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NOTE

Photoinhibition in benthic diatom assemblages under light stress

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ABSTRACT: Microphytobenthos are frequently subjected to light intensities higher than those required to saturate photosynthesis, which consequently can cause photoinhibition. Photosystem II (PSII) protein D1 (the main target of photoinhibition) and xanthophyll cycle pigments were quantified in epipelic benthic diatom assemblages under high irradiance, in the presence of inhibitors and promoters of photoprotection mechanisms. Levels of D1 protein were significantly lower under high irradiance (1 h, 1500 µmol photons m^{-2} s^{-1}) than under low light (80 µmol photons m^{-2} s^{-1}), corresponding to a photoinhibition of 22 to 29%. Photoinhibition increased to 44 and 80% in the presence of lincomycin (inhibitor of chloroplast-protein synthesis) and dithiothreitol (inhibitor of the xanthophyll cycle), respectively. High light treatment had no significant effect on D1 protein concentrations in the presence of added glutathione and ascorbate, scavengers of reactive oxygen species (ROS). In contrast, the ROS promoter methylviologen increased photoinhibition to 63%. Under light stress, the functional stability of PSII reaction centres of the studied epipelic benthic diatoms was more dependent on xanthophyll cycle activation than on D1 protein recycling mechanisms, and our results substantiate the role of antioxidants in photoprotection via ROS scavenging.

KEY WORDS: Microphytobenthos · Xanthophyll cycle · D1 protein · Reactive oxygen species · Photoprotection · Antioxidants

INTRODUCTION

Diatoms have developed several photoprotective mechanisms to cope with high light and minimize photoinhibition. The main physiological process involved in photoprotection in diatoms is the thermal dissipation of harmful excess energy through the diadinoxanthin (Ddx) cycle (Olaizola et al. 1994, Lavaud et al. 2004). Pigment conversion of Ddx to diatoxanthin (Dtx) induces non-photochemical quenching (NPQ) in the antenna pigment–protein complexes that decrease the excitation rate of Photosystem (PS) II reaction centres (Müller et al. 2001). Diatoms have been shown to form NPQ 3 to 5 times larger than in higher plants (Ruban et al. 2004).

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Additional photoprotection mechanisms include photorespiration, cyclic electron transport and photoreduction of molecular oxygen by PSI (see review by Niyogi 1999). The latter mechanism, associated with the scavenging of damaging reactive oxygen species (ROS) via the water–water cycle (see Asada 2006), has been shown to play an important role in the photoprotection of a planktonic diatom (Waring et al. 2010). Effective antioxidant systems employing several molecules (e.g. tocopherols, ascorbate and glutathione) and scavenging enzymes limit ROS lifetime and accumulation in the chloroplasts (Niyogi 1999, Foyer et al. 2006).

If the above-mentioned photoprotective mechanisms are insufficient to counteract overexcitation of PSII and accumulation of ROS, damage to the photosynthetic apparatus occurs. This damage is particularly relevant at the level of the PSII reaction centre protein D1 (Ohad et al. 1990, Aro et al. 1993). Fast turnover of multiple PSII subunits, including D1 protein, and the induction of transcriptional processes involved in the protection of cellular structures have been shown in diatoms at an early phase of high light exposure (Nymark et al. 2009, Wu et al. 2011). If photoinactivation exceeds the rate of repair, then photoinhibition of photosynthesis occurs because the pool of D1 protein and active PSII reaction centres decline.

Benthic microalgae (microphytobenthos) have been shown to play key roles in the productivity, trophic dynamics and sediment stability of shallow coastal ecosystems (MacIntyre et al. 1996). In intertidal sand and mudflats, diatom-dominated communities form dense surface biofilms despite the extreme variability in various key environmental parameters, including fluctuating light regimes and punctuated exposure to high light levels. Photoinhibition of PSII in microphytobenthos has been typically assessed indirectly by chlorophyll fluorescence techniques (e.g. Kromkamp et al. 1998, Perkins et al. 2010, Serôdio et al. 2012). After a light stress, the slow recovery of the quantum yield of PSII is ascribed to the photoinhibitory quenching component of NPQ, qI. However, this parameter is poorly characterized and might be due to a combination of photoprotection and photodamage (Niyogi 1999 and references therein, Müller et al. 2001).

In this study, we directly assessed photoinhibition by quantifying D1 protein immunochemically in suspensions of epipelic benthic diatoms subjected to light stress. Specific inhibitors were used to determine the relevance of the xanthophyll cycle, D1 protein recycling and ROS scavenging as photoprotection mechanisms.

MATERIALS AND METHODS

Sampling and diatom suspensions

Intact sediment cores (8 cm diameter) were collected in July (Expt 1) and September (Expt 2) 2012 during low tide from Trancão intertidal mud flats (38° 47′ 46″ N, 09° 05′ 33″ W), located in the Tagus Estuary, Portugal. Samples were brought to the laboratory and exposed to low light (80 µmol photons m^{-2} s^{-1}) provided by a halogen lamp through fiberoptics 460-F (Walz). The epipelic fraction of the microphytobenthos community was collected using the lens tissue method (Eaton & Moss 1966). Two pieces of lens tissue (Whatman International) were placed on the surface of the sediment, and after ca. 1 h, the upper piece of lens tissue was removed and cells resuspended in filtered site water. Microscopic examination revealed that on both occasions, the epipelic community was composed exclusively of diatoms, dominated by specimens of the genera *Navicula*. Other genera represented in the microphytobenthos community included *Staurophora*, *Cylindrotheca*, *Gyrosigma*, *Nitzschia*, *Plagiotropis* and *Entomoneis*. Chlorophyll *a* concentrations in the diatom suspensions obtained ranged between 0.8 and 1.5 µg ml^{-1}.

Light and chemical treatments

Expt 1

Two different light treatments of 1 h at 20°C were applied to the diatom suspensions: low light (LL, 80 µmol photons m^{-2} s^{-1}) or high light (HL, 1500 µmol photons m^{-2} s^{-1}) using a temperature-controlled chamber (Fytoscope FS130, Photon Systems Instruments). The Fytoscope light source is composed of LED panels emitting in the visible and near infrared (400–775 nm). Diatom suspensions subjected to HL stress were previously subjected to 15 min of the following chemical treatments: Control (no addition); lincomycin (LINC, 0.9 mM); dithiothreitol (DTT, 2 mM); and DTT+LINC. LINC is an inhibitor of chloroplast protein synthesis, thereby preventing D1 protein recycling, and does not have short-term side effects in the concentrations used (Campbell & Tyystjärvi 2012). DTT is an inhibitor of the functioning of the xanthophyll cycle (Demmig-Adams & Adams 1993, Grouneva et al. 2008). After chemical and light treatments, diatom suspensions were rapidly filtered using syringes, filter holders and 25 mm GF/F What-

man filters. For each diatom suspension, 20 ml were filtered for protein D1 quantification and 5 ml for pigment analysis. Filtration was done under the light source of the Fytoscope, and filters were immediately frozen in liquid nitrogen.

Expt 2

LL and HL treatments were applied as described above. Diatom suspensions exposed to HL stress were previously subjected to 15 min of the following chemical treatments: Control (no addition); glutathione (GSH, 5 mM) + ascorbate (AsA, 2.5 mM); and methylviologen (MV, 1 µM). GSH and AsA are scavengers of ROS (e.g. Noctor & Foyer 1998), while MV promotes the formation of ROS species (e.g. Mano et al. 2001 and references therein). Diatom suspensions were filtered and frozen as described above.

Protein extraction and immunodetection of D1

Diatoms were scratched off the filters into Eppendorf tubes containing 1 ml of extraction buffer (sodium phosphate 10 mM, pH 7.4; EDTA 1 mM; 0.2% Tween 20, v/v) supplemented with freshly prepared phenylmethylsulphonyl fluoride (1 mM) and DTT (2 mM). Extracts were homogenized with a vortex and frozen in liquid nitrogen. Samples were then incubated at 80°C for 5 min, sonicated (Bransonic 220, Branson) for 1 min and vortexed. The freeze–thaw cycle was repeated 4 times. To eliminate cell debris, the samples were centrifuged at 10 000 × *g* (20 min at 4°C). Protein concentrations were determined with Bradford microassay (Bio-Rad) using bovine serum albumin (Sigma-Aldrich) as a standard.

For each replicate, 2 µg of total proteins were separated by SDS-PAGE in a 12% polyacrylamide gel using the mini-protean 3 system from Bio-Rad. Different amounts of purified PsbA/D1 protein (Agrisera) were also loaded in the gel in order to build a calibration curve to determine D1 concentrations. A protein standard (Novex, Life Technologies) was used to calculate protein size and control migration. Protein transfer to nitrocellulose membrane was performed in a Trans-Blot Semi-Dry Electrophoretic Transfer Cell (Bio-Rad) for 1 h at 140 mA in buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol, pH 8.3). Protein loading was checked by Ponceau S staining. Membranes were blocked in PBS-T buffer supplemented with defatted powdered milk (5% w/v) for 1 h at room temperature. A rabbit anti-PsbA antibody (Agrisera) was used for detection of D1, at 1:20 000 dilution in blocking buffer for 1 h. Horseradish peroxidase (GE Healthcare) coupled secondary anti-rabbit IgG antibody was incubated for 1 h at a dilution of 1:40 000. Four 15 min washings with PBS-T were performed after antibody incubation. Chemoluminescence detection was done using an ECL Advance Western Blotting Detection Kit (GE Healthcare). Developed films (Hyperfilm ECL, GE Healthcare) were imaged with a Gel Doc XR imaging system to quantify band intensities by densitometry, using Quantity-One software (all Bio-Rad). Photoinhibition was calculated as $(1 - D1_T / D1_{LL}) \times 100$, where $D1_T$ is the concentration of D1 at the different light/chemical treatments and $D1_{LL}$ is the concentration of D1 at low light.

Pigment analysis

Filters for pigment analysis were homogenized in 95% cold buffered methanol (2% ammonium acetate) using a glass rod. Samples were then sonicated for 30 s, briefly vortexed and transferred to −20°C for 30 min. Supernatants were collected after centrifugation at 1100 × *g* (5 min at 4°C), and filtered through 0.2 µm Fluoropore membrane filters (Millipore). Extracts were then immediately injected into an HPLC system (Shimadzu) with a photodiode array detector (SPD-M10AVP). Chromatographic separation was carried out using a Supelcosil C18 column (25 cm length; 4.6 mm diameter; 5 µm particles; Sigma-Aldrich) for reverse-phase chromatography and a 35 min elution programme. The solvent gradient followed Kraay et al. (1992), with an injection volume of 100 µl and a flow rate of 0.6 ml min^{-1}. Pigments were identified from absorbance spectra and retention times, and their concentrations were obtained from the signals in the photodiode array detector. Calibration curves were performed using pure crystalline standards from DHI. The de-epoxidation state (DES) was calculated as DES = Dtx / (Ddx + Dtx), where Dtx and Ddx are the concentrations of diatoxanthin and diadinoxanthin, respectively.

Statistical analysis

The existence of significant differences was tested using one-way analysis of variance (ANOVA) for effects of light/chemical treatments, using in all cases (DES and D1 protein levels) 4 replicate suspensions

for each treatment. Data were transformed whenever necessary to comply with ANOVA assumptions. Post hoc comparisons were made with Tukey HSD tests. Statistical analyses were carried out using Statistica 10.0 (StatSoft).

RESULTS AND DISCUSSION

Exposure of diatom suspensions to an irradiance of 1500 µmol photons m^{-2} s^{-1} (HL) caused significantly (Tukey HSD, $p < 0.01$) lower D1 protein levels (74.1 ± 5.7 [SD] and 59.6 ± 5.6 fmol μg^{-1} protein) relative to LL (94.6 ± 3.6 and 84.4 ± 5.5 fmol μg^{-1} protein), corresponding to 22% (Expt 1) and 29% (Expt 2) of photoinhibition (see Figs. 1 & 2, respectively). Addition of LINC inhibited D1 protein recycling, causing a significant (Tukey HSD, $p < 0.001$) decrease in the concentrations of D1 (52.7 ± 4.0 fmol μg^{-1} protein; Fig. 1). Photoinhibition was increased from 22 to 44% in the presence of LINC.

Most phototrophs have developed a rapid D1 turnover repair cycle, involving proteolytic release of the damaged D1, de novo synthesis and incorporation of the protein into reassembled PSII complexes (Mattoo et al. 1984, Aro et al. 1993). However, significant photoinactivation by net loss of D1 protein can be attained by blocking chloroplast protein synthesis with LINC (Campbell & Tyystjärvi 2012). Recently, Domingues et al. (2012) reported significant degradation and re-synthesis of D1 protein under low irradiances in the diatom model species *Phaeodactylum tricornutum*, but increased turnover rates under high light. Fast turnover of multiple PSII subunits, including D1 protein, has been shown to play an important role in the resistance of small planktonic diatoms to high irradiances (Wu et al. 2011, 2012).

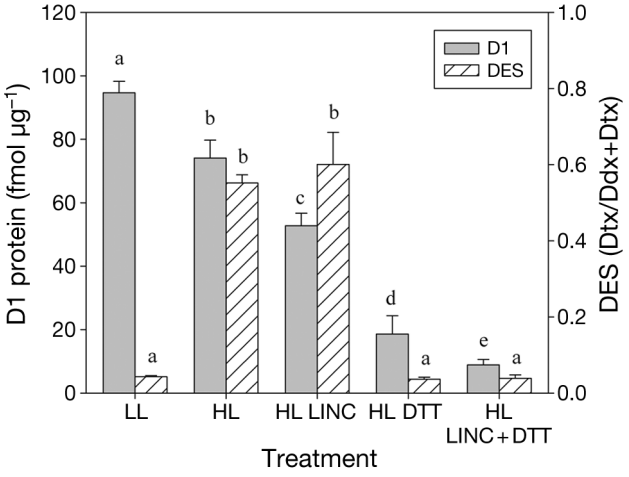

Fig. 1. Photosystem II protein D1 content (fmol μg^{-1} protein) and de-epoxidation state (DES) in suspensions of benthic diatoms (mean ± SD, n = 4) in Expt 1. LL: low light, 80 µmol photons m^{-2} s^{-1}; HL: high light, 1500 µmol photons m^{-2} s^{-1}; LINC: lincomycin; DTT: dithiothreitol; Dtx: diatoxanthin; Ddx: diadinoxanthin. Different letters indicate significant differences between treatments ($p < 0.01$)

When exposed to DTT, an inhibitor of the xanthophyll cycle, D1 protein levels were significantly (Tukey HSD, $p < 0.001$) lower (18.6 ± 5.8 fmol μg^{-1} protein) than in the presence of LINC or under LL, corresponding to a photoinhibition of 80% (Fig. 1). No significant differences were found for DES between LL and HL treatments in the presence of DTT, showing full inhibition by DTT of Ddx conversion to Dtx. DES was significantly (Tukey HSD, $p < 0.001$) lower in LL than in both HL and HL+LINC treatments, showing activation of the xanthophyll cycle caused by HL exposure (Fig. 1). The lowest D1 protein levels (8.9 ± 1.7 fmol μg^{-1} protein) were observed when both inhibitors (LINC and DTT) were used simultaneously (Fig. 1), corresponding to a photoinhibition of 91%.

These results indicate that the functional stability of PS II in the studied epipelic benthic diatoms under high irradiances was more dependent on xanthophyll cycle activation than on D1 protein recycling. Previous works have shown the key role of the Ddx cycle in the photoprotection of planktonic diatoms through thermal dissipation of harmful excess energy (e.g. Olaizola et al. 1994, Lavaud et al. 2004, Ruban et al. 2004). While some studies have shown that intertidal microphytobenthos depend greatly on the xanthophyll cycle during daylight emersion periods (Chevalier et al. 2010, Cartaxana et al. 2011), others estimated its contribution for photoprotection as minimal (Perkins et al. 2010, Serôdio et al. 2012).

D1 protein concentrations were not significantly different between LL and HL treatment with the addition of GSH and AsA (82.2 ± 10.1 fmol μg^{-1} protein; Fig. 2). DES was significantly (Tukey HSD, $p < 0.001$) lower in LL than in all HL treatments, showing activation of the xanthophyll cycle upon exposure to high irradiances (Fig. 2). These results show full protection of PSII protein D1 under high irradiances through a combination of energy dissipation by the Ddx cycle and ROS scavenging by the added antioxidants GSH and AsA. In the chloroplasts, GSH and AsA are capable of directly quenching several ROS species (singlet oxygen, $^1O_2$; superoxide anion radical, O_2^-; and hydroxyl radical, OH•) and are involved in the regeneration of other antioxidant molecules (e.g. α-tocopherol; Noctor & Foyer 1998, Niyogi 1999, Foyer et al. 2006).

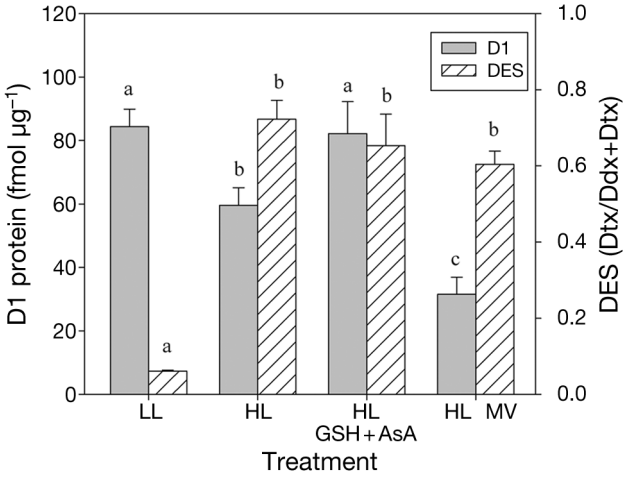

Fig. 2. Photosystem II protein D1content (fmol µg^{-1} protein) and de-epoxidation state (DES) in suspensions of benthic diatoms (mean ± SD, n = 4) in Expt 2. LL: low light, 80 µmol photons m^{-2} s^{-1}; HL: high light, 1500 µmol photons m^{-2} s^{-1}; GSH+AsA: glutathione + ascorbate; MV: methylviologen; Dtx: diatoxanthin; Ddx: diadinoxanthin. Different letters indicate significant differences between treatments ($p < 0.01$)

Diatoms can photoreduce molecular oxygen using the Mehler reaction driven by PSI, thereby protecting PSII (Waring et al. 2010). This is an effective photoprotection mechanism provided the cells can deal with the ROS produced by a set of reactions that comprise the water–water cycle (Asada 2006). In this cycle, O_2^- is rapidly converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD) and reduced to H_2O by AsA, in a reaction catalysed by ascorbate peroxidase (APX). AsA is regenerated with the involvement of GSH and a set of enzymes comprising the GSH-AsA cycle. Waring et al. (2010) showed higher rates of oxygen photoreduction and increased SOD and APX activities at saturating light levels in the planktonic *Thalassiosira pseudonana* when compared to the benthic *Nitzschia epithemioides*.

Addition of MV functioned in the opposite direction, significantly (Tukey HSD, $p < 0.001$) promoting D1 protein degradation (31.5 ± 5.4 fmol µg^{-1} protein) and increasing photoinhibition under HL from 29 to 63% (Fig. 2). MV accelerates the photoproduction of O_2^- in PSI and simultaneously inhibits the photoreduction of monodehydroascorbate to AsA, inactivating APX (Mano et al. 2001). This leads to the accumulation of H_2O_2 and amplified oxidative damage.

Most available data on D1 recycling in diatoms is for planktonic, mostly centric species (e.g. Key et al. 2010, Wu et al. 2011, 2012). Studying a panel of 7 centric marine diatoms with a cell volume span of 10^1 to 10^7 µm^3, Key et al. (2010) found that D1 per total protein increased with cell size and that larger species showed slower repair rates for PSII. Further work is needed on protein D1 quantification in benthic epipelic diatoms to better characterize their response to light stress. However, a comparison with the results presented by Key et al. (2010) suggests that the species that compose the studied microphytobenthos community may allocate a high proportion of their total protein to D1 and have slow repair rates, similarly to larger planktonic diatoms.

The response of natural microphytobenthos communities to light stress is probably more complex than that of planktonic communities, due to the capacity of benthic epipelic species to migrate vertically within the sediment profile. When exposed to high irradiances, intertidal microphytobenthos have been found to migrate downwards, avoiding photoinhibitory light levels (e.g. Kromkamp et al. 1998). However, the photoprotective nature frequently attributed to this photophobic behaviour has only recently been addressed experimentally using a diatom motility inhibitor to obtain intact non-migratory biofilms (Cartaxana & Serôdio 2008, Perkins et al. 2010, Cartaxana et al. 2011). We are currently extending D1 protein quantification to sediment samples to directly assess photo-oxidative damage in intact microphytobenthos biofilms and further characterize the relevance of migration as a 'behavioural' photoprotection mechanism.

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