Antioxidants, a radical solution?

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Oxidative stress responses in the marine Antarctic diatom *Chaetoceros brevis* (Bacillariophyceae) during photoacclimation

Paul J. Janknegt, Willem H. Van de Poll, Ronald J. W. Visser, Jan W. Rijstenbil and Anita G. J. Buma

Abstract

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* Schütt during transition to excess (including ultraviolet radiation) and limiting irradiance conditions. Over a four day period cellular responses of 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 found for cultures transferred to low irradiance. This acclimation process was completed after two days in the new irradiance environment. SOD activity increased significantly after the first day regardless of the new irradiance environment but returned to pre-exposures 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.
Photoacclimation in a diatom

Introduction

Reactive oxygen species (ROS) are produced as byproducts 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 principle site for ROS production (Pinto et al. 2003). When the consumption of the reduced equivalents cannot match their production, electron transport chains 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 ultraviolet radiation (UVR: 280–400 nm) stimulate radical formation (Hideg & Vass 1996). Reduction of O₂ occurs through a series of univalent electron transfer (Taube 1965). The first one-electron reduction generates the superoxide radical (O₂⁻). Addition of a second electron generates H₂O₂ either by spontaneous or an enzymatic reaction catalyzed by superoxide dismutase (SOD; Gregory & Fridovich 1973a, b; Halliwell & Gutteridge 1984). Both reactive oxygen species are moderately harmful but in the presence of unchelated bivalent cations (i.e. Fe²⁺/³⁺, Cu⁺/²⁺), O₂⁻ is able to reduce H₂O₂ thereby generating the highly reactive hydroxyl radical (HO•; Kehrer 2000, Cadenas 1989, Halliwell & Gutteridge 1984). If the production of HO• is not limited, adverse effects can be expected, ranging from the temporary impairment of photosynthesis to viability loss, possibly related with membrane damage due to lipid peroxidation (Van de Poll et al. 2006, Halliwell 2006).

Marine microalgae experience excess irradiance and oxidative stress conditions particularly near the water surface (Llabrés & 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. 1998a, 2003). Exposure to UVR causes additional stress and is responsible for significant decreases of the photosynthetic activity of Antarctic microalgae (Smith et al. 1992, Holm-Hanssen 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 towards the photosystems by adjusting the composition of the light harvesting and photoprotective pigments (Falkowski & 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 superoxide dismutase (SOD) holds a key position in this network because it is able to scavenge the superoxide radical by which proliferation of ROS is prevented (Wolfe-Simon et al. 2005). 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 found 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 (Janke & White 2002, Rijstenbil 2003), irradiance (Lesser & Shick 1996b) and UVR (Lesser 1996a, b; 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 pre-cultured at 180 µmol·m⁻²·s⁻¹ and transferred to low and periodic excess irradiance (including UVR). Growth, PSII efficiency, pigment composition, and SOD activity were followed for four days after the transition. In addition, the possible variability in these responses during the diurnal cycle was investigated.

**Materials & methods**

**Experimental design**

Chaetoceros brevis (Schütt, Center for Culture of Marine Phytoplankton isolate 163) was cultured for three weeks in duplicate UV transparent 2.2 L polymethylmethacrylate boxes containing modified f/2 enriched seawater (Veldhuis & Admiraal 1987) of 34.5 PSU at 4°C. The pre-cultures received 180 µmol photons·m⁻²·s⁻¹ photosynthetically active radiation (PAR, initial condition) during a 16:8 h light dark-cycle. To avoid nutrient limitation, medium was replenished every ten days. The duplicate cultures were split into 4 UV transparent boxes (making 8 boxes in total) and supplied with 2 L fresh medium. After 3 more days of growth duplicate boxes, each originating from one pre-culture, were transferred to low irradiance (LL, 20 µmol photons·m⁻²·s⁻¹), high irradiance conditions (HL, 1350 µmol photons·m⁻²·s⁻¹ PAR, 24.2 W·m⁻² ultraviolet A (UVAR: 315 – 400 nm), and 2.1 W·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 hours per day after which the cultures were transferred back to the CTRL conditions. This treatment was repeated for 4 days. 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 4 replicates for each irradiance condition.

**Irradiance**

PAR was provided by Biolux fluorescence tubes (Osram, GmbH, Munich, Germany), whereas UVR was provided by 4 UVA 340 fluorescent tubes (Q-panel, Cleveland, Ohio, USA). PAR irradiance was measured with a spherical irradiance meter (QSL-100, Biospherical Instruments) in air. UVR was measured with 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 four days 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 (Ø 47mm; Osmonics Inc., USA) and stored at -80°C for one month. SOD analyses were done using the 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 KH₂PO₄, pH 7.8, 0.1 mM EDTA, 0.1% Triton X-100 (Aldrich, Steinheim Germany), 2% PVP (Acros, New Jersey USA) and Complete protease inhibitor cocktail (Roche, Mannheim Germany). Subsequently, cells were disrupted by sonication (2 x 30 s, at A=30) at 4°C after which the extracts were centrifuged for 25 min (20,000 g) 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 Riboflavin/NitroBlue Tetrazolium (RF/NBT) method (Beauchamp & Fridovich 1971, Fryer et al. 1998). This assay is based on the competition between SOD and NBT for O₂⁻ generated by the illuminated Riboflavin. SOD activity was defined as the amount of sample required to achieve 50% inhibition of the NBT reduction (V₅₀). NBT reduction was measured (in duplicate) in 4 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 KH₂PO₄, pH 7.8, 0.1 mM EDTA, and 0.25% Triton X-100 (Sigma)), 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 4 fluorescence tubes (Philips TLD/18W, 30 cm distance) giving 199 µmol photons m⁻²·s⁻¹. Afterwards, absorbance was measured at 560 nm on a Cary 3E UV/VIS double beam spectrophotometer (Varian, Middelburg, The Netherlands). Fifty percent inhibition (V₅₀) was calculated by regression using the linear part of a natural semi-log curve. The specific activity of SOD (S.A.) was calculated according to 1 / (V₅₀*[protein]).

**Pigments**

Samples for pigment analysis were filtered over GF/F filters (Ø 25mm) and stored at -80°C for one month. Pigments were analysed as described by Van Leeuwe et al. (2006). In short, filters were handled under dim light, 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) with a C₁₈ 5 μm DeltaPak reversed-phase column (Milford, MA, USA) and identified by retention time and diode
array spectroscopy. Quantification was done using standard dilutions of Chlorophyll \(a, c_2,\) fucoxanthin, diadinoxanthin, diatoxanthin, and \(\beta\) carotene. Cell counts were done 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 Thiobarbituric acid reactive substances (TBARS)**

Thiobarbituric acid reactive substances (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 done after Heath & 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, 20000 g). 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. Chlorophyll fluorescence was measured with a PAM 2000 pulse amplitude modulated fluorometer (Walz, Effeltrich, Germany). Maximum quantum yield \(\frac{F_v}{F_m}\) was determined after 5 min of dark acclimation.

**Statistical analyses**

Differences in growth rates were analysed using One-way ANOVA. Other differences were tested for significance with a Repeated Measures ANOVA (RM ANOVA). Significant differences were further analysed using a LSD post-hoc test. Tests were performed using STATISTICA 7 for Windows.
Results

Growth
Under the pre-experimental conditions, where irradiance was the same as the experimental control, *Chaetoceros brevis* grew exponentially at an average rate of 0.45 divided per day (Fig. 1). After transfer to the experimental irradiance conditions all cultures continued to grow exponentially. The control (CTRL) cultures grew fastest (0.42 divided per day) followed by HL (0.34 divided per day), HL+UV (0.28 divided per day) and LL (0.12 divided per day). The growth rates of the control and pre-experimental 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).

![Figure 1: Growth rates of *Chaetoceros brevis* cultured at 180 µmol photons m⁻² s⁻¹ PAR (Initial) before and after transition to low irradiance PAR (LL, 20 µmol photons m⁻² s⁻¹), high PAR irradiance (HL, 1350 µmol photons m⁻² s⁻¹), and high PAR + UV irradiance (UV, 1350 µmol photons m⁻² s⁻¹ + 24 W m⁻² UVAR and 2.1 W m⁻² UVBR). 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).](image)

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 (± 0.012 SD) (Fig. 2a). During the following four days, 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 around 0.7 (Fig. 2a). No significant changes were observed for LL during the diurnal cycle. Lowest values were found 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, F_v/F_m of HL+UV was significantly lower than HL (RM ANOVA, p<0.01).
The diurnal cycles on the fourth day showed significant recovery (RM ANOVA, p<0.0005) two hours after the HL or HL+UV exposure, with Fv/Fm increasing to 89% and 79% of the CTRL values, respectively.

Figure 2: Photosystem II efficiency before (Initial, 180 µmol photons·m⁻²·s⁻¹ PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m⁻²·s⁻¹), high PAR irradiance (HL, 1350 µmol photons·m⁻²·s⁻¹), and high PAR + UV irradiance (1350 µmol photons·m⁻²·s⁻¹ + 24 W·m⁻² UVAR and 2.1 W·m⁻² UVBR). The control culture (CTRL) was kept at the same irradiance condition as the initial irradiance condition. a) during four days 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 till t=24) represents the night period. The daily data points of all cultures were measured at t=10 h. Error bars represent standard deviations.

The following 12 h caused an additional increase of approximately 2% and 5%, respectively. The Fv/Fm of HL+UV remained significantly lower (RM ANOVA, p<0.05) than that of the HL exposed cultures until after the dark period. Yet, the recovery rates of both cultures (1.14 h⁻¹) were not significantly different.

**Pigment analyses**

After three weeks of pre-culturing at CTRL conditions, the ratio of photoprotective xanthophyll pigments to photosynthetic pigments (DD+DT/Fuco+ChLα) 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.

**Figure 3:** Light protection pigments (DD+DT) relative to light harvesting pigments (Fuco+Chl.a) before (Initial, 180 µmol photons·m⁻²·s⁻¹ PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m⁻²·s⁻¹), high PAR irradiance (HL, 1350 µmol photons·m⁻²·s⁻¹), and high PAR + UV irradiance (1350 µmol photons·m⁻²·s⁻¹ + 24 W·m⁻² UVAR and 2.1 W·m⁻² UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. **a** during four days of photoacclimation **b** during the diurnal cycle on day four. LL cultures were kept at low irradiance for the entire photo period. During their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to new irradiance environment (light dashed area) at t=6h and returned to CTRL conditions at t=10h. The dark dashed area (t=16 till t=24) represents the night period. The daily data points of all cultures were measured at t=10h. Error bars represent standard deviations a: significant UVR effect (p<0.05).

This 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
Chapter 3

diurnal cycles of HL and HL+UV cultures showed significantly higher pigment ratio’s (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 two hours later. During the exposure period, the ratio of the HL culture increased mainly due to significant reduction of light harvesting pigments (RM ANOVA, p<0.05). This response was postponed in the HL+UV culture until after the exposure period (Table 1a, b).

Table 1: a) cellular pigment content of Chaetoceros brevis before (Initial, 180 µmol photons·m⁻²·s⁻¹ PAR) and after a shift to low PAR irradiance (Low light, 20 µmol photons m⁻²·s⁻¹), high PAR irradiance (High light, 1350 µmol photons m⁻²·s⁻¹), and high PAR + UV irradiance (High light+UVR, 1350 µmol photons m⁻²·s⁻¹ + 24 W·m⁻² UVAR and 2.1 W·m⁻² UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. b) shows the cellular pigment content during their diurnal cycle, HL and UV cultures were grown at CTRL conditions, transferred to new irradiance environment at t=6h and returned to CTRL conditions at t=10h. Night period was between t=16 till t=24. The daily data points of all cultures were measured at t=10h. a) Chlorophyll a + fucoxanthin b) Diatoxanthin + diadinoxanthin. LL cultures were kept at low irradiance for the entire photoperiod. Values of pigments are means.cell⁻¹ ± SD shown for four replicas. T = time in days; t = time in hours. a: triplicate; b: duplicate.

a) Concentration Chlorophyll a + fucoxanthin per cell (x10² pg/cell).

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<th>T = 2</th>
<th>T = 3</th>
<th>T = 4</th>
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<td>1.78 ± 0.30</td>
<td>1.37 ± 0.18</td>
<td>1.41 ± 0.22</td>
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<td>Low light</td>
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b) Concentration diatoxanthin + diadinoxanthin (x10 pg/cell).

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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 two hours of recovery, the DES of both cultures returned to pre-exposure levels (Fig. 4b). Cultures growing at LL contained no detectable level of DT (Figs. 4a, b).

**Figure 4:** De-epoxidation status (DES = DT / DT+DD) of the photoprotective xanthophyll pigments before (Initial, 180 µmol photons·m⁻²·s⁻¹·PAR) and after a shift to low PAR irradiance (LL, 20 µmol photons·m⁻²·s⁻¹·PAR), high PAR irradiance (HL, 1350 µmol photons·m⁻²·s⁻¹·PAR), and high PAR + UV irradiance (1350 µmol photons·m⁻²·s⁻¹·PAR + 24 W·m⁻²·UVAR and 2.1 W·m⁻²·UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. a) during four days 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 at t=6h and returned to CTRL conditions at t=10h (dashed area). The daily data points of all cultures were measured at t=10h. Error bars represent standard deviations, grey area represents dark period.

**SOD activity**

During the experiment, the SOD activity of the CTRL culture was on average 135 U/mg 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 U/mg 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 U/mg protein, SD=29) than CTRL (average=106 U/mg protein, SD=15), HL (average=110 U/mg protein, SD=13) and HL+UV (average=116 U/mg protein, SD=18) cultures (RM ANOVA, p<0.05), which were all three similar to each other throughout the 24 h.

![Figure 5: Specific activity (SA) of Superoxide dismutase (SOD) before (Initial, 180 µmol photons m⁻² s⁻¹ PAR) and after a shift to low PAR irradiance (LL 20 µmol photons m⁻² s⁻¹), high PAR irradiance (HL, 1350 µmol photons m⁻² s⁻¹), and high PAR + UV irradiance (1350 µmol photons m⁻² s⁻¹ + 24 W m⁻² UVAR and 2.1 W m⁻² UVBR). 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.]

**TBARS content**

Four h after the irradiance transitions, the cellular TBARS content of all four 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).
Photoacclimation in a diatom

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Initial</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBARS content (µmol/cell)</td>
<td>0.0</td>
<td>3.0e-8</td>
<td>6.0e-8</td>
<td>9.0e-8</td>
<td>1.2e-7</td>
</tr>
</tbody>
</table>

Figure 6: Cellular TBARS content before (Initial, 180 µmol photons·m⁻²·s⁻¹ PAR) and after a shift to low PAR irradiance (LL 20 µmol photons·m⁻²·s⁻¹), high PAR irradiance (HL, 1350 µmol photons·m⁻²·s⁻¹), and high PAR + UV irradiance (1350 µmol photons·m⁻²·s⁻¹ + 24 W·m⁻² UVAR and 2.1 W·m⁻² UVBR). The control culture (CTRL) was kept at the same irradiance condition as the Initial irradiance condition. a) during four days 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 new irradiance environment (light dashed area) at t=6h and returned to CTRL conditions at t=10h. The dark dashed area (t=16 till t=24) represents the night period. The daily data points of all cultures were measured at t=10h. Error bars represent standard deviations. a differs significantly from all other irradiance conditions (p<0.005) on the same day.

Discussion

In this study we investigated oxidative stress responses in the marine diatom *Chaetoceros brevis* during photoacclimation. Prior to the experiment cells grew exponentially under saturating irradiance conditions (180 µmol·m⁻²·s⁻¹, 16:8 h light dark 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 ultraviolet A (UVAR: 315-400 nm) and B (UVBR: 280-315 nm) with a spectral ratio of 128:11.3:1 W·m⁻² (PAR:UVAR:UVBR). This approximates natural Antarctic irradiance ratio’s 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.
After transition to excess irradiance, light harvesting activity of HL and HL+UV cultures probably exceeded light processing activity, which over-reduced photosynthetic electron transport chains and subsequently enhanced ROS formation (Nishiyama et al. 2001, Karentz et al. 1991b). The relatively low maximal quantum yield of CTRL cultures (Fv/Fm = 0.4) indicated sub-optimal photosynthetic performance. Yet, the growth rate of the CTRL cultures was highest of all treatments, suggesting that the low Fv/Fm was not caused by photoinhibitory damage. Highest PSII efficiency was measured in LL cultures (average Fv/Fm 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 of xanthophyll pigments and a simultaneous increase in light harvesting pigments. In accordance with Post et al. (1985), this 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 was also found in other studies on responses of diatoms to irradiance shifts (Willemoës & Monas 1991, Olaiola & Yamamoto 1994, Mewes & 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 and coworkers (1994), we found 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 found previously (Van de Poll et al. 2006, Lesser et al. 1996, Vassiliev et al. 1994). 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. This could indicate partial UVR induced photodamage to the PSII reaction centre D1 protein, as found elsewhere (Bouchard et al. 2005a, b; Nishiyama et al. 2001). In addition, the DD+DT/fuco+Chl.\(a\) ratio of HL+UVR exposed cells was significantly lower than that of the HL culture. This is contrary to other studies where UVR did not affect pigment ratios or even caused enhancement of xanthophyll pools and pigment ratios (Mewes & Richter 2002, Goss et al. 1999, Buma et al. 2000). 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), Pfundel et al. (1992), and Mewes & Richter (2002). This reduction in xanthophyll cycle activity has been linked to the increased production of ROS (Bischof et al. 2002b). More specifically, Rijstenbil and coworkers (2003), and Mallick & Mohn (2000) suggested that diatoxanthin can act as an antioxidant by which the xanthophyll pigment is re-epoxidized to diadinoxanthin. Mewes & Richter (2002) proposed that UVR enhanced thylakoid membrane permeability, resulting in a lower \(\Delta pH\) gradient, which increases the activity of diatoxanthin epoxidase.

The SOD activity of the CTRL culture remained unchanged throughout the experiment. This 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 & 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 its scavenging counterpart (Bowler et al. 1992, Malanga & Puntrulo 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 found.

To 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), after an acclimation period (Rijstenbil 2003, 2001, Lesser 1996a, b; Lesser & Shick 1989b) or after a shift in growth-phase (Sigaud-Kutner et al. 2002, Malanga & Puntrulo 1995). Other studies described a UVR induced increase in SOD activity, although no effect or even decreased activity was also found (Van de Poll et al. 2006, Malanga et al. 1999, Zhang et al. 2005). 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, Mittler et al. 2004, Kohen & Nyska 2002) and the intracellular location of O$_2^-$ production (Wolfe-Simon et al. 2005). Because the cellular redox status depends on the amount of pro- and antioxidants, all genes involved in ROS scavenging influence each others expression level. Therefore, SOD genes could be down-regulated in one species (Wang et al. 2004) while up-regulated in another (Turpaev 2002, Mackerness et al. 1999, Mackerness 2000) as a response to oxidative stress.

The rapid decrease of 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 towards the photosystems was diminished and 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 & Lee 2005), or the lipid protecting α-tocopherol (Malanga & Puntarulo 1995, Malanga et al. 1997) which might be energetically more favourable 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 is responsible for growth inhibition but rather other factors like a change in cellular metabolism as a response to irradiance transition (Falkowski & LaRoche 1991, Post et al. 1985). In contrast to the other irradiance conditions where TBARS remained unchanged, the cellular TBARS content of the LL culture gradually increased as from the second day after its irradiance shift. This could be due to an increased synthesis of light harvesting complex proteins and accommodating membrane lipids (Falkowski & LaRoche 1991, Evens et al. 2001). These metabolic changes (i.e. increased level of cellular Fe$^{2+}$/3+ content and the accompanied level of Haber-Weiss/ Fenton cycling) could therewith lead to an increased production of ROS and an 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 *Chaetoceros 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 has progressed and other, perhaps more cost-effective or efficient ROS prevention mechanisms are installed.