Short-term antioxidative responses of 15 microalgae exposed to excessive irradiance including ultraviolet radiation

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Short-term photosensitivity and oxidative stress responses were compared for three groups of marine microalgae: Antarctic microalgae, temperate diatoms and temperate flagellates. In total, 15 low-light-acclimated species were exposed to simulated surface irradiance including ultraviolet radiation (SSI). Photosensitivity was assessed as the rate of recovery of Fv/Fm in the hours following SSI treatment. Before, during and after the SSI treatment, oxidative stress responses were assessed by following xanthophyll content and cycling, and activities of superoxide dismutase, ascorbate peroxidase and glutathione reductase, and glutathione redox status. When acclimated to low irradiance, antioxidant levels were not group specific. Superoxide dismutase activity was positively correlated with cell size, whereas in general, ascorbate peroxidase activity appeared to be lower and glutathione redox status appeared to be higher in the Antarctic than in the temperate species. After SSI exposure, the strong inhibition of PSII was followed by variable rates of recovery, although four species remained photosynthetically inactive. SSI tolerance appeared unrelated to geographic or taxonomic background, or to cell size. PSII recovery was enhanced in species with decreasing superoxide dismutase activity, glutathione redox status and increased xanthophyll cycle activity. We conclude that antioxidant responses are highly species specific and not related to the geographic or taxonomic background. Furthermore, xanthophyll cycling seems more important than antioxidants. Finally, it can be hypothesized that glutathione could function as a stress sensor and response regulator.

Key words: ascorbate peroxidase, glutathione, glutathione reductase, microalgae, photo-induced oxidative stress, photosensitivity, photosystem II efficiency, pigments, superoxide dismutase, xanthophyll cycle

Introduction

Marine microalgae can be exposed to high irradiance conditions such that the light-harvesting capacity exceeds light-processing capacity. As a result, over-reduced electron transport chains of the photosystems can leak electrons onto O_2, thereby generating reactive oxygen species (ROS; Mehler, 1951; Asada *et al.*, 1974; Gechev *et al.*, 2006). The presence of ultraviolet radiation (UVR: 280–400 nm) has detrimental effects on metabolic pathways and therefore stimulates ROS accumulation (Bischof *et al.*, 2003; Halliwell, 2006).

ROS can cause severe damage in marine microalgae leading to decreased photosynthetic performance, or even viability loss (Van de Poll *et al.*, 2005). Marine microalgae possess a suite of mechanisms to prevent or counteract ROS accumulation. To restrain the production of ROS, cells regulate the energy flow towards the photosystems by adjusting the protective/harvesting pigment ratio (Falkowski & LaRoche, 1991) and by activating the xanthophyll cycle (Demers *et al.*, 1991; Olaizola *et al.*, 1994; Demming-Adams & Adams, 1996). Moreover, several larger ($> 20\,\mu m$) microalgal species are known to synthesize UVR-absorbing compounds, such as mycosporine-like amino acids (MAAs), which are mostly present at the periphery of the cell (Karentz *et al.*, 1991*b*; Helbling *et al.*, 1996; Buma *et al.*, 2006).

When the prevention mechanisms are insufficient, superoxide ($O_2^{\bullet-}$) is the first radical species formed in the photosystems (Yu, 1994; Dat *et al.*, 2000) and can be converted into hydrogen peroxide (H_2O_2). Both species are moderately harmful, but in the presence of unchelated bivalent cations (i.e. $Fe^{2+/3+}, Cu^{1+/2+}$), $O_2^{\bullet-}$ is able to reduce H_2O_2, thereby generating the hydroxyl

radical (HO$^\bullet$) (Kehrer, 2000). HO$^\bullet$ is one of the most reactive species known to chemistry causing lipid peroxidation, DNA damage and protein oxidation (Halliwell, 2006). Microalgae are thought to counteract ROS by means of their antioxidant network (Mallick & Mohn, 2000; Asada, 2006) in which the enzyme superoxide dismutase (SOD) catalyses the conversion of $O_2^{\bullet-}$ into H_2O_2 (Gregory & Fridovich, 1973*a*, *b*; Klug & Rabani, 1972). Because SOD is the only enzyme capable of $O_2^{\bullet-}$ removal, thereby preventing uncontrolled production of HO$^\bullet$, it holds a key position within the antioxidant network (Bowler, 1992). Based on their metal co-factor, several SOD isozymes can be distinguished: Fe-, Mn- and Cu/ZnSOD, which occupy specific intracellular sites (Wolfe-Simon et al., 2005). Furthermore, SOD isozymes vary in amino acid composition, spatial structure and cellular content.

H_2O_2, formed by SOD activity, can diffuse out of the cell or be scavenged by a suite of other enzymes such as ascorbate peroxidase (APX; Shigeoka et al., 2002; Asada, 2006). During scavenging, APX oxidizes ascorbic acid, a low molecular weight antioxidant, which is also used for regeneration of other oxidized antioxidants and non-enzymatic ROS scavenging. Ascorbic acid can be regenerated enzymatically, using glutathione as a substrate, which can also act as a non-enzymatic antioxidant (de Kok & Stulen, 1993; Noctor et al., 2002). Oxidized glutathione (GSSG) is eventually reconverted into its reduced form (GSH) by glutathione reductase (GR). Therefore, GR is responsible for fueling the antioxidant network (Apel & Hirt, 2004), controlling the glutathione redox status (GRS; ratio of GSH over total amount of glutathione).

Antioxidant responses to excess irradiance, including UVR, have been investigated extensively in plants (Strid, 1993; Willekens et al., 1994; Ledford & Niyogi, 2005) and macroalgae (Aguilera et al., 2002*a*, *b*; Dummermuth et al., 2003; Shiu & Lee, 2005) while much less has been reported for microalgae. Data regarding microalgae are scattered and difficult to compare; experiments differ widely in set up, exposure time, spectral composition, irradiance intensity, acclimation and growth conditions, or the species under consideration (single species or whole communities; Butow et al., 1997; Malanga et al., 1999; Lesser et al., 2002; Rijstenbil, 2002; Sigaud-Kutner et al., 2002). Our aim was to elucidate if (irradiance-induced) antioxidative responses were related to geographic or taxonomic background, or cell size. Therefore, 15 species, ranging from small Antarctic flagellates to large temperate diatoms were exposed to excess irradiance including UVR (SSI). To this end, several studies have demonstrated cell size related UVR vulnerability of phytoplankton (Karentz et al., 1991*a*; Laurion & Vincent, 1998; Helbling et al., 2001). Therefore, we examined the influence of specific cell size (cell surface area/volume) on irradiance stress (indicated by PSII recovery) and antioxidative processes (UV absorbing compounds, SOD activity and isozyme composition, APX activity, GR activity, GRS, xanthophyll cycling). Secondly, we compared temperate with polar species since polar microalgae are less likely to be adapted to increased levels of UVR due to their evolutionary history (Helbling et al., 1992; Martínez, 2007). Moreover, O_2 solubility is between 1.5- and 2-fold higher in low (0°C) temperature seawater than in temperate (20°C) seawater, and low-temperature organisms contain more poly-unsaturated fatty acids, both potentially increasing the risk of oxidative damage (Louanchi et al., 2001; Lesser, 2006). Furthermore, since diatoms and phytoflagellates are thought to use different strategies to cope with excess irradiance we compared responses of distinct taxonomic groups (Davidson & Marchant, 1994; Karentz, 1994; Wagner et al., 2006).

Materials and methods

Culturing conditions

Experiments were conducted with the Antarctic species *Chaetoceros brevis*, *Chaetoceros dichaeta*, *Chaetoceros* sp., *Nitzschia frigida*, and *Pyramimonas* sp., and the temperate species *Odontella sinensis*, *Navicula salinarum*, *Navicula pelliculosa*, *Nitzschia ovalis*, *Thalassiosira weissflogii*, *Tetraselmis suecica*, *Prorocentrum micans*, *Fibrocapsa japonica*, *Emiliania huxleyi*, and *Porphyridium purpureum* (general information see Table 1). Of these 15 species four were benthic and 11 pelagic. It is important to note that all cultures were maintained and grown for years under laboratory conditions without being exposed to UVR. Prior to the experiments, all cultures were grown in triplicate at $25\,\mu mol\,m^{-2}\,s^{-1}$ photosynthetically active radiation (PAR) in 200 ml modified f/2 enriched seawater (Veldhuis & Admiraal, 1987) at 34.5 practical salinity units (PSU). *Navicula salinarum* was cultured at a salinity of 25 PSU. Light was provided by Biolux fluorescent tubes (Osram) and the cultures were grown in a 16-h:8-h light–dark cycle, shaken manually each day, and medium was replenished every 10 days. After 3 months, 150 ml of each culture was transferred to a UV transparent 2.2 l polymethylmethacrylate bottle containing 1,500 ml of fresh medium, after which the cultures were placed back at $25\,\mu mol\,m^{-2}\,s^{-1}$ PAR for 1 week.

Simulated surface irradiance

The polymethylmethacrylate bottles, containing optically dilute cultures, were exposed to 3 h of Simulated Surface Irradiance (SSI: $1,300\,\mu mol\,m^{-2}\,s^{-1}$

Table 1. Cell size, pigment and culture isolate characteristics of 15 microalgal species.

Species	Class	S/V ratio	Major harv. pig.	Major prot. pig.	Isolate/strain no. and first author
Antarctic microalgae					
Chaetoceros brevis	Bacillariophyceae	1.22	Chl *a*, Fuco	dd, dt	CCMP163 Schütt
Pyramimonas sp.	Prasinophyceae	1.21	Chl *a*, Chl *b*	VAZ	RUGCC, isolated from Weddell Scotia Confluence Schmayda
Chaetoceros sp.	Bacillariophyceae	1.05	Chl *a*, Fuco	dd, dt	RUGCC, isolated from Weddell Scotia Confluence Ehrenberg
Nitzschia frigida	Bacillariophyceae	0.72	Chl *a*, Fuco	dd, dt	NPICC isolated from Barentz Sea by Tamelander Grunow
Chaetoceros dichaeta	Bacillariophyceae	0.31	Chl *a*, Fuco	dd, dt	CCMP 1751 Mangin
Temperate diatoms					
Nitzschia ovalis	Bacillariophyceae	1.29	Chl *a*, Fuco	dd, dt	CCY 0205 Arnott
Navicula pelliculosa	Bacillariophyceae	1.19	Chl *a*, Fuco	dd, dt	CCMP 543 (Brébisson and Kützing) Hilse
Navicula salinarum	Bacillariophyceae	1.1	Chl *a*, Fuco	dd, dt	FDCC L1262 Grunow
Thalassiosira weissflogii	Bacillariophyceae	0.82	Chl *a*, Fuco	dd, dt	CCMP 1049 (Grunow) Fryxell and Hasle
Odontella sinensis	Bacillariophyceae	0.23	Chl *a*, Fuco	dd, dt	CCY 0380 (Greville) Grunow
Temperate flagellates					
Porphyridium purpureum	Rhodophyceae	1.33	Chl *a*	Not found	SAG 1380.1a (Bory de Saint-Vincent) Drew and Ross
Emiliania huxleyi	Haptophyta	0.95	Chl *a*, Fuco	dd, dt	Strain L. isolated from Oslo fjord by Paasche (Lohmann) Hay and Mohler
Tetraselmis suecida	Prasinophyceae	0.70	Chl *a*, Chl *b*	VAZ	NIOZCC (Kylin) Butcher
Fibrocapsa japonica	Raphidophyceae	0.30	Chl a, Fuco	VAZ	CCRUG W420 Toriumi and Takano
Prorocentrum micans	Dinophyceae	0.18	Chl *a*, Chl *b*2	dd, dt	T135MC1 Ehrenberg

Notes: specific cell size is indicated as surface/volume (S/V) ratio. Major harvesting pigments (Major harv. pig.) consisted of chlorophyll *a* (Chl *a*), chlorophyll b (Chl *b*) chlorophyll b2 (Chl *b*2) and fucoxanthin (Fuco). Major protective pigments (Major prot. pig.) consisted of diadinoxanthin (DD), diatoxanthin (DT), violaxanthin (V) antheraxanthin (A) and zeaxanthin (Z). Species were acquired from Goettingen University culture collection (SAG), Netherlands Institute of Ecology culture collection (CCY), University of Groningen culture collection (RUG), Bigelow Laboratory for Ocean Sciences culture collection of marine phytoplankton (CCMP), Loras College freshwater diatom culture collection (FDCC), Norwegian Polar Institute culture collection (NPICC) and the Royal Netherlands Institute for Sea Research culture collection (NIOZCC).

PAR, $42 \, W \, m^{-2}$ UVA and $0.47 \, W \, m^{-2}$ UVB) as in Van de Poll *et al.* (2005), 5 h after the beginning of light period. These irradiance conditions are comparable with solar irradiance at noon at mid-latitude (Van de Poll *et al.*, 2005). The temperature was controlled by a water bath at 4 or 12°C. After the SSI treatment the bottles were transferred to the pre-exposure conditions ($25 \, \mu mol \, m^{-2} \, s^{-1}$ PAR) for 21 h (including a dark period from $t = 13$ until $t = 21$) during which recovery was studied. SSI exposure and subsequent recovery was repeated with the three replicates of each species.

Sampling

Samples for SOD Native PAGE gel electrophoresis, SOD activity, APX activity, GR activity, glutathione content, pigment composition, UV-absorbing compounds and Fv/Fm measurements were obtained before the SSI treatment ($t = 0$), after 3 h of SSI treatment ($t = 3 \, h$), and after 3 h ($t = 6 \, h$) and 21 h ($t = 24 \, h$) of recovery. Samples for SOD Native PAGE gel electrophoresis, SOD, APX, and GR activity (70 ml), and glutathione content (100 ml) were filtered over $2.0 \, \mu m$ pore size polycarbonate filters (47 mm; Osmonics Inc., USA) and stored at −80°C for a maximum of 6 months. Samples for pigment analysis and UV-absorbing compounds (30 ml) were filtered over GF/F filters (25 mm) and stored at −80°C for a maximum of 6 months.

Samples for Fv/Fm measurements (30 ml) were processed immediately (see *PSII efficiency*).

Analytical procedures

PSII efficiency. Measurements for Fv/Fm, calculated as $(F_m–F_0)/Fm$ were performed according to Van de Poll *et al.* (2007) and Janknegt *et al.* (2007). In brief, samples for Fv/Fm measurements were filtered over GF/F filters (11.3 mm; Whatman, Maidstone, UK) by mild vacuum. Filtration was stopped before the filter dried. The filter was transferred to a temperature controlled seawater-filled cuvette and chlorophyll fluorescence was measured with a PAM 2000 pulse amplitude modulated fluorometer after 5 min of dark acclimation (Walz, Effeltrich, Germany). Measurements were performed at either 4°C or 12°C depending on the cultivation temperature of the species.

SOD activity and isozyme composition. SOD analyses were performed using the riboflavin/Nitro Blue Tetrazolium (RF/NBT) method modified for algal material, as described in Janknegt *et al.* (2007). In brief, filters were disrupted by sonication in 0.75 ml protein extraction buffer X (50 mM KH_2PO_4, pH 7.8; 0.1 mM EDTA; 0.1% Triton X-100; 2% PVP and complete protease inhibitor cocktail). After spinning down

cell debris, supernatants were transferred to a clean 1.5-ml Eppendorf tube and 150 μl was used for duplicate quantification of the protein concentration according to Bradford (1976). SOD activity was defined as the amount of sample required to achieve 50% inhibition of 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. Absorption was measured at 560 nm on a Cary 3E UV/VIS double beam spectrophotometer (Varian, Middelburg, The Netherlands) equipped with a temperature-controlled cell attached to a water bath. Fifty percent inhibition (V_{50}) was calculated by regression using the linear part of a semi natural-log plot. The specific activity (S.A.) of SOD was calculated according to $1/(V_{50}*[\text{protein}])$.

SOD activity and isozyme composition was visualized by native-PAGE gel electrophoresis as described in Janknegt *et al.* (2007). Fifteen micrograms protein from each extract was loaded on a non-denaturating 12% polyacrylamide gel using 5 μl protein loading buffer. After electrophoresis (80 V), SOD activity was visualized by soaking the gel in 50 ml staining buffer containing 0.25 mM RF (Sigma, Steinheim Germany), 5 mM NBT (Sigma, Steinheim Germany), and 0.5 mM Tetramethylethylenediamine (TEMED; Amresco, Solon USA) for 30 min in the dark, at 4°C or 12°C depending on the species analysed. After 15 min of illumination, SOD bands appeared as light bands on a blue/purple background. To determine isozyme composition based on their metal cofactor, samples were synchronically separated on three distinct native-PAGE gels. Of these, the first gel was soaked in normal staining buffer, the second was soaked in staining buffer containing 3 mM H_2O_2, inhibiting FeSOD and Cu/ZnSOD activity and the third was soaked in staining buffer containing 5 mM KCN which inhibits Cu/ZnSOD.

APX and GR activity. Supernatants for both APX and GR activity assays were prepared and protein concentrations were determined as described for the *SOD activity assay* (see above). APX activity was determined spectrophotometrically by measuring the consumption of its substrate, ascorbic acid, over a 3 min. interval, in a 3-ml quartz cuvette containing 2.4 ml buffer A (50 mM KH_2PO_4, pH7; 0.1 mM EDTA), 150 μl, 10 mM ascorbic acid (Merck, Darmstadt Germany), 400 μl cell extract and 30 μl, 20 mM H_2O_2 (Merck, Darmstadt Germany). Before H_2O_2 was added, the reaction mixture was incubated for 5 min at the appropriate temperature. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/VIS double beam spectrophotometer. Specific APX activity (U/mg prot) was calculated according to:

$$(-\text{Slope} \times \text{volume}_{\text{tot}}/\varepsilon \times \text{volume}_{\text{extr}})/[\text{protein}]$$

of which $\varepsilon = 2.8\,\text{mM}^{-1}\,\text{cm}^{-1}$. Determination of GR activity was based on its catalytic property to convert GSSG into its reduced form, GSH, thereby consuming NADPH as a reducing agent. NADPH consumption was measured spectrophotometrically by determining its decrease over a 3 min interval in a 3 ml quartz cuvette containing 1 ml buffer A; 150 μl, 10 mM GSSG (Merck,

Darmstadt Germany), 300 μl cell extract and 50 μl, 2.5 mM NADPH (Merck, Darmstadt Germany). Before NADPH was added, the reaction mixture was incubated for 5 min in a cryostat at the appropriate temperature. The rate of NADPH consumption was measured at 450 nm on a Cary 3E UV/VIS double beam spectrophotometer. Specific activity of GR (in U/mg prot) was calculated according to:

$$(-\text{Slope} \times \text{volume}_{\text{tot}}/\varepsilon \times \text{volume}_{\text{extr}})/[\text{protein}]$$

of which $\varepsilon = 6.22\,\text{mM}^{-1}\,\text{cm}^{-1}$.

Glutathione content. Filters were re-suspended in 0.4 ml glutathione extraction buffer containing 50 mM sulfosalicylic acid, 1 mM EDTA, and 0.15% ascorbic acid after which the cells were disrupted by sonication (30 s, at A = 30) at 4°C. Extracts were centrifuged for 25 min (20, 000 × g) at 4°C and supernatants were transferred to a clean 1.5-ml Eppendorf tube. The total amount of glutathione (TAG) was determined using the SIGMA Glutathione Assay Kit (CS0260; Sigma, Saint Louis, MI, USA); all chemicals were purchased from Sigma unless indicated otherwise. The kit is based on the principle that two molecules of GSH react with the Ellman reagents 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to release 5-thio-2-nitrobenzoic acid (TNB) and GSSG. By adding glutathione reductase, all GSSG is continuously reduced to two molecules of GSH by which the conversion of DTNB to TNB continues as well. As a result, the production rate of TNB is proportional to the concentration of the total amount of glutathione. The assay was performed in 96-well microtitre plates (Cayman chemical) containing 150 μl working mixture 1 (100 mM KH_2PO_4, pH7; 100 mM EDTA, GR, 0.170 U/ml; 0.043 mg/ml DTNB), and 10 μl cell extract. After 5 min of incubation at room temperature, 50 μl of a 0.16 mg/ml NADPH solution was added. TNB production was then followed spectrophotometrically for 10 min at 405 nm using a Victor$^{\text{tm}}$ 1420 multilabel counter plate reader (PerkinElmer, Waltham, MA, USA) reading at 1-min intervals. To determine glutathione content in 10 μl algal extract, a reference line was included based on 0.5, 0.25, 0.125, 0.0625 and 0.0312 nmoles GSH (Sigma, Saint Louis, MI, USA) in 10 μl sample. The TNB production rate was calculated for 1 nmole of glutathione by extrapolation. The TAG (in nmol) in 1 ml algal sample was calculated according to: slope$_{\text{sample}}$ × dilution factor original sample/slope$_{\text{1nmoleGSH}}$ × 0.01 ml sample). The amount of GSH was measured by the same principle as TAG but without adding GR and NADPH by which only GSH reacts with DTNB. This assay was performed in 96-well microtitre plates containing 100 μl working mixture 2 (500 mM KH_2PO_4, pH7; 500 mM EDTA; 0.065 mg/ml DTNB), and 100 μl cell extract. After 10 min of incubation at RT the total production of TNB was determined spectrophotometrically at 405 nm. A reference line was included based on 2, 1, 0.5, 0.25, and 0.125 nmoles GSH in 100 μl sample. The amount of GSH in the reaction mixture was calculated in accordance with the reference line. The amount of GSH (in nmol) in

1 ml algal extract was calculated according to: GSH in reaction mixture × dilution factor original sample/0.1 ml sample.

The GRS was expressed in sulphur atoms and calculated according to:

$$GSH/(GSH + 0.5\,GSSG)$$

of which $GSSG = TAG - GSH$.

Determination of pigments. Pigments were analysed as described by van Leeuwe *et al.* (2006). 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, darkness). Pigments were resolved using HPLC (Waters 2690 separation module, 996 photodiode array detector) 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 Chlorophyll *a*, Chlorophyll *b*, Chlorophyll *b2*, fucoxanthin, diadinoxanthin (DD), diatoxanthin (DT), violaxanthin, antheraxanthin and zeaxanthin.

UV-absorbing compounds. UV-absorbing compounds were analysed as described by Tartarotti & Sommaruga (2002). Filters were suspended in 2 ml, 25% aqueous methanol (v/v) at 45°C for 2 h. Thereafter, extracts were filtered through a 0.2-μm disposable cellulose acetate filter (Whatman, Dassel, Germany). The absorption of the filtrate was measured spectrophotometrically between 280 and 700 nm. Baseline correction was by subtracting background absorption using 25% aqueous methanol as a blank. UV-absorbing compounds were expressed as absorption peak normalized to Chl *a*.

Calculation of volume-specific cell surface area

Volume-specific cell surface area (cell surface area/volume; SA/V) was calculated from volume and surface area, which were determined according to Hillebrand *et al.* (1999). Length and width of 15 cells of each species were measured using an Olympus IMT-2 inverted light microscope (Olympus, Centre Valley, PA, USA). Depth was only determined for *Odontella sinensis*, *Navicula salinarum*, *Nitzschia frigida*, *Nitzschia ovalis* and *Pyramimonas* sp.

Statistical analyses

Measurements were done on three replicates unless indicated otherwise. Statistical analyses were performed using SPSS 12.0.1 for windows. Differences between species and responses to the different treatments (3 h SSI, 3 and 21 h recovery in low irradiance) were tested with One-Way ANOVA. Intraspecific differences were analysed using paired-samples *t*-test. The influence of volume-specific cell surface area, geographic region taxonomic background on SOD activity, APX activity, GR activity, GRS, PSII efficiency, pigment content and ratios were tested in an univariate nested ANOVA. In this analysis, species, as a random factor, was nested into the factors specific surface area, geographic region and taxonomic background. Here, geographic region and taxonomic background were introduced as fixed factors and specific surface area was tested as a covariance. Statistical error was determined as error type I. r^2 was obtained by plotting the covariance against the parameter in a regression analysis. Nonparametric Spearman's rho analysis was performed on mean values ($n = 3$) of SOD activity, APX activity, GR activity, GRS, PSII efficiency, pigment content and ratios to test data for correlations.

Results

Photosystem II efficiency

In order to compare interspecific changes in Fv/Fm, values were normalized to the species-specific initial value. The 3 h of SSI exposure ($t = 3$) caused a significant decrease of Fv/Fm in all cultures (*t*-test; $p < 0.0005$, Fig. 1) down to 0.5–15% of the initial values (averaging 0.6 +/– 1.5). Fv/Fm recovery during 21 h ($t = 3$ till $t = 24$) under low irradiance ($25\,\mu mol\,m^{-2}\,s^{-1}$) was species specific. After the first 3 h, Fv/Fm of *N. frigida*, *Pyramimonas* sp., *O. sinensis*, *E. huxleyi* and *P. purpureum* recovered poorly (< 12% of their initial Fv/Fm). In contrast, *P. micans* (23%), *F. japonica* (30%), *N. ovalis* (36%), *C. dichaeta* (43%), *N. salinarum* (45%), *T. weissflogii* (50%), *N. pelliculosa* (57%), *C. brevis* (59%), *Chaetoceros* sp. (61%) and *T. suecica* (74%) showed stronger short-term recovery of their Fv/Fm. After 21 h, Fv/Fm of *N. frigida*, *O. sinensis*, *E. huxleyi* and *P. purpureum* was still less than 38% of their initial values while all other species regained 67–94% of their initial F_v/F_m. No clear relation was observed in PSII responses based on volume specific cell surface area, taxonomic or geographic grouping.

Pigment analyses

The investigated species contained either the diato–diadinoxanthin cycle (DT/DD cycle) or the viola–anthera–zeaxanthin cycle (VAZ cycle); except for the red alga *P. purpureum* which possessed no xanthophyll cycle pigments (Table 1). After growing under low irradiance ($25\,\mu mol\,m^{-2}\,s^{-1}$) for 3 months, the ratio of light-protective relative to light-harvesting pigments (LP/LH) ratio; $DT + DD/Chl\,a + Fuco$ or $VAZ/Chl\,a + Chl\,b$) reached values between 0.03 (*F. japonica*) and 0.13 (*C. brevis*; Table 2). Before and during the experiment, differences between these ratios could not be attributed to volume-specific cell surface area, geographic background or taxonomic group although species containing the VAZ cycle had a significantly lower ratio than those equipped with the DT/DD cycle (One-Way ANOVA, $p < 0.005$).

In response to the SSI treatment, all species showed an increase in their LP/LH ratio
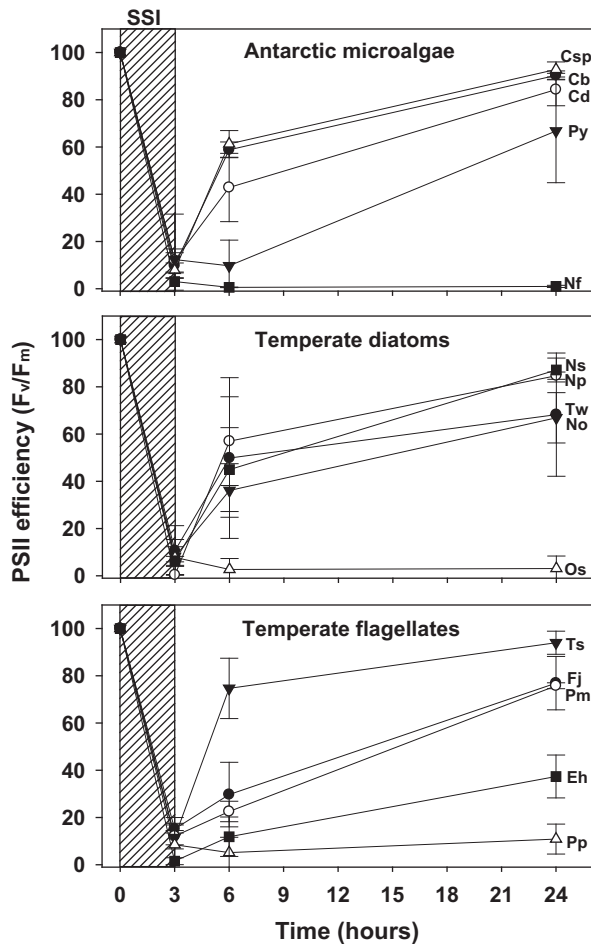
Fig. 1. Photosystem II efficiency (Fv/Fm) of 15 microalgae when acclimated to low-irradiance conditions ($t = 0$; $25\,\mu mol\,m^{-2}\,s^{-1}$ PAR), after 3 h exposure to SSI conditions ($t = 3$; $1,300\,\mu mol\,m^{-2}\,s^{-1}$ PAR $+ 42\,W\,m^{-2}$ UVA $+ 0.47\,W\,m^{-2}$ UVB) and during the subsequent 21-h recovery period under low irradiance conditions ($t = 3$ until $t = 24$; $25\,\mu mol\,m^{-2}\,s^{-1}$ PAR), which included a dark period from $t = 13$ to $t = 21$. Fv/Fm was expressed as percentage of the initial ($t = 0$) value and error bars represent standard deviations ($n = 3$). The dashed area ($t = 0$ until $t = 3$) represents the 3-h exposure period. Abbreviaitons: Cb: *Chaetoceros brevis*; Cd: *Chaetoceros dichaeta*; Csp: *Chaetoceros* sp.; Eh: *Emiliania huxleyi*; Fj: *Fibrocapsa japonica*; Nf: *Nitzschia frigid*; No: *Nitzschia ovalis*; Np: *Navicula pelliculosa*; Ns: *Navicula salinarum*; Os: *Odontella sinensis*; Pm: *Prorocentrum micans*; Pp: *Porphyridium purpureum*; Py: *Pyramimonas* sp; Ts: *Tetraselmis suecica*; Tw: *Thalassiosira weissflogii*.

(Table 2), which they achieved in two different ways. The species showing poor PSII recovery (*N. frigida, O. sinensis*, and *E. huxleyi*) exhibited decreased pigment concentrations in particular of the light harvesting pigments (Table 2). As a result, their LP/LH ratio increased by 68%, 98% and 141%, respectively. All other cultures showed slight fluctuations in their harvesting pigment content and increased xanthophyll pigment concentrations thereby increasing the LP/LH ratio on average by 27%. *N. frigida, O. sinensis* and *E. huxleyi* showed consistently declining pigment

concentration during the recovery period ($t = 3$ until $t = 24$). All other species had significantly higher LP/LH ratios after 21 h recovery in low irradiance (ANOVA, $p < 0.05$; except for *Pyramimonas* sp. and *N. ovalis*) than before the SSI treatment (on average 34% increased LP/LH ratio).

Xanthophyll cycle activity

Before, during and after the SSI treatment, (changes in) xanthophyll cycle activity depended neither on volume-specific cell surface area, geographic region, or taxonomic affiliation. Throughout the experimental period ($t = 0$ until $t = 24$), *N. frigida, O. sinensis* and *P. purpureum* (which did not contain an active xanthophyll cycle) did not show changes in de-epoxidation status (DEPS: DT/DD $+$ DT or zea- $+$ antheraxanthin/viola- $+$ anthera- $+$ zeaxanthin) at all (Fig. 2). As a response to the SSI treatment, all cultures with an active xanthophyll cycle significantly (t-test; $p < 0.005$) increased their DEPS, on average between 27% (*N. ovalis*) and 76% (*F. japonica*). DEPS decreased significantly (t-test; $p < 0.005$) during recovery. Correlation analyses (Table 3) showed a positive and significant relation ($r = 0.747, p < 0.005$) between the increase in DEPS after the SSI treatment and recovery of Fv/Fm after 3 h in low irradiance.

Superoxide dismutase

Quantitative analyses of SOD on non-denaturating PAGE gel showed a unique isozyme pattern for all species (except *Chaetoceros* sp. and *C. brevis*; Fig. 3). The composition differed both in number of detected bands and in their quantitative presence. The total brightness of the bands did not coincide with the quantity of SOD activity measured by the RF/NBT enzyme assays. The native page gel showed higher SOD activity for *N. ovalis* than for *O. sinensis* or *P. micans*, while actual measurements showed the contrary. Within these patterns, no bands were found to be specific for volume-specific cell surface area, taxonomic or geographic background. Despite the wide variety of isozymes, none of the bands was exclusively reserved for a certain taxonomic group (polar or temperate, diatoms or flagellates). Species of the genus *Chaetoceros* demonstrated a high level of similarity (in contrast to *Navicula* or *Thalassiosira*), but their bands were also shared with other genera. Inhibition experiments indicated that MnSOD was most abundant for all species (results not shown). A majority of the species also contained one or two FeSOD isozymes, but their appearance was much less pronounced than MnSOD. With the exception of *N. pelliculosa*, FeSOD was found in all diatoms and was absent

Table 2. Ratio of light-protective pigment to light-harvesting pigment (LP/LH ratio) of 15 microalgae when acclimated to low-irradiance conditions (Initial; 25 µmol m^{-2} s^{-1} PAR), after 3 h exposure to SSI conditions (after 3-h SSI; 1,300 µmol m^{-2} s^{-1} PAR + 42 W m^{-2} UVA + 0.47 W m^{-2} UVB) and after the subsequent 21 h recovery period (after 21-h recovery) under low irradiance conditions (25 µmol m^{-2} s^{-1} PAR) including a dark period from $t = 13$ until $t = 21$.

	Initial LP/LH ratio	After 3h SSI			After 21h recovery		
		LH	LP	LP/HP ratio	LH	LP	LP/LH ratio
Antarctic microalgae							
Chaetoceros brevis	0.116	−3.6	4.7	0.125*	10.8	68.8	0.175*
Pyramimonas sp.	0.051	14.4	23.7	0.055*	−3.2	−2.3	0.052
Chaetoceros sp.	0.127	7.0	17.4	0.139*	17.5	76.2	0.186*
Nitzschia frigida	0.051	−32.9	−16.0	0.074	−54.3	−22.7	0.090*
Chaetoceros dichaeta	0.113	−17.7	2.5	0.144*	−25.9	26.8	0.163*
Temperate diatoms							
Nitzschia ovalis	0.078	−4.9	16.3	0.095*	−9.4	6.0	0.090
Navicula pelliculosa	0.124	35.9	42.2	0.130	91.1	141.2	0.158*
Navicula salinarum	0.060	3.2	18.2	0.071	−4.8	22.1	0.075*
Thallasiosira weissflogii	0.056	−13.6	5.5	0.068*	−12.2	11.1	0.069*
Odontella sinensis	0.097	−48.8	−30.0	0.129	−93.9	−100	0
Temperate flagellates							
Porphyridum purpureum	n.a.	−34	n.a.	n.a	−44.4	n.a.	n.a
Emiliania huxleyi	0.086	−47.5	−17.5	0.156	−95.9	−100	0
Tetraselmis suecica	0.032	23.1	33.4	0.035	6.3	34.7	0.041*
Fibrocapsa japonica	0.032	0.5	74.9	0.055*	−6.2	33.0	0.044*
Prorocentrum micans	0.098	21.9	34.4	0.108*	−6.8	31.4	0.138*

*Increased significantly from Initial (*t*-test, $p < 0.05$). n.a.: not applicable.
Note: All separate changes of light harvesting (LH) and light protective pigments (LP) are indicated as a percentage of the initial amount of pigment.

in all flagellates. None of the species studied showed the presence of Cu/Zn-SOD.

Quantitative SOD activity varied significantly (One-way ANOVA, $p < 0.0005$) between the 15 species ranging from 140 (*C. brevis*) to 509 U/mg protein (*P. micans*; Figs 3, 4). This variation was significantly (univariate nested ANOVA, $p < 0.005$; $r^2 = 0.47$) related to volume specific cell surface area; the smaller the surface area/ volume (SA/V) quotient, the higher the specific activity of SOD (Fig. 4a). To compare responses of different species, antioxidant activity was expressed as relative changes (Table 4). After SSI exposure, different SOD responses were observed, both in direction and magnitude of change (Fig. 5, Table 4). The (relative) response to the SSI treatment was significantly influenced by cell size (univariate nested ANOVA, $p < 0.01$; $r^2 = 0.41$; Fig. 4b); species with the smallest volume specific cell surface area showed the largest decrease, while species with the highest specific surface area showed the largest increase. Correlation analyses showed that the initial SOD activity ($t = 0$) was significantly and negatively related to short-term (3 h) recovery (Table 3); species with lower initial SOD activity showed higher recovery after SSI exposure. During the recovery period ($t = 3$ till $t = 24$), all 15 species showed a wide variety of responses (Table 4) which were not dependent on volume specific cell surface area, geographic background or taxonomic group.

Other antioxidant parameters

At the beginning of the experiment ($t = 0$), APX activity, GR activity and the glutathione redox status (GRS; GSH/GSH + 0.5 GSSG) showed significant (One-Way ANOVA, $p < 0.0005$) and large differences between the 15 microalgal species (Fig. 5). APX activity varied between 0.27 (*C. brevis*) and 3.14 U/mg prot (*O. sinensis*), GR activity varied between 0.002 (*T. weissflogii*) and 0.19 U/mg prot (*Chaetoceros* sp.), and the GRS varied between 0.13 (*T. weissflogii*) and 0.61 (*C. dichaeta*). At the beginning of the experiment, Antarctic species were found to have significantly lower APX activity (univariate nested ANOVA, $p < 0.05$) and significantly higher GRS values (univariate nested ANOVA, $p < 0.05$) than temperate species. None of these antioxidant parameters showed any volume-specific cell surface area dependency. After SSI exposure, differences in APX and GRS as well as in GR were not explained by cell size, geographic background or taxonomic group. There was a positive relation between GR activity and short-term (3 h) recovery of Fv/Fm while APX activity and GRS showed negative relations with recovery (Table 3). The latter relation was particularly reflected in the response of the poorly recovering species *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi* which significantly (*t*-test; $p < 0.05$) increased their GRS while all other cultures showed a significant (*t*-test;
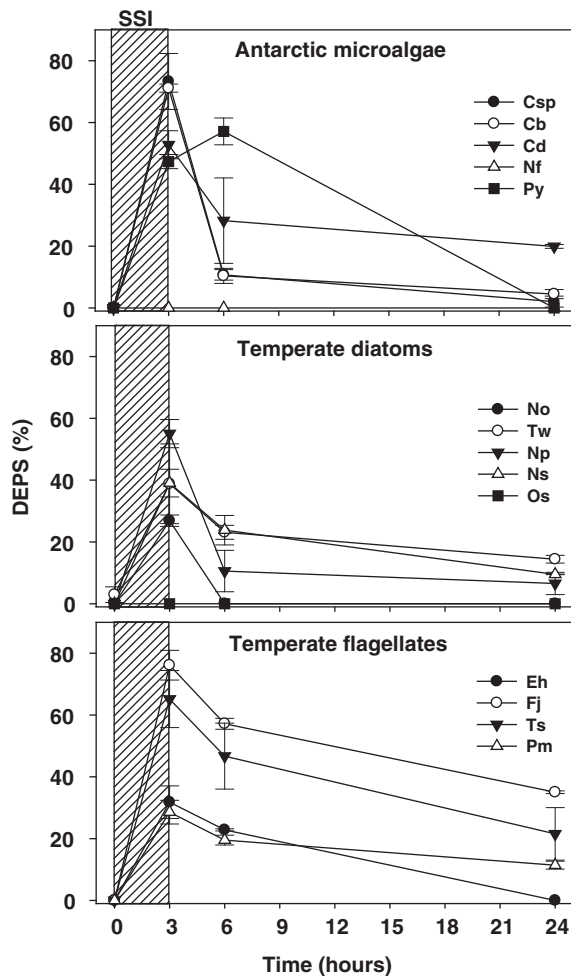
Fig. 2. De-epoxidation state $(DEPS = DT/DT + DD)$ of the photoprotective xanthophyll pigments of 15 microalgae when acclimated to low-irradiance conditions $(t = 0;$ $25 \, \mu mol \, m^{-2} \, s^{-1}$ PAR), after 3 h exposure to SSI conditions $(t = 3;\ 1,300 \, \mu mol \, m^{-2} \, s^{-1}\ PAR + 42 \, W \, m^{-2}\ UVA + 0.47$ $W \, m^{-2}$ UVB) and during the subsequent 21-h recovery period under low irradiance conditions $(t = 3$ until $t = 24;$ $25 \, \mu mol \, m^{-2} \, s^{-1}$ PAR), which included a dark period from $t = 13$ to $t = 21$. *Porphyridium purpureum* did not have detectable amounts of xanthophyll pigments and therefore is not represented. Error bars represent standard deviations $(n = 3)$. The dashed area $(t = 0$ until $t = 3)$ represents the 3-h exposure period. Abbreviations as in Fig. 1.

ANOVA, $p < 0.05$) decrease or no significant change at all (Fig. 5). Correlation analyses showed that the relative change in GRS had a significant and negative relation to the relative change in GR activity, DEPS of xanthophyll pigments and short-term (3 h) recovery of Fv/Fm (Table 3). Also, the relative change in APX activity was significantly and positively correlated with the relative change in SOD activity. During recovery, changes in APX activity, GR activity and GRS values were species specific (Table 4) and independent of volume-specific cell surface area, geographic background or taxonomic group.

Table 3. Correlations between parameters superoxide dismutase (SOD) activity, de-epoxidation state of xanthophyll pigments (DEPS), ascorbate peroxidase (APX) activity, glutathione reductase (GR) activity, glutathione redox status (GRS) and short-term recovery capacity of Fv/Fm (3 h PSII recovery).

Parameters		Correlation data $(n = 15)$	
X-axis	Y-axis	r	p
SOD $(t = 0)$	3 h Fv/Fm recov.	-0.639	< 0.05
SOD $(t = 3)$	3 h Fv/Fm recov.	-0.714	< 0.005
DEPS $(t = 3)$	3 h Fv/Fm recov.	0.747	< 0.005
APX $(t = 3)$	3 h Fv/Fm recov.	-0.386	$> 0.05*$
GR $(t = 3)$	3 h Fv/Fm recov.	0.632	< 0.05
GRS $(t = 3)$	3 h Fv/Fm recov.	-0.614	< 0.05
ΔGRS	3 h Fv/Fm recov.	-0.732	< 0.005
ΔGRS	ΔGR	-0.593	< 0.05
ΔGRS	DEPS	-0.613	< 0.05
ΔSOD	ΔAPX	-0.700	< 0.005

*: Not significant.

Notes: Analyses were performed using average values $(n = 3)$ of 15 microalgae when acclimated to low-irradiance conditions $(t = 0;$ $2 \, \mu mol \, m^{-2} \, s^{-1}$ PAR) and after 3 h exposure to SSI conditions $(t = 3;\ 1300 \, \mu mol \, m^{-2} \, s^{-1}\ PAR + 42 \, W \, m^{-2}\ UVA + 0.47 \, W \, m^{-2}$ UVB). Responses (Δ) were analysed (Spearman's rho correlation test) by correlating percentage changes during the SSI treatment expressed as percentages.

UV-absorbing compounds

UV-absorbing compounds were found in the three large species *P. micans*, *F. japonica* and *C. dichaeta*. The first two species did not significantly change their UV-absorbing capacity during SSI exposure and recovery, which remained on average between 0.038 UV abs/Chl a $(n = 12, SD = 0.004)$ and 0.18 UV abs/Chl a $(n = 12, SD = 0.008)$, respectively. *C. dichaeta* did not increase its UV-absorbing compounds/Chl a ratio as an immediate response to the SSI treatment, but did significantly (t-test, $p < 0.05$) increase its UV abs/Chl a ratio from 0.093 $(n = 4, SD = 0.014)$ to 0.128 $(n = 4, SD = 0.007)$ after 3 h of recovery.

Discussion

In this study, we demonstrated that transition of low-light acclimated microalgae to high irradiance including UVR resulted in highly species-specific responses of photosystem II efficiency (Fv/Fm), pigment composition and antioxidant activities. Studies addressing irradiance induced physiological responses, often use malondialdehyde (product of lipid peroxidation) levels as a stress indicator (Rijstenbil, 2002; Zhang et al., 2005; Buma et al., 2006). Yet, changes in these stress metabolites are subject to rapid metabolic interference by which they are less suitable for this short-term study (Janero, 1990; Janknegt et al., 2008). Although Fv/Fm recovery is an overall stress indicator,
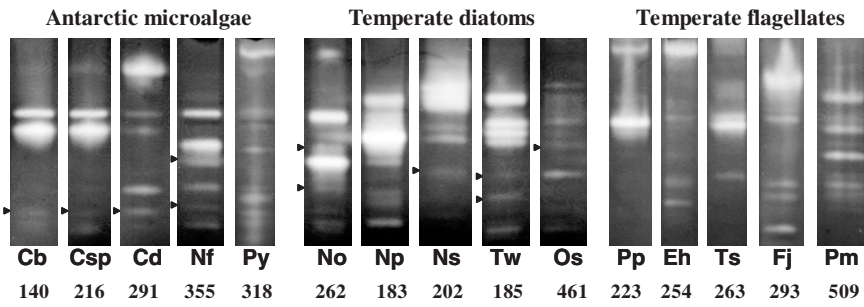
Fig. 3. SOD isozyme composition of 15 microalgae visualized on a 12.5%-native polyacrylamide gel. Species were low-irradiance acclimated (25 μmol m^{-2} s^{-1} PAR). Inhibition analysis identified MnSOD and FeSOD (indicated by the arrowheads). Numbers beneath species abbreviations represent SOD activity values as measured using the RF/NBT SOD activity assay. Abbreviations as in Fig. 1.
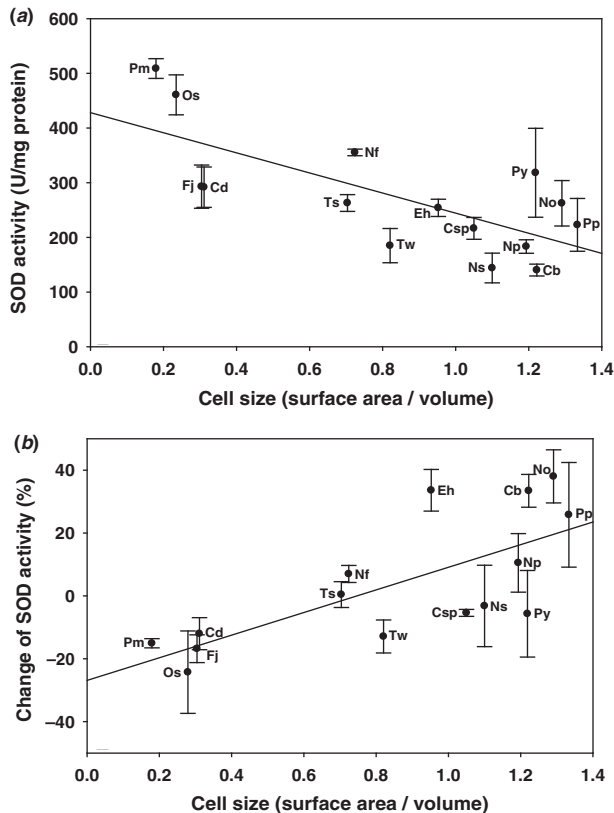

Fig. 4. Cell-size-related activity of superoxide dismutase (SOD). (a) Activity of 15 microalgae when acclimated to low-irradiance conditions (25 μmol m^{-2} s^{-1} PAR) is significantly related to cell size (univariate nested ANOVA, $p < 0.005$; $r^2 = 0.47$). (b) Percentage change in SOD activity after 3 h exposure to SSI conditions (1,300 μmol m^{-2} s^{-1} PAR + 42 W m^{-2} UVA + 0.47 W m^{-2} UVB) compared with initial ($t = 0$) activity is significantly related to cell size (nested ANOVA, $p < 0.01$; $r^2 = 0.41$). Error bars represent standard deviations of the percentage change ($n = 3$). Abbreviations as in Fig. 1.

it does respond rapidly and accurately and is liable to irradiance shifts and therefore, was more appropriate as a measure for photosensitivity than levels of stress metabolites in our study.

After 3 h SSI exposure, the Fv/Fm of all cultures was dramatically reduced whilst the DEPS of

xanthophyll pigments was simultaneously increased. After 3 h of recovery, most cultures showed a significant decrease in DEPS, accompanied by an increased Fv/Fm. *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi*, on the other hand, did not show substantial PSII recovery, probably because of irreversible damage (Bouchard *et al.*, 2005*a*, *b*). The overall inverse relation between DEPS and Fv/Fm demonstrates the involvement of an active xanthophyll cycle in rapid and reversible PSII down-regulation during light stress (Falkowski & LaRoche, 1991; Goss *et al.*, 1999; Mewes & Richter, 2002). Its significance was further emphasized by the positive relation between the induction of DEPS during the SSI treatment and the subsequent recovery of Fv/Fm under low irradiance. The photoprotective role of the xanthophyll cycle was also apparent in changes of the pigment composition as all cultures had increased their light protective/light harvesting (LP/LH) ratio as a result of the SSI treatment (Table 2). These findings agree with other studies that confirmed the positive relation between high irradiance acclimation and increasing xanthophyll content as well as their DEPS (Buma *et al.*, 2006; van de Poll *et al.*, 2006; Dimier *et al.*, 2007). During recovery in low irradiance, all photosynthetically active cultures increased their LP/LH ratio, probably due to *de novo* synthesis of xanthophyll pigments. In contrast, the LP/LH ratio of *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi* increased due to faster degradation of the light-harvesting pigments than xanthophyll pigments. The difference in LP/LH ratio between species containing the VAZ pool and the DT + DD cycle has been reported before (Van Leeuwe *et al.*, 2005; Wagner *et al.*, 2006). Because there was no relationship between type of xanthophyll cycle and PSII recovery, this could not explain the differences in SSI sensitivity.

Although not directly measured in this study, the photosensitivity of *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi*, could be explained by

Table 4. Differences (%) in activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR), and glutathione redox status (GRS) during three subsequent irradiance regimes.

	SOD			APX			GR			Glu		
	3 h SSI	3 h rec	18 h rec	3 h SSI	3 h rec	18 h rec	3 h SSI	3 h rec	18 h rec	3 h SSI	3 h rec	18 h rec
Antarctic microalgae												
Cb	33 ± 5	−4 ± 4	7 ± 4	67 ± 18	−8 ± 10	7 ± 20	−4 ± 4	75 ± 30	35 ± 22	−14 ± 2	−4 ± 6	29 ± 5
Py	−6 ± 14	2 ± 13	10 ± 15	85 ± 64	−17 ± 9	−3 ± 14	−74 ± 7	−3 ± 38	−5 ± 45	−25 ± 5	−10 ± 13	−27 ± 63
Csp	−5 ± 1	−4 ± 4	14 ± 8	−14 ± 22	35 ± 10	−9 ± 14	7 ± 8	13 ± 4	−1 ± 4	2 ± 18	−13 ± 7	31 ± 14
Nf	7 ± 3	4 ± 4	−3 ± 12	44 ± 8	−2 ± 10	15 ± 2	−55 ± 22	−13 ± 3	48 ± 99	50 ± 3	20 ± 10	−38 ± 5
Cd	−12 ± 5	4 ± 7	−10 ± 1	35 ± 17	11 ± 7	7 ± 6	−35 ± 12	49 ± 8	21 ± 12	−24 ± 6	26 ± 13	7 ± 1
Temperate diatoms												
No	38 ± 8	−10 ± 5	34 ± 4	38 ± 16	24 ± 21	17 ± 10	−56 ± 4	88 ± 33	25 ± 5	4 ± 6	−1 ± 7	1 ± 3
Np	11 ± 9	5 ± 14	−13 ± 6	26 ± 13	6 ± 11	−15 ± 27	29 ± 8	−12 ± 2	6 ± 13	−24 ± 2	−4 ± 7	16 ± 17
Ns	−3 ± 12	24 ± 9	−10 ± 6	36 ± 31	1 ± 6	−6 ± 14	−22 ± 2	−16 ± 5	29 ± 7	−30 ± 13	−16 ± 23	21 ± 33
Tw	−13 ± 5	−14 ± 15	−17 ± 3	−10 ± 4	23 ± 1	−3 ± 2	−5 ± 5	−12 ± 10	40 ± 23	17 ± 19	1 ± 1	−1 ± 19
Os	−24 ± 13	28 ± 8	−6 ± 13	34 ± 12	17 ± 25	19 ± 11	−36 ± 5	16 ± 13	−11 ± 3	17 ± 19	−39 ± 6	40 ± 26
Temperate flagellates												
Pp	26 ± 17	−4 ± 12	11 ± 9	14 ± 13	22 ± 23	13 ± 18	−48 ± 19	−9 ± 9	20 ± 8	37 ± 14	7 ± 16	−25 ± 13
Eh	34 ± 7	28 ± 1	−19 ± 1	106 ± 39	−72 ± 3	190 ± 19	−39 ± 3	−80 ± 2	469 ± 44	43 ± 28	−16 ± 8	−2 ± 2
Ts	0.5 ± 4	−20 ± 1	9 ± 3	−9 ± 10	−37 ± 14	22 ± 14	39 ± 41	33 ± 69	−7 ± 2	−28 ± 4	63 ± 3	−6 ± 3
Fj	−17 ± 4	−28 ± 10	34 ± 23	−6 ± 24	25 ± 8	16 ± 6	−15 ± 11	11 ± 13	−17 ± 15	30 ± 29	35 ± 17	57 ± 64
Pm	−15 ± 1	−10 ± 2	23 ± 8	14 ± 9	−9 ± 13	9 ± 14	−48 ± 4	60 ± 16	−9 ± 19	−10 ± 10	18 ± 12	9 ± 22

Abbreviations: Cb: *Chaetoceros brevis*; Cd: *Chaetoceros dichaeta*; Csp: *Chaetoceros* sp.; Eh: *Emiliania huxleyi*; Fj: *Fibrocapsa japonica*; Nf: *Nitzschia frigid*; No: *Nitzschia ovalis*; Np: *Navicula pelliculosa*; Ns: *Navicula salinarum*; Os: *Odontella sinensis*; Pm: *Prorocentrum micans*; Pp: *Porphyridium purpureum*; Py: *Pyramimonas* sp; Ts: *Tetraselmis suecica*; Tw: *Thalassiosira weissflogii*.
Notes: Low-irradiance-acclimated cultures ($25\,\mu mol\,m^{-2}\,s^{-1}$ PAR) were exposed to SSI conditions ($1300\,\mu mol\,m^{-2}\,s^{-1}$ PAR + $42\,W\,m^{-2}$ UVA + $0.47\,W\,m^{-2}$ UVB) for 3 h (3 h SSI). SSI exposed cultures recovered under low irradiance conditions ($25\,\mu mol\,m^{-2}\,s^{-1}$ PAR) for 3 h (3 h rec). Cultures recovered for an additional 18 h under low-irradiance conditions including a dark period from $t = 13$ until $t = 21$ (18 h rec). ±: standard deviation ($n = 3$).
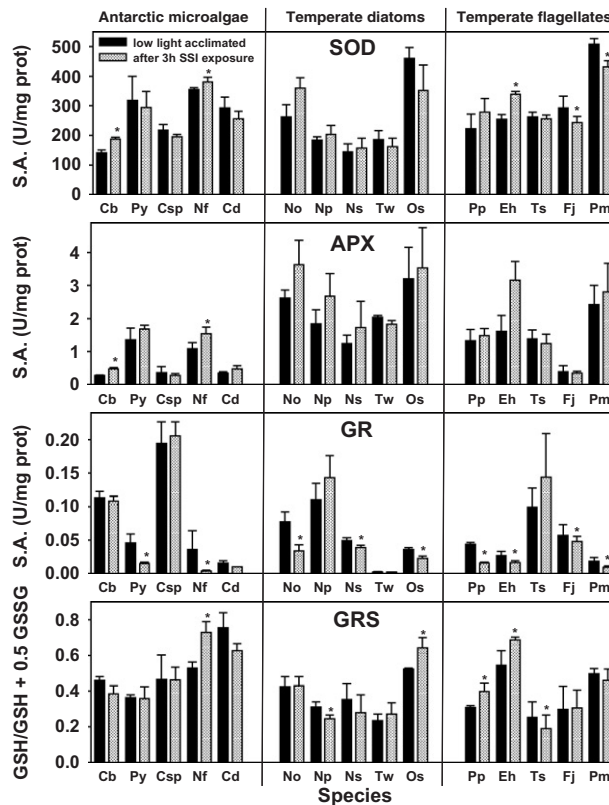

Fig. 5. Activity of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione redox status (GRS) of 15 microalgae when acclimated to low-irradiance conditions (black bars; $25\,\mu mol\,m^{-2}\,s^{-1}$ PAR) and after 3 h exposure to SSI conditions (dashed bars; $1,300\,\mu mol\,m^{-2}\,s^{-1}$ PAR + $42\,W\,m^{-2}$ UVA + $0.47\,W\,m^{-2}$ UVB). Error bars represent standard deviations ($n = 3$). Abbreviations as in Fig. 1. *Differs significantly from $t = 0$ (*t*-test, $p < 0.05$).

uncontrolled generation of reactive oxygen species (ROS; Ishida *et al*., 1997; Shiu & Lee, 2005). The synergistic effects of photo-induced damage and (the subsequent) production of ROS could inhibit photosynthesis and cause viability loss (Nishiyama *et al*., 2001; Gechev & Hille, 2005; Van de Poll *et al*., 2005). To avoid damage from (photo-induced) oxygen radicals, cells monitor ROS formation and regulate antioxidant systems accordingly. Because glutathione plays an important role in H_2O_2 detoxification, changes in cellular pro- and antioxidants are reflected in the GRS (De Kok & Stulen, 1993). Therefore, it has been suggested that glutathione acts as a ROS sensing and regulating agent (Wingate *et al*., 1988; Georgiou, 2002; Noctor *et al*., 2002). From this perspective it is striking that the photosensitive species: *N. frigida*, *O. sinensis*, *P. purpureum* and *E. huxleyi* significantly increased GRS after SSI exposure. Moreover, we observed that a decrease in GRS coincided with an increase in DEPS. Previously, it has been reported that increased GRS inhibited xanthophyll cycle activity in plants and therewith increased photoinhibition and oxidative damage (Creissen *et al*., 1999; Xu *et al*., 2000). Thus, it can be speculated that a decrease in GRS caused up-regulation of de-epoxidation activity of the xanthophyll cycle to constrain ROS production. This would avoid PSII reaction centre damage and subsequently enhances Fv/Fm recovery after the SSI treatment. Besides DEPS, we also found a negative relation between the change in GRS and the change in GR activity, its regenerating enzyme, as was reported for plants and macroalgae (Wingsle & Karpinski, 1996; Karpinski *et al*., 1997; Shiu & Lee, 2005).

Only a few studies have determined SOD isozyme composition in microalgae (Asada *et al*., 1977; Okamoto & Colepicolo, 1998; Janknegt *et al*., 2007). Intensive studies on plants revealed the presence of three types of SOD (Mn, Fe and Cu/ZnSOD; Slooten *et al*., 1995; Kliebenstein *et al*., 1998; Gómez *et al*., 2004). In this study we only found Fe- and Mn-type SOD isozymes, of which the latter appeared to dominate in microalgae. Its prevalence in microalgae could be explained due to its association with the lumen side of photosystems and mitochondria; the major sites of superoxide formation (Wolfe-Simon *et al*., 2005). The absence of Cu/ZnSOD (activity) in (most) eukaryotic microalgae was reported previously, and was ascribed to early evolutionary processes (Asada *et al*., 1977; Wolfe-Simon *et al*., 2005; Lesser, 2006). Yet, it cannot be completely discounted that the low amount of protein applied (16 µg) caused Cu/ZnSOD activity to remain beneath its detection limit. Furthermore, it must be noted that SOD inhibition studies are

not always unambiguous. Besides *Chaetoceros* sp. and *C. brevis*, each species had a unique SOD activity pattern, probably due to the combined effects of intracellular enzyme distribution, the associated target signals, use of the co-metal, membrane solubility, quaternary structure and species-specific amino acid substitution (Fridovich, 1975; Allen, 1995; Rao, 1996; Wolfe-Simon *et al*., 2005). Especially the latter property may cause the species specific SOD band patterns. Species singularity of the SOD isozyme patterns was also found by others (Asada *et al*., 1977; Okamoto & Colepicolo, 1998; Lesser, 2006).

Initial SOD activity was the only antioxidant parameter that correlated (negatively) with short-term Fv/Fm recovery after the SSI treatment. This relationship was also apparent directly after SSI exposure. Moreover, we found that increased DEPS coincided with decreased SOD activity. We also showed that volume-specific cell surface area related positively with SOD activity, but negatively with the response to SSI exposure (Fig. 4, Table 3). As reported earlier (Raven, 1991; Garcia-Pichel *et al*., 1994), induction of UV-screening compounds, such as MAAs, would be more cost effective in larger cells (>10 µm radius), than in smaller cells (1–10 µm), primarily because internal self-shading would be higher in larger cells as compared with smaller cells. In other words, the amount of cellular volume protected by a single MAA molecule increases with increasing cell size by which the MAA production costs per unit of protected volume decreases with increasing cell size. Similarly, other compounds (e.g. SOD) that would help to avoid UV damage could operate more effectively in larger cells, thus giving a lower cost-benefit ratio for larger cells. However, this would obviously only apply to SOD and not to the other antioxidants as investigated in our study (Fig. 5). Also, no relationship was observed between UV vulnerability and volume-specific cell surface area, as suggested previously (Garcia-Pichel, 1994). Therefore, any cell-size-related differences in internal self shading did not significantly determine UV vulnerability in our study.

Although SOD activity was related to photosensitivity and volume-specific cell surface area, there was no relationship between volume-specific cell surface area and PSII recovery, which could be for several reasons. First of all, cellular characteristics like shape, morphology and cell wall influence irradiance transmission into the cell and therewith its SSI sensitivity (Karentz *et al*., 1991a; Laurion & Vincent, 1998). In addition, UV-absorbing compounds in large microalgae (SA/V <0.5 µm^2 µm^3), such as MAAs, may have contributed to SSI tolerance (Karentz *et al*., 1991b; Helbling *et al*., 1996; Laurion & Vincent, 1998). Accordingly, we found

that three of the four largest species, *P. micans*, *C. dichaeta* and *F. japonica*, contained UV-absorbing compounds and recovered well after SSI exposure, while *O. sinensis*, with no UV-absorbing compounds, did not recover at all.

In contrast to our expectations, Antarctic microalgae had lower APX activity and higher GRS than temperate species, indicating that the former experienced less oxidative stress when acclimated to low irradiance conditions. Although speculative, this might be due to their lower metabolic rate (Atkin & Tjoelker, 2003) by which the rate of photosynthesis and the consumption of the reduced equivalents could be lower as well. In other words, in order to tune the production of reducing power to the rate of their consumption, Antarctic microalgae would theoretically require a lower amount of photosystems. As a result, less potential electron leakage could occur. As a result, polar microalgae would be less susceptible to ROS formation and therefore would need less antioxidant activity (Dohahue *et al.*, 1997).

Photosensitivity to the SSI treatment decreased with an increasing level of DEPS and GR activity, but with a decreasing level of SOD activity, APX activity (although not significant) and GRS (as discussed above). This contrasts with studies that suggest that stress tolerance increases with increasing levels of antioxidant activity (Donahue *et al.*, 1997; Aguilera *et al.*, 2002b). Responses of SOD and APX activity were probably positively correlated, because of the SOD dependent generation of H_2O_2; a product that is scavenged by APX. H_2O_2 can also act as a signalling molecule, regulating antioxidant activity, including APX, at a genetic and mRNA level (Yoshimura *et al.*, 2000; Neill *et al.*, 2002; Ledford & Niyogi 2005).

The wide variety of antioxidant responses is probably due to the tightly regulated and balanced synergy of the antioxidant network (Kubo *et al.*, 1999; Apel & Hirt, 2004). Accordingly, several studies demonstrated that experimentally imposed up-regulation of SOD activity, or GRS, did not lead to a higher tolerance level against stress (Slooten *et al.*, 1995; Mallick & Mohn, 2000). In addition to H_2O_2 and GRS, there are many regulation factors such as singlet oxygen, superoxide and the hydroxyl radical that can interfere with antioxidant activities (Allen, 1995; Mallick & Mohn, 2000; Laloi *et al.*, 2004). Furthermore, the intracellular location of ROS formation is of importance as well by which antioxidant activity might increase at the site of production, while it decreases at other locations (Willekens *et al.*, 1994; Yosimura *et al.*, 2000). We conclude that volume-specific cell surface area, regional and taxonomic background play a minor role in determining the photosensitivity of microalgae. Like others, we found that differences in excess irradiance tolerance were highly species specific, and depend on the synergy of protection mechanisms and cell characteristics, rather than on volume-specific cell surface area, geographical and taxonomic background (Karentz *et al.*, 1991a; Laurion & Vincent, 1998). Furthermore, our study indicated that GRS plays a crucial role in the sensing and regulation system that counteracts photo-induced stress. Although speculative, the presence of ROS decreases GRS which in turn increases activity of GR and the activity of xanthophyll de-epoxidase. As a consequence, excess energy is dissipated as heat by which the formation of $O_2^{\bullet-}$ is constrained. Therefore, an increase of DEPS not only enhances the efficiency of PSII during recovery, it also reduces production of H_2O_2, which in turn down regulates the activities of SOD and APX. Thus, our study suggests that photosensitivity decreases with increasing xanthophyll cycle activity but decreasing levels of SOD.

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