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Antioxidative Responses of Two Marine Microalgae During Acclimation to Static and Fluctuating Natural UV Radiation

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ABSTRACT

Photoacclimation properties were investigated in two marine microalgae exposed to four ambient irradiance conditions: static photosynthetically active radiation (PAR: 400–700 nm), static PAR + UVR (280–700 nm), dynamic PAR and dynamic PAR + UVR. High light acclimated cultures of *Thalassiosira weissflogii* and *Dunaliella tertiolecta* were exposed outdoors for a maximum of 7 days. Dynamic irradiance was established by computer controlled vertical movement of 2 L bottles in a water filled basin. Immediate (< 24 h), short-term (1–3 days) and long-term (4–7 days) photoacclimation was followed for antioxidants (superoxide dismutase, ascorbate peroxidase and glutathione cycling), growth and pigment pools. Changes in UVR sensitivity during photoacclimation were monitored by measuring UVR-induced inhibition of carbon assimilation under standardized UV conditions using an indoor solar simulator. Both species showed immediate antioxidant responses due to their transfer to the outdoor conditions. Furthermore, upon outdoor exposure, carbon assimilation and growth rates were reduced in both species compared with initial conditions; however, these effects were most pronounced in *D. tertiolecta*. Outdoor UV exposure did not alter antioxidant levels when compared with PAR-only controls in both species. In contrast, growth was significantly affected in the static UVR cultures, concurrent with significantly enhanced UVR resistance. We conclude that antioxidants play a minor role in the reinforcement of natural UVR resistance in *T. weissflogii* and *D. tertiolecta*.

INTRODUCTION

Marine microalgae typically experience fluctuating irradiance conditions due to co-occurring changes in intensity of incoming irradiance (daily and seasonally), cloud coverage and their changing position in the water column (1–4). In a shallow upper mixed layer (UML; 4) vertical mixing can mediate phytoplankton transport through a gradient of photosynthetically active radiation (PAR; 400–700 nm) ranging from saturating irradiance at depth to excess (detrimental) irradiance close to the water surface (3,4). In contrast, UV radiation (UVR; 280–400 nm) is attenuated much faster and can be significantly low at the bottom of the UML. UVR may inhibit repair pathways and therefore algae can benefit from periods with low UVR levels to recover from photodamage (3). Therefore, the net effect of UVR on microalgae in a shallow UML depends on the optical properties of water, mixing depth and speed (3,5).

The detrimental action of excess irradiance including UVR is mostly mediated through the formation of reactive oxygen species (ROS; 6–7). ROS production can be initiated by over-reduction of the photosystems. Over-reduced photosystems can leak electrons onto oxygen and therewith initiate generation of the moderately dangerous superoxide ($O_2^{\bullet-}$). These radicals can donate their electron to other $O_2^{\bullet-}$ radicals *via* unchelated bivalent cations (*i.e.* $Fe^{3+/2+}$) thereby generating hydrogen peroxide (H_2O_2) *via* the Haber–Weiss/Fenton reaction, these two oxygen intermediates are able to produce the most destructive hydroxyl radical ($HO^{\bullet}$; 8). ROS may damage DNA, phospholipids and proteins such as photosystem II (PSII) reaction centers. As a result, ROS may lead to decreased photosynthetic efficiency or even viability loss (9,10).

To prevent irradiance-induced formation of ROS, microalgae apply immediate regulatory mechanisms such as nonphotochemical quenching *via* xanthophyll cycling (11–13). On a longer time-scale, photoacclimation occurs during which photosynthesis and its regulatory mechanisms are adjusted to the prevailing irradiance (9,14). To counteract ROS accumulation, oxygen radicals are scavenged by a network of antioxidants which is aimed to prevent $HO^{\bullet}$ formation. In this network, $O_2^{\bullet-}$ is converted into H_2O_2 by superoxide dismutase (SOD; 15,16) which is subsequently scavenged by peroxidases like ascorbate peroxidase (APX; 17,18). During this process, APX consumes ascorbic acid which is regenerated using glutathione as a reducing agent, followed by glutathione reduction by glutathione reductase (GR; 19,20).

Several laboratory studies have shown that microalgae adjust their cellular antioxidant levels during photoacclimation to altered PAR or UVR (11,21,22). Yet, virtually nothing is known about antioxidant responses in marine microalgae under ambient UVR, let alone under natural fluctuating irradiance regimes. The unpredictable nature of fluctuating

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irradiance may hamper adequate photoacclimation by microalgae. For example, Van de Poll *et al*. (23) found a low irradiance type acclimation response in a diatom under a fluctuating light regime when compared with static conditions when applying an identical daily dose. As a result, cells could be more vulnerable to ROS production in a fluctuating irradiance regime, for instance, due to lower cell quota of protective pigments or antioxidants.

In the present study, we investigated antioxidant acclimation strategies of the diatom *Thalassiosira weissflogii* and the green phytoflagellate *Dunaliella tertiolecta* under static and fluctuating natural UVR, the latter by applying artificial vertical mixing. We chose a diatom and a phytoflagellate because representatives of these taxa have been demonstrated to fundamentally differ in pigment composition, fluctuating irradiance responses as well as in excess irradiance vulnerability including UVR (24–26). It has been suggested that taxon-specific photoprotection strategies depend on the deployment of additional energy dissipation mechanisms (24–27). In contrast to diatoms, green flagellates depend less on their xanthophyll cycle but contain supplementary quenching mechanisms such as rapid redistribution of energy *via* state transitions and cyclic PSI electron transport, the latter potentially leading to enhanced ROS formation (28). Moreover, these two taxa make up an important component of the microalgal assemblages found in Patagonian coastal waters during summer (29).

To unravel antioxidant responses in representatives of these two taxa during acclimation to stable and fluctuating natural UVR, we formulated the following hypotheses: (1) antioxidant levels are enhanced as a result of prolonged natural UVR exposure when compared with PAR only conditions; (2) antioxidant levels are lower in prolonged fluctuating UVR as opposed to static UVR regimes; (3) the diatom has or induces higher antioxidant activity and higher UV resistance during prolonged natural UVR exposure compared with the green flagellate.

MATERIALS AND METHODS

Culturing conditions. *Dunaliella tertiolecta* (Dunal) Teodoresco (Chlorophyceae) and *Thalassiosira weissflogii* (Grunow) G. Fryxell et Hasle (Bacillariophyceae) were obtained from the Algal Culture Collection of Estación de Fotobiología Playa Unión. *Thalassiosira weissflogii* and *D. tertiolecta* were grown at 250 μmol photons m^{-2} s^{-1} (PAR: 400–700 nm; 12/12 h, l per daily cycle) without UVR (280–400 nm). Pre-experimental cultivation was carried out in f/2 enriched autoclaved sea water at a salinity of 34.5 in a culture cabinet (Sanyo MLR 350) at 20°C for approximately 8 weeks prior to the experiments. Cultures were kept in the exponential growth phase by regular dilution with fresh medium.

Experimental design. In total, six outdoor experiments were carried out, with experiments 1 and 2 lasting 7 days, and experiments 3–6 for 1 day only. Experiment 1 (60 min mixing cycle) was performed from 3 to 10 February 2006. Experiment 2 (10 min mixing cycle) was performed from 10 to 17 February 2006. The short-term experiments (Exps. 3–6) were carried out on 31 January and 16 February 2006 for *D. tertiolecta*, and on 15 and 17 February for *T. weissflogii*. During the 7-day experiments, the two species as well as conditions of ± UVR exposure and static *versus* fluctuating irradiance were tested (Fig. 1). Thus, eight experimental bottles (2 L working volume) were incubated simultaneously (Fig. 1). During the short-term experiments, only one species was tested simultaneously. Due to space restriction, no replicates could be run in each experiment. Therefore, experiments were repeated, despite possible differences in natural irradiance conditions (see Results and Discussion).

Static cultures were incubated 15 cm below the surface (static) as this depth represented the mean irradiance for the mixed water column. Mixed bottles were moved up and down through the water column between the water surface and 80 cm water depth (Fig. 1) using a home-made computer-controlled mixing simulator. Sinusoidal vertical transport of the bottles was established by a DC motor (Maxon Motor, Switzerland). The speed of the motor and thus that of the bottles in the water was frequency controlled and adjusted to the required mixing cycles. Note that static and dynamic irradiance conditions did not give identical daily PAR and UVR doses (Table 1 and Discussion).

Radiation treatments. The PAR (400–700 nm) only exposure was carried out in UV transparent 2.2 L polymethylmethacrylate (PMMA) bottles wrapped in 395 nm cut-off filter foil (Ultraphan, UV Opak Digefra, Germany; for transmission characteristics see [30]). The PAR + UVR (PUV: 280–700 nm) samples were incubated in UV transparent 2 L PMMA bottles (for transmission characteristic see [31]). Enclosure of the cultures in the PMMA bottles reduced PAR exposure by *ca* 10% (31) while the 395 nm cut-off foil further reduced PAR exposure by another 5–8%. Hereafter, we will refer to the combination of mixing/static conditions and radiation treatments as (1) mixed PAR, mixed PAR + UVR (mixed PUV), static PAR and static PAR + UVR (static PUV). In the evening before the start of the long-term experiments, 500 mL of culture of *D. tertiolecta* and *T. weissflogii* were transferred to the PMMA bottles, diluted with fresh medium up to a total 2 L of algal culture per bottle and placed outside. During the subsequent 7 days of experimentation, the daily sampling volume (see below, Sampling during 7-day experiments

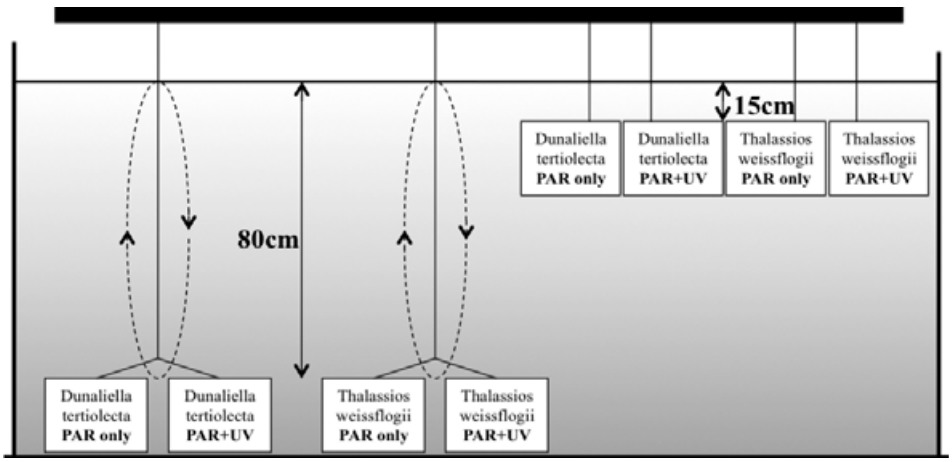

Figure 1. Experimental set-up for the outdoor incubations. During the 7-day experiments, eight bottles were incubated simultaneously: four static (for both species PAR and PAR + UVR), four fluctuating (for both species PAR and PAR + UVR). PAR, photosynthetically active radiation.

Table 1. (a) Daily average irradiance doses (kJ m^{-2}) during short-term and long-term exposure periods of experiment 1 and 2; (b) Calculated daily average outdoor doses (kJ m^{-2}) experienced by the *Dunaliella tertiolecta* and *Thalassiosira weissflogii* PUV cultures for PAR, UVA radiation (315–400 nm) and UVB radiation (280–315 nm)

(a)

	Exp. 1	Exp. 2
Short term		
PAR	6667 ± 2079	7784 ± 521
UVA	776 ± 233	859 ± 59
UVB	17 ± 6	19 ± 1
Long term		
PAR	7944 ± 919	7254 ± 564
UVA	900 ± 92	789 ± 52
UVB	20 ± 2	17 ± 1

(b)

Response time	Condition	PAR	UVA	UVB
Preculturing	Indoors	2200	–	–
Immediate	*Dunaliella tertiolecta* static	8163	958	22
	D. tertiolecta mixed	4897	460	10
	Thalassiosira weissflogii static	6264	673	15
	T. weissflogii mixed	5632	514	11
Short term	Static	7226	818	18
	Mixed	5984	555	12
Long term	Static	7599	845	19
	Mixed	6257	564	12

(a) Values represent irradiance doses experienced by static cultures. Short term: averaged over first 3 days ± SD of the outdoor exposures. Long term: averaged over days 4–7 ± SD of the outdoor exposures. There were no significant differences in photosynthetically active radiation (PAR), UVA (315–400 nm) and UVB radiation (280–315 nm) between the two experiments.
(b) Immediate: day 1 between sunrise and 5:00 P.M. Short term: averaged over first 3 days of the outdoor exposures. Long term: averaged over days 4–7 of the outdoor exposures.

[1 and 2]) was replenished with fresh medium; as a result, the cultures were growing under semicontinuous conditions. In the case of the short-term experiments (Exps. 3–6), the set-up was the same but both species were incubated on different days, and cultures were not replenished.

The experiments were performed in an outside water-filled basin (depth: 1.1 m, diameter: 5 m). Fine sediments of the nearby Chubut river were added to enhance light attenuation and thus to reproduce a bigger portion of the euphotic zone in the basin. Due to the characteristics of the sediments almost no variation in attenuation coefficients was observed for PAR UVAR (315–400 nm) and UVBR (280–315 nm). Irradiance attenuation was measured three times a day, at three water depths, 0.22, 0.44 and 0.8 m, and turbidity was adjusted when necessary using mechanical resuspension of sediments.

Irradiance measurements. Irradiance (PAR, UVAR and UVBR) was continuously recorded as the average of 1 min intervals using a broadband ELDONET radiometer (Real Time Computers Inc.) located on the roof of the institute. Attenuation of UVR and PAR in the reservoir was determined with a USB diode array spectrophotometer (HR 2000CG-UV-NIR; Ocean Optics, Duneclin) with a 10 m fiber optic cable and cosine diffuser. Attenuation coefficients were used to estimate the irradiance for the cultures during mixed and static regimes. By considering mixing depth and mixing speed, average irradiance levels in the mixed cultures were calculated.

Sampling during 7-day experiments (1 and 2). Pre-experimental samples (Initial) were taken for GR (120 mL), glutathione (160 mL), APX (120 mL), SOD (120 mL), pigments (100 mL), UV sensitivity (100 mL) and cell counts (2 mL). During the outdoor incubation, samples were taken daily just before sunrise and 7:30 A.M. for GR, glutathione, pigments, UV sensitivity and cell counts (same sample volumes as Initial). On the days 3 and 7, additional samples were taken for APX. It must be emphasized that photoprotection properties, as described above, might potentially vary during the day in response to actual irradiance conditions. In accordance, other studies described diurnal variations in antioxidant and pigment levels as a response to increased irradiance (11,32). Yet, our study focused on photoacclimation responses (variation in photoprotective pools) and not on diurnal photoregulation. Thus, to avoid the influence of actual photoregulatory processes, samples were taken before sunrise.

Sampling during 1-day experiments (Exps. 3–6). During the 1-day experiments, samples for APX, SOD, GR and glutathione were obtained at 9:00 A.M. In addition, we also sampled at 1:00 P.M. and 5:00 P.M. to collect additional diurnal data.

Processing of samples. Samples for SOD, APX and GR activity and glutathione content were filtered using polycarbonate filters (diameter: 47 mm, 2.0 μm pore size; Osmonics Inc.), frozen in liquid nitrogen and stored at −80°C. Samples for pigment analysis were immediately filtered over GF/F filters (diameter, 25 mm) in a darkened room, immediately frozen in liquid nitrogen and stored at −80°C. Samples for cell counts were fixed with formalin (0.1%, vol/vol) and stored at 4°C in darkness. Samples for the assessment of UV sensitivity were processed immediately (see below).

Cell counts and growth rates. Cell counts were carried out on a Coulter MCL flow cytometer (Beckmann-Coulter) as described in Van de Poll *et al*. (23). For estimation of increase in cell density, cell counts were corrected for the dilution factor. Growth rates were determined by measuring the slope of the natural log (Ln) of corrected cell numbers as a function of time. Growth rates of the initial cultures were determined by measuring the cell concentrations over four successive days, prior to experimentation.

Measurement of UV sensitivity. In order to determine if the UVR sensitivity of the cells changed as the experiment progressed, incubations were carried out using an indoor solar simulator (Hönle, Sol 1200, Germany) and measured carbon incorporation. The use of a constant light source (as the solar simulator) allowed the comparison of samples collected at different times during the experiments under controlled conditions. In contrast, if samples were incubated under solar radiation, the incorporation of carbon would be variable under variable radiation conditions (*i.e.* cloudy *vs* sunny conditions) and thus the sensitivity could not be compared directly. Samples were collected from all outdoor cultures, transferred to quartz tubes (50 mL, in duplicate), spiked with ^{14}C sodium bicarbonate (3.75 μCi) and incubated under the solar simulator for 1.5 h in a water bath inside a temperature-controlled chamber (20°C). Half of the tubes were covered with 395 nm cut-off foil (receiving simulated PAR only) while the others remained uncovered (receiving simulated PUV). The lamp was covered with cellulose acetate film to avoid UVC exposure. The applied radiation was 183, 69.9 and 1.7 W m^{-2} for PAR, UVAR and UVBR, respectively. After exposure, samples were filtered on 25 mm GF/F filters (Whatman), stored in scintillation vials and exposed to concentrated HCl fumes overnight to remove unbound ^{14}C sodium bicarbonate. The activity was quantified using liquid scintillation counting; ^{14}C incorporation, corrected for dark controls and normalized to chlorophyll *a* (Chl *a*) concentration measured at the beginning of the exposure to provide carbon assimilation numbers. Chl *a* concentration was determined by filtering 50 mL culture on 25 mm GF/F filters followed by extraction in (100%) methanol. The samples were sonicated (20 min) and extracted for at least 2 h in darkness at 4°C. After centrifugation (10 min, 2500 ***g***), the Chl *a* concentration of the supernatant was determined by measuring the fluorescence of the extract before and after acidification, using a calibrated Turner Designs TD700 fluorometer. Chl *a* was also determined later using HPLC (see below) at the University of Groningen. There was an excellent agreement between both techniques as found earlier (29). UV sensitivity was determined by calculating the relative difference in carbon assimilation between PAR-only and PUV exposure, expressed as percentage UVR inhibition.

SOD activity. Superoxide dismutase analyses were carried out using the riboflavin/nitro blue tetrazolium (NBT) method modified for algal material, as described in Janknegt *et al*. (33). Filters were disrupted by sonication in 0.75 mL protein extraction buffer X (50 mM KH_2PO_4, pH7.8, 0.1 mM EDTA, 0.1% Triton X-100, 2% polyvinylpyrrolidone and complete protease inhibitor cocktail). After spinning down cell

debris, supernatants were transferred to a clean 1.5 mL Eppendorf tube. SOD activity was defined as the amount of sample required to achieve 50% inhibition of the NBT reduction (V_{50}). NBT reduction was measured (in duplicate) in four reaction mixtures containing 0 (blank, no inhibition), 12.5, 25 and 50 μL extract. 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. V_{50} was calculated by regression using the linear part of a natural semilog curve. The specific activity (S.A.) of SOD was calculated according to:

$$\text{S.A.} = 1/(V_{50} \times [\text{protein}])$$

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. 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, pH 7, and 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). Before H_2O_2 was added, the reaction mixture was incubated for 5 min at 20°C. The rate of ascorbate consumption was measured at 290 nm on a Cary 3E UV/VIS double beam spectrophotometer. Specific APX activity (U mg^{-1} protein) was calculated according to:

$$\text{S.A.} = -\text{slope} \times \text{volume}_{\text{tot}}/\varepsilon \times \text{volume}_{\text{extr.}}/[\text{protein}]$$
$$\text{where } \varepsilon = 2.8\ \text{mM}^{-1}\ \text{cm}^{-1}$$

Determination of GR activity was based on its catalytic property to convert oxidized glutathione (GSSG) into its reduced form (GSH) thereby consuming NADPH (reduced forms of nicotinamide adenine dinucleotide phosphate) as a reducing agent. The 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), 300 μL cell extract and 50 μL, 2.5 mM NADPH (Merck). Before NADPH was added, the reaction mixture was incubated for 5 min in a cryostat at the appropriate temperature. Specific activity of GR (in U mg^{-1} protein) was calculated according to:

$$\text{S.A.} = -\text{slope} \times \text{volume}_{\text{tot}}/\varepsilon \times \text{volume}_{\text{extr.}}/[\text{protein}]$$
$$\text{where } \varepsilon = 6.22\text{mM}^{-1} \cdot \text{cm}^{-1}$$

Glutathione content. Filters were suspended in 0.4 mL glutathione extraction buffer containing 50 mM sulfosalicylic acid, 1 mM EDTA and 0.15% ascorbic acid followed by cell disruption 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; St. Louis, MO); all chemicals were purchased from Sigma unless indicated otherwise. The assay was performed in 96-well microtiter plates (Cayman Chemicals) containing 150 μL working mixture 1 (100 mM KH_2PO_4, pH 7, 100 mM EDTA, GR, 0.170 U mL^{-1} and 0.043 mg mL^{-1} 5,5′-dithiobis-2-nitrobenzoic acid) and 10 μL cell extract. After 5 min of incubation at room temperature, 50 μL of a 0.16 mg mL^{-1} NADPH solution was added. 5-Thio-2-nitrobenzoic acid (TNB) production was then followed spectrophotometrically for 10 min at 405 nm using a Victor™ 1420 Multilabel plate reader (PerkinElmer, Waltham, MA) using 1 min intervals. To determine the 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 nmols GSH (Sigma) in 10 μL sample. The TNB production rate was calculated for 1 nmol of glutathione by extrapolation. The TAG (in nmol) in 1 mL algal sample was calculated according to:

$$\text{TAG} = \frac{\text{slope}_{\text{sample}} \times \text{dilution factor original sample}}{\text{slope}_{1\text{nmoleGSH}} \times 0.01\ \text{mL sample}}$$

The amount of GSH was measured by the same principle as TAG but without adding GR and NADPH by which only GSH reacts with Ellman reagents 5,5′-dithiobis-2-nitrobenzoic acid (DTNB). This assay was performed in 96-well microtiter plates containing 100 μL working mixture 2 (500 mM KH_2PO_4, pH 7, 500 mM EDTA and 0.065 mg mL^{-1} DTNB) and 100 μL cell extract. After 10 min of incubation at room temperature, 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 nmols GSH in 100 μL sample. The amount of GSH in the reaction mixture was calculated in accordance to the reference line. The amount of GSH (in nmol) in 1 mL algal extract was calculated according to:

$$\text{GSH in reaction mixture} \times \text{dilution factor original sample}/0.1\ \text{mL sample}$$

The glutathione redox status (GRS) was expressed in sulfur atoms and calculated according to:

$$\text{GSH}/(\text{GSH} + 0.5\text{GSSG}) \quad \text{where GSSG} = \text{TAG} - \text{GSH}$$

Pigments. HPLC pigment analysis was carried out as described by Van Leeuwe *et al*. (34). In short, filters were handled under dim light, freeze-dried (48 h) after which pigments were extracted in 4 mL 90% cold acetone (vol/vol, 48 h, 5°C). 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) and identified by retention time and diode array spectroscopy. Quantification was carried out using standard dilutions of Chl *a*, Chl *b*, fucoxanthin, diadinoxanthin, diatoxanthin, violaxanthin, antheraxanthin and zeaxanthin. Cellular pigment concentrations were calculated from cell counts and extraction volume.

Data analysis and statistics. Differences in irradiance conditions and species were tested using a paired *t*-test. As no significant differences were found in irradiance conditions between the two 7-day experiments (Table 1) we pooled the corresponding days of Exps. 1 and 2. Also we pooled days 1–3 and days 4–7 from Exps. 1 and 2 (Table 1), giving short- and long-term acclimation patterns. Immediate responses were derived from the pooled data of the 1-day experiments (Exps. 3–6). Intraspecific differences between response periods and irradiance treatments (static PUV, static PAR, mixed PUV and mixed PAR) for SOD activity, APX activity, GR activity, GRS, total glutathione content and pigment composition and growth were tested by performing a multivariate ANOVA. When significant differences were found, an LSD *post hoc* test was applied to specify these differences. Statistical analyses were performed using STATISTICA 7 for Windows.

RESULTS

Irradiance conditions

When comparing experiments 1 and 2, the average daily radiation doses between the two short-term and the two long-term periods did not differ significantly (Table 1a). Therefore, the corresponding cultures were exposed to comparable radiation conditions during both experiments. This similarity was reflected in the response parameters of both experiments (UV sensitivity; SOD, GR and APX activity; cellular glutathione content and the glutathione redox potential) which showed highly corresponding trends in time. Therefore, average values of the two experiments were pooled.

During the immediate (< 24 h), short-term (1–3 days) and long-term (3–7 days) periods, the average daily outdoor radiation dose showed fluctuating irradiance conditions (Table 1b). Attenuation coefficients (K_d) in the experimental basin were 0.91 (PAR), 2.74 (UVAR) and 3.95 (UVBR) so that the irradiance intensities at 0.15 m were reduced to 87%, 66% and 55% respectively. At 0.8 m, irradiance intensities were reduced to 48%, 11% and 4%, respectively, compared with surface irradiance. Regardless of the mixing speed, cultures subjected to the simulated mixing regime received on average

79% (PAR), 50% (UVAR) and 36% (UVBR) of the daily radiation dose compared with the static cultures.

Growth

Before their transfer to the experimental basin, the growth rate of *D. tertiolecta* was higher than that of *T. weissflogii* (Table 2). During the first 3 days (short term) of outdoor exposures mean growth rates of *D. tertiolecta* cultures remained at a similar level compared to initial values after which they all decreased significantly ($P < 0.05$) by 37–66% (Tables 2–4). Except for the mixed PAR cultures, growth rates of *T. weissflogii* were already reduced significantly ($P < 0.05$) during the first 3 days (short term) by 14–26% and did not change significantly thereafter (Tables 2–4). Growth rates of both species were significantly ($P < 0.05$) affected by UVR (Tables 2–4). Eventually, *D. tertiolecta* was significantly ($P < 0.05$) more affected by long-term (7 days) outdoor irradiance exposure than *T. weissflogii*.

UVR sensitivity

During the entire experimental period, *D. tertiolecta* showed significantly ($P < 0.05$) higher assimilation numbers than *T. weissflogii* when exposed to PAR under the solar simulator (Fig. 2a,b). However, *D. tertiolecta* showed a significantly ($P < 0.05$) larger reduction in carbon assimilation on the short and long term than the corresponding cultures of *T. weissflogii* (Fig. 2a,b; Table 4). After long-term (7 days) outdoor exposure, assimilation numbers of all cultures of both species had decreased significantly ($P < 0.005$) compared with pre-experimental values (Table 4). Although both species showed lowest assimilation numbers for the static PUV cultures, there were no significant differences between the four outdoor irradiance conditions for both species.

Dunaliella tertiolecta was significantly ($P < 0.05$) more UV sensitive (expressed as percentage UV inhibition) than *T. weissflogii* (Fig. 2c,d; Table 4). In addition, UVR sensitivity of the static PUV cultures of both *T. weissflogii* and *D. tertiolecta* decreased significantly ($P < 0.01$) over time, becoming significantly ($P < 0.0001$) lower than the other (PUV mixed, PAR static and mixed) cultures. UV sensitivity decreased for both species following mixed PAR > static PAR > mixed PUV > static PUV.

Superoxide dismutase and ascorbic peroxidase

Species specific differences were observed in antioxidant enzyme activity and responses. When acclimated to pre-experimental irradiance (250 μmol photons m^{-2} s^{-1}), SOD activity of *D. tertiolecta* (103 U mg^{-1} protein, SD = 3.0) was significantly ($P < 0.05$) higher than *T. weissflogii* (68 U mg^{-1} protein, SD = 10.6; Table 3). APX activity of *D. tertiolecta* (0.89 U mg^{-1} protein, SD = 0.2) was significantly ($P < 0.05$) lower than that of *T. weissflogii* (3.6 U mg^{-1} protein, SD = 0.6; Table 3). During the first day of the outdoor exposure, no significant differences were found between samples taken during the daily cycle (9:00 A.M., 1:00 P.M. and 5:00 P.M.) and therefore these data were pooled. Also, there were no significant differences in SOD and APX activities between the four radiation conditions during short- and long-term exposure. Therefore, these data were pooled as well. *Thalassiosira weissflogii* showed no significant responses in SOD and APX activity regardless of radiation condition or exposure duration (Tables 3 and 4). In contrast, *D. tertiolecta* showed a slight but significantly

Table 2. Growth rates of *Dunaliella tertiolecta* and *Thalassiosira weissflogii*.

Initial	*Dunaliella tertiolecta* 0.53 (0.040)		*Thalassiosira weissflogii* 0.42 (0.035)	
	Short term	Long term	Short term	Long term
Mixed PAR	0.57 (0.088)	0.35 (0.008)	0.42 (0.037)	0.37 (0.180)
Mixed PUV	0.57 (0.075)	0.22 (0.052)	0.31 (0.031)	0.28 (0.021)
Static PAR	0.57 (0.062)	0.33 (0.229)	0.36 (0.012)	0.34 (0.066)
Static PUV	0.57 (0.085)	0.18 (0.094)	0.33 (0.036)	0.26 (0.055)

PAR, photosynthetically active radiation; PUV, PAR + UV.
Initial: acclimated to 250 μmol photons m^{-2} s^{-1} PAR. Short term: exposed for 3 days (1–3 days) to outdoor irradiance conditions. Long term: days 4–7 of outdoor irradiance. In brackets, SE of the linear regression of log-transformed cell numbers.

Table 3. Superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity (U mg^{-1} protein) of *Dunaliella tertiolecta* and *Thalassiosira weissflogii*.

	Thalassiosira weissflogii		*Dunaliella tertiolecta*			
	SOD	APX	SOD	APX	Treatment	Immediate APX
Initial	67.9 (10.6)	3.6 (0.60)	103.4 (3.0)	0.89 (0.21)	S. PAR	0.51*[a] (0.01)
Immediate	n/a	4.4 (1.20)	111.4 (19.8)	See ⇒	S. PUV	0.54*[a] (0.04)
Short term	66.9 (0.8)	3.9 (0.29)	110.9 (3.1)*	0.83 (0.11)	M. PAR	0.83 (0.13)
Long term	64.1 (9.9)	4.4 (0.38)	114.7 (2.5)*	0.91 (0.05)	M. PUV	0.88 (0.13)

PAR, photosynthetically active radiation; PUV, PAR + UV; S. PAR = static PAR culture; S. PUV = static PUV culture; M. PAR = mixed PAR culture; M. PUV = mixed PUV culture; n/a = not available.
Initial: acclimated to 250 μmol photons m^{-2} s^{-1} PAR. Immediate: cultures exposed for 1 day (from sunrise till 5:00 P.M.) to outdoor irradiance conditions. Significant differences were found between the immediate APX activity values of the four treatments (S. PAR, S. PUV, M. PAR, M. PUV) of *D. tertiolecta* (See ⇒). Short term: exposed for 3 days (1–3 days) to outdoor irradiance conditions. Long term: days 4–7 of outdoor irradiance. Values in brackets indicate SD; Initial, Short term and Long term, $n = 4$; Immediate APX (except for *D. tertiolecta*), $n = 12$; Immediate SOD, $n = 6$; Immediate APX activity responses of each of the four *D. tertiolecta* treatments, $n = 3$.
*Differs significantly from initial value.
[a]Differs significantly from other treatments.

Table 4. Long-term (4–7 days) effects on cellular parameters for both species, expressed as percent change relative to initial conditions.

	Thalassiosira weissflogii				*Dunaliella tertiolecta*			
	Mixed PAR	Mixed PUV	Static PAR	Static PUV	Mixed PAR	Mixed PUV	Static PAR	Static PUV
Growth	−12	−33	−19	−38	−34	−58	−37	−66
Ass no.	−44*	−33*	−39*	−54*	−50*	−53*	−52*	−61*
UV inhibition	−7	−21*	−18	−33*	−5	−7*	−5	−16*
APX	+9	+26	+34	+20	−4	+5	−2	+7
SOD	−2	+10	+1	−7	+11*	+11*	+8*	+13*
GR	−16	−17	−14	−13	+4	+16	−9	−2
Glut/cell	+17	+28	+16	+35	−41*	−43*	−31*	−42*
GRS	−5	−2	+3	+10	−30*	−39*	−37*	−39*
Prot/harv	−28	−25	−20	−24	+105*	+115*	+82*	+92*
Harv/cell	−33*	+4	−42*	−11	−71*	−67*	−73*	−64*
Prot/cell	−46*	−13	−48*	−24	−47*	−40*	−54*	−38*

PAR, photosynthetically active radiation; PUV, PAR + UV; Glut, glutathione; GRS, glutathione redox status; GR, glutathione reductase; Prot/Harv, protective over harvesting; APX, ascorbate peroxidase; SOD, superoxide dismutase.
*Differs significantly from initial value.

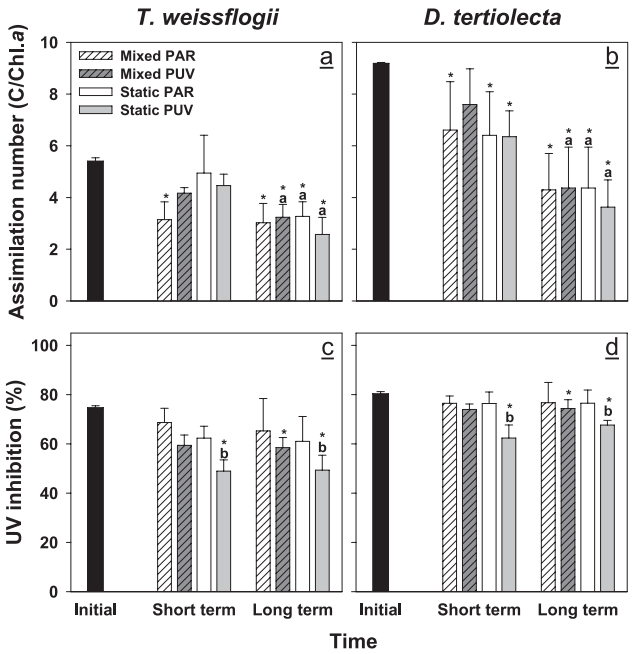

Figure 2. Assimilation number (from solar simulator PAR condition only) and UVR-induced inhibition of ^{14}C incorporation of *Thalassiosira weissflogii* (a,c) and *Dunaliella tertiolecta* (b,d). Short term: cultures exposed up to 3 days. Long term: cultures exposed to outdoor irradiance from days 4 to 7. Bars show mean values of three (Short term) or four (Initial and Long term) replicates, error bars represent SD. *Differs significantly from the initial value ($P < 0.05$); [a]differs significantly from the previous exposure period ($P < 0.05$); [b]differs significantly from the other irradiance conditions within the same exposure period ($P < 0.05$). PAR, photosynthetically active radiation.

($P < 0.05$) enhanced SOD activity after 3 days which remained on this level until day 7 (Tables 3 and 4). Regarding APX activity, only static cultures of *D. tertiolecta* showed an immediate (1 day) significant ($P < 0.05$) decrease by which they were significantly ($P < 0.05$) lower than the cultures exposed to a mixed irradiance regime. After 3 days their APX activities had returned to initial values and remained on this level until day 7 (Tables 3 and 4).

Glutathione and glutathione reductase

After acclimation to 250 μmol photons m^{-2} s^{-1} GR activity of *D. tertiolecta* (0.19 U mg^{-1} protein; SD = 0.014) was significantly ($P < 0.05$) higher than that of *T. weissflogii* (0.052 U mg^{-1} protein, SD = 0.006; Fig. 3a,b). Both species contained comparable amounts of cellular glutathione (Fig. 3c,d; 18.1 nmol per 10^6 cells, SD = 2.1 and 15.9 nmol per 10^6 cells, SD = 1.6 respectively) and a similar GRS (GRS = GSH/GSH + 0.5GSSG; Fig. 2e,f; 0.21, SD = 0.024 and 0.20, SD = 0.014 respectively). During the first day of the outdoor exposure, no significant differences were found during the daily cycle, and therefore these data were pooled. When exposed to outdoor radiation GR activity of *T. weissflogii* showed no response regardless of irradiance condition or duration of exposure (Fig. 3a; Table 4). Static *D. tertiolecta* cultures immediately (1 day) increased their GR activity reaching significantly ($P < 0.05$) higher values than those of the mixed cultures. (Fig. 3b). Subsequently, both mixed cultures increased their GR activity significantly ($P < 0.005$) to similar enzyme activities as the static cultures (3 day) response. During the following 4 days (7 days) all four cultures decreased their GR activity significantly ($P < 0.05$) to initial values (Fig. 3b; Table 4).

Regardless of the outdoor irradiance conditions, all *T. weissflogii* cultures immediately (1 day) responded significantly ($P < 0.0005$) by doubling their glutathione content (Fig. 3c). This was accompanied by a significant ($P < 0.01$) decrease in their GRS (Fig. 3e). Throughout the following 3 days (short term), both parameters returned to their initial values and remained on this level for the rest of the experiments (7 days; Table 4). *Dunaliella tertiolecta* responded by immediately increasing the cellular amount of glutathione of both static cultures significantly ($P < 0.05$) by which they were significantly ($P < 0.05$) higher than both mixed cultures (Fig. 3d). Throughout time, the cellular glutathione content of all cultures dropped significantly ($P < 0.01$) to comparable levels which were significantly ($P < 0.005$) lower than the initial values (Table 4). During the first day outside, the GRS of all *D. tertiolecta* cultures decreased significantly ($P < 0.005$) by which the static cultures had dropped to a significantly ($P < 0.05$) lower level than the mixed cultures

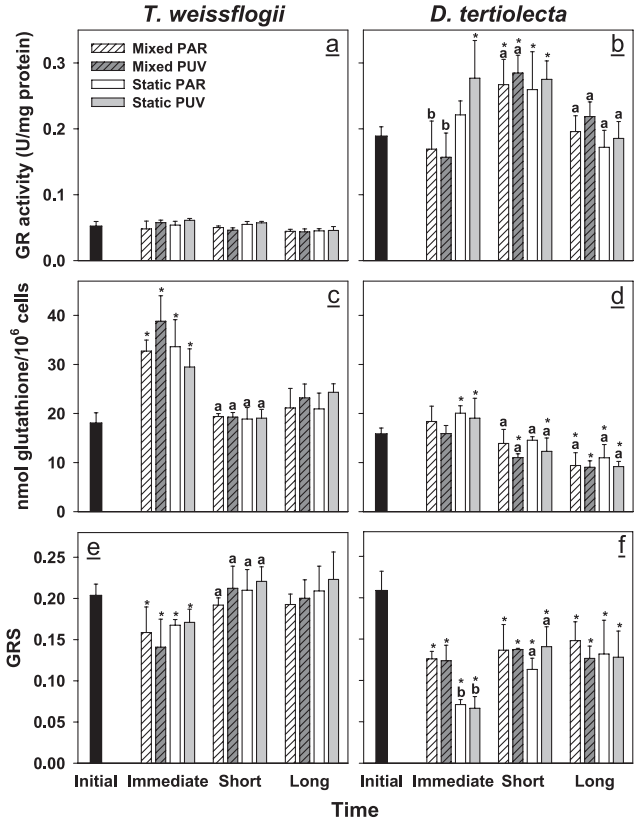

Figure 3. Antioxidant responses of *Dunaliella tertiolecta* and *Thalassiosira weissflogii*. (a,b) Glutathione reductase (GR) activity. (c,d) Cellular amount of glutathione and (e,f) glutathione redox status (GRS). Immediate: cultures exposed for 1 day (from sunrise till 5:00 P.M.) to outdoor irradiance conditions. Short term: cultures exposed for 3 days to outdoor irradiance conditions. Long term: cultures exposed to outdoor irradiance during the 4 subsequent days. Bars show mean values of three (Immediate and Short term) or four (Initial and Long term) replicates, error bars represent SDs. *Differs significantly from the initial value ($P < 0.05$); [a]differs significantly from the previous exposure period ($P < 0.05$); [b]differs significantly from the other irradiance conditions within the same exposure period ($P < 0.05$).

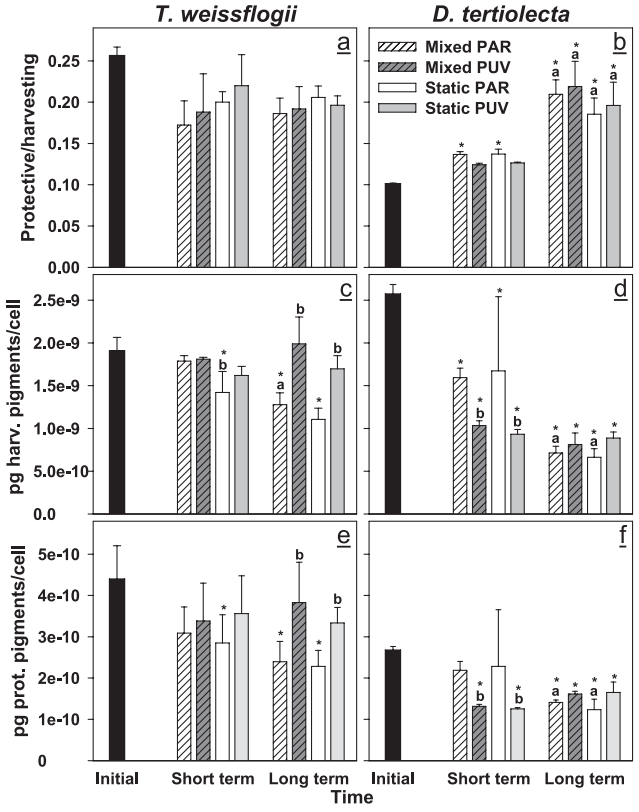

Figure 4. Pigment dynamics of *Thalassiosira weissflogii* and *Dunaliella tertiolecta*. (a,b) Photoprotective overharvesting ratio. (c,d) Cell quota of harvesting and (e,f) photoprotective pigments. Short term: cultures exposed for 3 days to outdoor irradiance conditions. Long term: cultures exposed to outdoor irradiance during the 4 subsequent days. Bars show mean values of three (Short term) or four (Initial and Long term) replicates, error bars represent SDs. *Differs significantly from the initial value ($P < 0.05$); [a]differs significantly from the previous exposure period ($P < 0.05$); [b]differs significantly from the other irradiance conditions within the same exposure period ($P < 0.05$).

(Fig. 3f). During the short-term response, the GRS of both static cultures increased again to similar values as the mixed cultures after which they remained on this same level, significantly lower than initial value (Fig. 3f; Table 4).

Pigments

Thalassiosira weissflogii contained Chl *a* and fucoxanthine (Fuco) as major photoharvesting pigments and the xanthophyll cycle pigments diatoxanthin and diadinoxanthin (DT/DD cycle). *D. tertiolecta* contained Chl *a* and Chl *b* as major light harvesting pigments and the xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin cycle. Before their transfer outdoors, *T. weissflogii* had a significantly ($P < 0.05$) lower content of light harvesting pigments than *D. tertiolecta* (Fig. 4c,d; 1.91 pg per cell, SD = 0.16 and 2.57 pg per cell, SD = 0.11, respectively) and significantly ($P < 0.05$) more protective pigments (Fig. 4e,f; 0.44 pg per cell, SD = 0.08 and 0.27 pg per cell, SD = 0.009 respectively). As a result, the ratio of protective overharvesting pigments (prot/harv ratio; Fig. 4a,b) of *T. weissflogii* (0.26, SD = 0.01) was significantly ($P < 0.0005$) higher than that of *D. tertiolecta* (0.10, SD = 0.001). At the end of the long-term period (7 days), PAR-only exposed *T. weissflogii* had significantly ($P < 0.05$) lower amounts of light harvesting and protective pigments when compared with PUV-exposed cells (Fig. 4c,e; Table 4). However, the prot/harv ratios did not differ significantly between PAR and PUV cultures, and between short- and long-term exposure periods (Fig. 4a).

During the first 3 days (short term) of outdoor exposure, all cultures of *D. tertiolecta* significantly ($P < 0.05$) decreased their cellular amount of harvesting and protective pigments with PUV-exposed cultures containing significantly ($P < 0.05$) less pigments than PAR-only exposed cultures (Fig. 4d,f). As a result of the long-term (7 days) exposure, PAR-only exposed cultures showed significant ($P < 0.05$) decreases in their cellular pigment contents, reaching similar values as cultures subjected to PUV. As a result, prot/harv ratio of *D. tertiolecta* increased significantly ($P < 0.01$; Fig. 4b; Table 4). Yet, no significant differences between the four irradiance conditions were found.

DISCUSSION

Indoor mixing set-ups allow the accurate control of irradiance, but UVR/PAR ratios, the spectral quality within wavelength bands, as well as the irradiance levels fundamentally diverge from those occurring in the water column. Therefore, we chose to perform our experiments under natural irradiance conditions, even though irradiance differences between experimental days were foreseen. The mixing speeds applied during our experiments (10 and 60 min) are naturally occurring conditions in Patagonian coastal waters (35). During the 1-day experiments (Exps. 3–6), weather conditions were rather variable. Yet, irradiance conditions during the two 7-day experiments (Exps. 1 and 2) were highly stable allowing for the comparison between experimental treatments.

Due to the transfer from indoor to outdoor conditions, daily PAR doses increased two- to four-fold (Table 1b), regardless of the irradiance treatments (static, mixed) and despite the high pre-experimental irradiance level (250 μmol photons m^{-2} s^{-1}). In addition, both static and mixed cultures received on average more than 250 μmol photons m^{-2} s^{-1} between 07:45 A.M. and 7:15 P.M., even when mixed cultures were at the lower end of the mixing cycle. Therefore, our experimental irradiance conditions allowed comparison of excess irradiance effects without generating periods of irradiance limitation during the day. UVR intensities, on the other hand, were significantly lower at the bottom than at the surface of the mixing cycle. Therefore, mixed cultures experienced periods of significantly low UVR irradiance as well as lower daily UVR doses (Table 1) when compared with the static cultures.

Intraspecific differences

Acclimation to the outdoor conditions did not lead to significantly different carbon assimilation numbers as determined in the solar simulator under PAR-only, when comparing the four irradiance treatments. This indicates that cultures that were acclimated to outdoor PUV did not exhibit a chronically obstructed photosynthetic machinery (36) when compared with the cultures acclimated to outdoor PAR-only. Similarly, shallow mixing did not chronically affect photosynthetic performance when compared with the static cultures. In contrast, prolonged exposure to outdoor UVR (static as well as mixed cultures) strongly affected growth rates and UVR sensitivity of both species. These results suggest that prolonged elevated excess irradiance induces increasing levels of photoprotection, therewith preventing photosystem damage and reduced carbon incorporation, however at the expense of growth, as found elsewhere (37–39). In support of this, static PUV cultures that received the highest outdoor UVR doses were significantly more resistant to solar-simulated UVR than the other three cultures. This indicates that UVR protection was effectively induced in static PUV cultures, however, at strongly depressed growth rates. Mixed PUV cultures on the other hand exhibited somewhat higher growth rates, yet their UV resistance was only slightly higher than those of the PAR-exposed cultures. In other words, the dynamic regimes increased growth rates, but decreased UVR resistance, possibly related with overall lower UVR levels when compared with the static PUV cultures (Table 1b).

Strikingly, exposure to outdoor UVR or irradiance fluctuations did not induce distinct antioxidant responses between the four treatments in either *T. weissflogii* or *D. tertiolecta* on the longer term (3–7 days). This implies that prolonged outdoor excess PAR rather than fluctuating irradiance or outdoor UVR affected antioxidant levels, if at all (see below). Several studies have stressed the importance of xanthophyll cycling for quenching of excess irradiance energy (40,41). Yet, protective overharvesting (prot/harv) ratios were not significantly different between irradiance treatments, despite the treatment-related changes in cellular pigment quantities. So, similar to the antioxidants, pigment ratios rather responded to the excess outdoor PAR than to additional UVR exposure or fluctuating irradiance. Therefore, irradiance-induced differences in UV sensitivity or growth rates could not be explained by explicit antioxidant or pigment responses. Clearly, other mechanisms must have been responsible for the observed increase in UVR resistance in the static PUV cultures. These could involve upregulation of repair processes (42), increased protein turnover (41), production of UV-absorbing compounds (42–44), *de novo* synthesis of D1 proteins (45), increased level of lipid protecting α-tocopherol (46,47) or rerouting of metabolic pathways (48,49). In conclusion, hypotheses I (antioxidant levels are enhanced as a result of prolonged natural UVR exposure compared with PAR-only conditions) and II (antioxidant levels are lower in dynamic UVR as opposed to static UVR cultures) were rejected.

Interspecific differences

Cell size or surface to volume (S/V) ratio may partly determine excess irradiance or UVR responses in marine microalgae (42,44,50). However, in our study, *T. weissflogii* and *D. tertiolecta* had a similar cell volume (367 and 368 μm^3, respectively) and S/V ratio's (0.82 and 0.69 respectively) and therefore cell size characteristics had a minimal impact on the observed responses. The phytoflagellate *D. tertiolecta* was found to be more sensitive to outdoor excess irradiance including UVR than the diatom *T. weissflogii* when considering carbon assimilation (Fig. 2b; Table 4), UVR resistance (Fig. 2d; Table 4) and growth rate (Tables 2 and 4). It must be noted that growth rates of *D. tertiolecta* during preculturing (250 μmol photons m^{-2} s^{-1}) and the short-term (3 days) outdoor exposures were maintained at a level of 0.53–0.57 per day before the strong reduction (4–7 days, Table 2) occurred. Other authors also found rapid acclimation of *D. tertiolecta* to elevated PAR levels (360–1000 μmol photons per m^2 s), with respect to growth rate (51,52). Havelková-Doušová *et al.* (53) showed increased growth (up to 0.61 per day) with increasing PAR dose during a 1-week period. However growth was slightly reduced at the highest total irradiance dose, being lower than our outdoor conditions (max. of 1750 μmol photons per m^2 s). This could indicate that prolonged exposure to PAR intensities exceeding 1000 μmol photons per m^2 s led to growth inhibition in *D. tertiolecta*. In contrast, growth rates of *T. weissflogii* decreased immediately but to a lesser extent when compared with *D. tertiolecta*. Also growth rates of *T. weissflogii* were not further reduced after 3 days.

During preculturing, *D. tertiolecta* showed higher SOD and GR activity compared with the diatom. Considering that both species showed similar values for GRS and cellular glutathione

content, this indicated that glutathione consumption by *D. tertiolecta* was much higher than by *T. weissflogii*. This implied enhanced levels of ROS in *D. tertiolecta* compared with *T. weissflogii* (22,52,54). APX activity, on the other hand, was nearly four times higher in *T. weissflogii* than in *D. tertiolecta*. Yet, because microalgae use various pathways to remove hydrogen peroxide (catalase, glutathione peroxidase, nonenzymatic antioxidants, diffusion), these results do not imply that *T. weissflogii* experienced enhanced hydrogen peroxide production.

Immediate antioxidant responses during the first day after their transfer indicated enhanced levels of oxidative stress in both species. On a longer term, *D. tertiolecta* increased its SOD and GR activity, decreased its GRS and progressively reduced its glutathione quota throughout the experimental period in contrast to *T. weissflogii*. As SOD is a $O_2^{\bullet-}$ scavenger and glutathione plays an important role in H_2O_2 detoxification (20,55), observed changes suggest prolonged and increased levels of ROS production during short- (3 day) and long-term (7 day) exposure periods. The stoichiometrically inverse relation between GR and GRS has been found before in plants and macroalgae (54,56) and was ascribed to a putative regulatory role of glutathione in controlling (antioxidant) gene expression (19,57,58). *Thalassiosira weissflogii*, on the other hand, did neither significantly change its glutathione parameters, nor its levels of SOD and APX during short- (3 days) and long-term (7 days) outdoor exposure. These responses show that antioxidants are especially important directly after an irradiance shift. As they did not correspond with the differences in growth rates or reduced photosynthetic activity under the solar simulator, antioxidant responses were not found to explain interspecific differences in irradiance sensitivity in our study.

Similar to the antioxidants, *T. weissflogii* did neither show differences in its prot/harv pigment ratio during short- (3 days) and long-term (7 days) exposure to outdoor irradiance conditions. The prot/harv pigment ratio of *D. tertiolecta*, on the other hand, increased gradually to similar values as *T. weissflogii* during exposure to outdoor irradiance. Yet, this increase was not achieved by increasing the cellular amount of protective pigments but by dramatically decreasing the cellular amount of light harvesting and protective pigments, on average 3.5- and 2-fold, respectively. So, despite its increased prot/harv ratio and antioxidant responses, *D. tertiolecta* still showed the largest adverse responses to the outdoor irradiance exposure. In other words, hypothesis III (the diatom has or induces higher antioxidant activity and higher UV resistance during prolonged natural UVR exposure compared with the green flagellate) was rejected. On the one hand, UVR resistance of *T. weissflogii* was higher than that of *D. tertiolecta*. This is in support of earlier studies where diatoms were found to be more resistant to high outdoor irradiance including UVR than flagellates (1,58–60). Yet, this higher resistance could not be related with higher antioxidant activity.

In conclusion, simulated shallow mixing may offer protection against harmful UVR but may increase UVR vulnerability. Second, the green phytoflagellate *D. tertiolecta* was more sensitive to excess irradiance including UVR than *T. weissflogii* as a result of insufficient photoprotection strategies. Third, antioxidants were primarily deployed as an initial response after the irradiance shift to excess outdoor PAR. Finally, antioxidant and pigment responses could not explain the observed differences in growth and UV sensitivity in both species.

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