression level. Therefore, SOD genes could be down-regulated in one species (Wang et al. 2004) while up-regulated in another (Mackerness et al. 1999, Mackerness 2000, Turpaev 2002) as a response to oxidative stress.

The rapid decrease in SOD activity after the first day in HL and HL + UV treatment could be due to the gradual increase of the DD + DT/chl *a* ratio, by which energy transfer toward the photosystems was diminished, also slowing down the formation of superoxide radicals. However, since UVR is known to stimulate radical formation and affect the DD + DT/ chl *a* ratio, this seems to explain the acclimation process only partially. Besides that, several studies have demonstrated UVR-induced increases of other antioxidant compounds such as the water-soluble glutathione and ascorbate (Dai et al. 1997, Costa et al. 2002, Shiu and Lee 2005), or the lipid protecting α -tocopherol (Malanga and Puntarulo 1995, Malanga et al. 1999), which might be energetically more favorable than SOD. Exposure to HL and HL + UV did not change the level of oxidative damage measured as TBARS relative to CTRL, but their growth rates were reduced significantly. Therefore, it seems that not ROS-induced damage, but rather other factors, such as a change in cellular metabolism as a response to irradiance transition, are responsible for growth inhibition (Post et al. 1985, Falkowski and LaRoche 1991). In contrast to the other irradiance conditions where TBARS remained unchanged, the cellular TBARS content of the LL culture gradually increased from the second day after its irradiance shift. This finding could be due to an increased synthesis of light-harvesting complex (LHC) proteins and accommodating membrane lipids (Falkowski and LaRoche 1991, Evens et al. 2001). These metabolic changes (i.e., increased level of cellular $Fe^{2+}/^{3+}$ content and the accompanying level of Haber-Weiss/Fenton cycling) could lead to increased production of ROS and increased activity of SOD. After this initial antioxidant response, other components of the network could take over,

coinciding with a decrease in SOD capacity after day 1.

We suggest that SOD is an important ROS-scavenging mechanism in *C. brevis* because the activity of SOD was rapidly (4 h) up-regulated in response to irradiance transitions. Nevertheless, the increase in SOD activity was only short lived, returning to initial values once photoacclimation had progressed and other, perhaps more cost-effective or efficient ROS-prevention mechanisms were installed.

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