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ORIGINAL ARTICLE

The role of O_2 as an electron acceptor alternative to CO_2 in photosynthesis of the common marine angiosperm *Zostera marina* L.

Pimchanok Buapet[1] · **Mats Björk**[2]

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Abstract This study investigates the role of O_2 as an electron acceptor alternative to CO_2 in photosynthesis of the common marine angiosperm *Zostera marina* L. Electron transport rates (ETRs) and non-photochemical quenching (NPQ) of *Z. marina* were measured under saturating irradiance in synthetic seawater containing 2.2 mM DIC and no DIC with different O_2 levels (air-equilibrated levels, 3 % of air equilibrium and restored air-equilibrated levels). Lowering O_2 did not affect ETR when DIC was provided, while it caused a decrease in ETR and an increase in NPQ in DIC-free media, indicating that O_2 acted as an alternative electron acceptor under low DIC. The ETR and NPQ as a function of irradiance were subsequently assessed in synthetic seawater containing (1) 2.2 mM DIC, air-equilibrated O_2; (2) saturating CO_2, no O_2; and (3) no DIC, air-equilibrated O_2. These treatments were combined with glycolaldehyde pre-incubation. Glycolaldehyde caused a marked decrease in ETR in DIC-free medium, indicating significant electron flow supported by photorespiration. Combining glycolaldehyde with O_2 depletion completely suppressed ETR suggesting the operation of the Mehler reaction, a possibility supported by the photosynthesis-dependent superoxide production. However, no notable effect of suppressing the Mehler reaction on NPQ was observed. It is concluded that during DIC-limiting conditions, such as those frequently occurring in the habitats of *Z. marina*, captured light energy exceeds what is utilised for the assimilation of available carbon, and photorespiration is a major alternative electron acceptor, while the contribution of the Mehler reaction is minor.

Keywords *Zostera marina* · Photorespiration · Mehler reaction · Chlorophyll fluorescence

✉ Pimchanok Buapet
pimchanok.b@psu.ac.th

[1] Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai 90112, Songkhla, Thailand

[2] Department of Ecology, Environment and Plant Sciences, Stockholm University, Stockholm SE-106 91, Sweden

Introduction

Photosynthetic organisms are constantly challenged by an imbalance between two vital processes: light energy capture and carbon fixation. When light energy exceeds what can be utilised by carbon assimilation, photochemically derived electrons can be transferred to alternative acceptors, such as O_2, via photorespiration and the Mehler reaction. In C3 plants, photorespiration constitutes a significant electron sink (Badger et al. 2000), as 20 % of electrons supporting carboxylation/oxygenation by Rubisco can be diverted to the oxygenation of RuBP (Ort and Baker 2002). Besides photorespiration, the direct photoreduction of O_2 at photosystem I (PSI) by the Mehler reaction could also sustain electron transport. This reaction is initiated by O_2 being reduced at PSI, forming superoxide, which is eventually degraded to water in a process called the water–water cycle (Mehler 1957; Asada 1999; Raven and Larkum 2007). In C3 plants, the contribution of the Mehler reaction has been demonstrated to be rather low compared with that of photorespiration, accounting for <5 % of the linear electron flow (Ruuska et al. 2000; Clarke and Johnson 2001; Driever and Baker 2011). Nevertheless, high rates of the Mehler reaction have been observed at high irradiances when photorespiration is suppressed (up to 10–20 % of the electron transport; Lovelock and Winter 1996), and it has been demonstrated

to be an important electron sink in certain C4 plant species (Laisk and Edwards 1998; Siebke et al. 2003), cyanobacteria (Badger et al. 2000), and diatoms (Waring et al. 2010). Although both photorespiration and the Mehler reaction have been regarded as undesirable processes brought about by high O_2 content in the atmosphere, it has been proposed that their non-assimilatory consumption of electrons might help dissipate excess light energy, thus protecting the photosynthetic apparatus (Osmond and Grace 1995). Moreover, it has been suggested that the Mehler reaction might have additional roles in sustaining the pH gradient across thylakoid membranes, a gradient required for generating non-photochemical quenching (NPQ) (Heber 2002) and supporting additional ATP production to meet the energy requirement for C4 photosynthetic carbon assimilation (Cardol et al. 2011; Kramer and Evans 2011).

As marine angiosperms (seagrasses) often inhabits areas with large fluctuations in DIC and pH (Beer et al. 2006; Semesi et al. 2009) and generally exhibit a typical C3 fixation pattern (Beer and Wetzel 1982; Touchette and Burkholder 2000), photorespiration and the Mehler reaction are expected to be present in this group of plants. However, like many aquatic photosynthetic organisms, seagrasses employ carbon-concentrating mechanisms (CCMs) consisting of the extracellular carbonic anhydrase-catalysed dehydration of HCO_3^- to CO_2 and stimulation of CO_2 formation at the diffusion boundary layer by H^+ extrusion (Beer and Rehnberg 1997; Hellblom et al. 2001; Beer et al. 2002). In addition, reported photorespiratory rates appear to be lower in seagrasses than in terrestrial plants (Black et al. 1976; Downton et al. 1976; Abel and Drew 1989; Beer 1989; Frost-Christensen and Sand-Jensen 1992). It is thought that their CCMs can maintain a saturating concentration of CO_2 in the photosynthetic cells, suggesting insignificant rates of oxygen-dependent electron flows (Touchette and Burkholder 2000; Beer et al. 2002). Consequently, these pathways have received little attention and been largely ignored when estimating seagrass primary production (Touchette and Burkholder 2000). However, a recent study using a gas-exchange technique demonstrated that although photorespiration in the seagrasses *Zostera marina* and *Ruppia maritima* was negligible in the presence of well-equilibrated dissolved inorganic carbon (DIC) levels, and photorespiratory activity was enhanced significantly when DIC levels were limiting, something that happens frequently in seagrass-dominated bays (Buapet et al. 2013a). This finding has changed the common view of the role of photorespiration in seagrasses. However, the capacity of photorespiration to sustain electron transport has not yet been determined. Far less is known about direct photoreduction via the Mehler reaction in seagrasses. A few studies have suggested its operation in some seagrass species (Mass et al. 2010; Silva et al. 2013), although as yet there is no conclusive evidence that this reaction occurs in seagrasses. The ecological relevance of alternative electron transport to O_2 in seagrass is associated with the significant natural fluctuation of DIC in their habitat where periodically low availability of inorganic carbon is rather common. Such a decrease in inorganic carbon availability driven by photosynthesis has been widely observed in nearshore habitats from tropical to temperate latitudes in which both daily and seasonal variations have been detected (Frankignoulle and Distèche 1984; Menéndez et al. 2001; Middelboe and Hansen 2007; Unsworth et al. 2012; Saderne et al. 2013; Buapet et al. 2013b). The photosynthetic organisms growing in these habitats are therefore prone to energy imbalance particularly when the light intensity is high. This paper describes the photosynthetic characteristics of the cosmopolitan marine angiosperm *Zostera marina* together with evidence for photorespiration and the Mehler reaction in this species. The aim is to assess the extent of their contribution to electron transport and to search for an initial indication of their photoprotective role. Chlorophyll fluorescence was used to determine the rates of electron transport through photosystem II (PSII) and excess energy dissipation as heat (i.e. non-photochemical quenching). By using a metabolic inhibitor, glycolaldehyde, to suppress RuBP regeneration in the carbon reduction cycle and by making measurements under both high and depleted DIC and O_2 conditions over a range of irradiances, the contributions of photorespiration and the Mehler reaction could be assessed.

Materials and methods

Shoots of *Z. marina* were collected from the Sven Lovén Centre for Marine Sciences situated at the mouth of the Gullmar Fjord on the Swedish Skagerrak coast. All specimens were transported to the laboratory at Stockholm University, where the experiments took place. The plants were allowed to acclimate in an aquarium natural seawater at a constant temperature of 18 °C for approximately 48 h before being used in the experiments; they were replaced with fresh specimens after 5 days. Simple synthetic seawater adapted from Beer and Rehnberg (1997) containing 450 mM NaC1, 30 mM $MgSO_4$, 10 mM KCI, and 10 mM $CaC1_2$ was used as the incubating medium throughout the experiments. As a source of DIC, $NaHCO_3$ was added to the desired concentration (2.2 mM total DIC). Seawater pH was subsequently adjusted to 8.1 by adding NaOH or HCl. This yielded a final concentration of 16.9 ± 0.5 μM CO_2 and 1973.5 ± 17.9 μM HCO_3^-.

Effect of DIC and O_2 availability on electron transport rate (ETR) and non-photochemical quenching (NPQ) under saturating light (400 µmol photons m^{-2} s^{-1})

This experiment consisted of two treatments: (1) synthetic seawater containing 2.2 mM DIC and (2) DIC-free synthetic seawater. The leaf segments of 3 cm length were fixed in a U shape within three 3-ml Hansatech chambers connected to the oxygen electrodes (Hansatech Instruments, King's Lynn, UK). The optical fibres of the multichannel submersible modulated fluorometer were fixed to the side of these chambers (Aquation Pty Ltd, Australia). The temperature was kept constant at 18 °C using an RC 20 refrigerated circulating water bath (LAUDA-Brinkmann, Lauda-Königshofen, Germany). Fluorescence parameters were assessed in three steps: (1) after subjected to 20 min of saturating irradiance under air-equilibrated O_2 condition, (2) after subjected to 20 min of saturating irradiance under low O_2 condition, and (3) after the O_2 level was restored for 20 min under saturating irradiance. Firstly, all leaf segments were subjected to 12–15 min of darkness and the maximum photochemical efficiency of PSII, i.e. $F_v/F_m = (F_m - F_0)/F_m$, was measured. This allowed us to assess the initial status of the plant and the leaves with the initial F_v/F_m below 0.7 were discarded. Then the actinic light of 400 µmol m^{-2} s^{-1} (saturating irradiance level obtained in the preliminary study, data not shown) was provided by a cold light source (KL 1500 LCD; Zeiss, Oberkochen, Germany) for 20 min before the quantum yield of electron transport through PSII, i.e. $\varphi_{PSII} = (F'_m - F)/F'_m$, was determined followed by another dark adaptation and F_v/F_m measurement. The seawater was then replaced and bubbled constantly with nitrogen gas (N_2) in order to keep the O_2 concentration at approximately 3 % of air equilibrium. The light was once again provided for 20 min before Φ_{PSII} was measured followed by 12–15 min of dark adaptation and F_v/F_m measurement. Finally, the seawater was replaced in order to restore the O_2 level and the same procedure was repeated.

The electron transport rate (ETR) at each light intensity was calculated by multiplying Φ_{PSII} by the light intensity, by 0.5 (assuming absorbed photons to be distributed equally between photosystems I and II), and by a leaf absorption factor (AF). The absorption factors were determined by measuring the incident irradiance from an LED light source before and after the Quantitherm PAR/Temperature Sensor (Hansatech, Norfolk, UK) was covered with the seagrass leaves. The AF of each leaf used in the experiment was calculated from the proportion of irradiance the leaf absorbed (Beer and Björk 2000). The average AF determined in this study was 0.69 ± 0.01 ($n = 100$) which was lower than the standard value of 0.84 preset in the commonly used pulsed amplitude modulated fluorometer (PAM fluorometer, Walz, Germany). Previous works have reported varying AF values for *Zostera marina*, e.g. 0.44 ± 0.02 (Beer et al. 1998) and 0.846 ± 0.004 (Ochieng et al. 2010). NPQ was calculated as $(F_m - F'_m)/F'_m$.

Effect of DIC, O_2 availability, and glycolaldehyde on the electron transport rates (ETRs) and non-photochemical quenching (NPQ) as a function of irradiance

In the experimental procedure (adapted from Proctor and Smirnoff 2011), measurements were made in three 3-mL incubation chambers connected to Clark-type O_2 electrodes (DW1/AD; Hansatech Instruments, Norfolk, UK). The temperature was kept constant at 18 °C using an RC 20 refrigerated circulating water bath (LAUDA-Brinkmann, Lauda-Königshofen, Germany). The measurements were made with three different treatments: (1) in synthetic seawater containing 2.2 mM DIC and air-equilibrated levels of O_2, representing normal seawater well equilibrated with air; (2) in synthetic seawater bubbled with N_2 + 1 % CO_2 (mixed locally at Stockholm University, Sweden) until the O_2 concentration reached 0–5 µM (measured using Hansatech DW1/AD O_2 electrodes), representing conditions under which carbon is the only electron acceptor and there is no competition with RuBP oxygenation at Rubisco; and (3) in DIC-free synthetic seawater containing air-equilibrated levels of O_2, representing conditions under which only O_2 is available to accept electrons. To study the effect of a RuBP regeneration inhibitor, 3-cm hand-cut segments of *Z. marina* were pre-incubated in experimental media containing 90 mM glycolaldehyde (Sigma-Aldrich) for 30 min under dim light (10 µmol photons m^{-2} s^{-1}) before the measurements. Glycolaldehyde inhibits phosphoribulokinase and subsequently prevents RuBP regeneration. Both the carbon fixation and photorespiration are thus suppressed due to the lack of substrate (Wiese et al. 1998). Untreated segments were also pre-incubated in experimental media for 30 min without glycolaldehyde. The glycolaldehyde concentration and incubation time used were determined using a preliminary test. The effect of glycolaldehyde at the concentration used in this experiment (90 mM) on the photosynthetic electron transport was subsequently assessed. This was conducted by adding glycolaldehyde to the isolated thylakoids of *Spinacia oleracea* L. and the seagrass *Thalassia hemprichii* in the presence of an artificial electron acceptor, 2,6-dichlorophenolindophenol (DCPIP). The absorbance of

DCPIP at 600 nm was measured by spectrophotometry (Panigrahi and Biswal 1979). The reduction of DCPIP over time under constant irradiance was similar both in the absence and in the presence of 90 mM glycolaldehyde, thus showing no inhibitory effect on photosynthetic electron transport (unpublished).

The ETR and NPQ in response to different light intensities were assessed using multi-channel submersible modulated fluorometers (Aquation, Umina Beach, NSW, Australia). Pre-incubated segments of seagrass leaves were fixed in a U shape within the chamber facing the tip of the optical fibres of the fluorometer. The samples were subjected to a series of actinic light exposures provided by the optical fibre ranging from 25 to 420 µmol photons m^{-2} s^{-1}. The quantum yield of electron transport through PSII (φ_{PSII}) was determined at each light intensity. Finally, the leaves were incubated in darkness for 10 min, after which the maximum photochemical efficiency of PSII (F_v/F_m) was measured. ETR and NPQ at each light intensity were calculated as described in the previous experiments.

The plots of ETR and NPQ as the functions of irradiance were fitted to a curve using the empirical equation of Platt et al. (1980). The following photosynthetic parameters were determined: the asymptotic maximum levels of ETR and NPQ, i.e. ETR_{max} and NPQ_{max}, respectively, the initial slope of the light response curve, α, and the minimum saturating irradiance, E_k.

Nitroblue tetrazolium (NBT) staining for superoxide

Hand-cut 3-cm segments of *Z. marina* were first incubated in a solution containing 2 mM nitroblue tetrazolium (NBT), 10 mM NaN_3, and 25 ppt NaCl in 10 mM potassium phosphate buffer (pH 7.8) for 20 min (method modified from Hsu and Lee 2010), after which the segments were subjected to the experimental conditions for 40 min. In the first experimental set-up, the plants, incubated in synthetic seawater containing 2.2 mM DIC and air-equilibrated levels of O_2, were exposed to irradiances of 500, 1000, and 2000 µmol photons m^{-2} s^{-1}. In the second set-up, the plants were incubated in synthetic seawater containing DIC concentrations of 0.55, 2.2, and 6.6 mM (adjusted by adding a stock solution of $NaHCO_3$ to DIC-free synthetic seawater) under an irradiance of 1500 µmol photons m^{-2} s^{-1}. The control leaf segments were incubated in synthetic seawater instead of NBT detection solution. NBT reacts with superoxide to form a dark blue formazan compound. The leaf segments were then bleached before imaging by boiling in a solution of lactic acid, glycerol, and ethanol (1:1:4 by volume). Stained leaves were scanned using an Epson Perfection 4990 Photo scanner (48-bit colour image, 800 dpi; Epsom, Suwa, Japan). The mean pixel intensity of the dark blue stain was determined using the ImageJ program (http://rsb.info.nih.gov/ij/). The image was converted to grayscale prior to the analysis and the threshold was set between 0 and 255 (0, white; 255, black).

Statistical analysis

In the first experiment, the effect of O_2 levels and the level of DIC on the electron transport rates (ETRs) and non-photochemical quenching (NPQ) was tested using repeated-measures ANOVA (O_2 level as the within-group factor and DIC level as the categorical factor). Fisher's least significant difference (LSD) test was used to compare the fluorescence parameters across the O_2 and DIC levels. As normality and homogeneity of variance were not achieved in the second experiment, the fluorescence data were analysed using non-parametric tests. Kruskal–Wallis ANOVA by rank tests and Kruskal–Wallis multiple comparison tests were conducted to analyse the variations in photosynthetic parameters (obtained by curve fitting) between the treatments. The relationships between the staining intensity and both irradiance and DIC concentration were tested using regression analysis. All analyses were performed using STATISTICA version 10 (StatSoft, Tulsa, OK, USA).

Results

Effect of DIC and O_2 availability on electron transport rate (ETR) and non-photochemical quenching (NPQ) under saturating light (400 µmol photons m^{-2} s^{-1})

As shown in Fig. 1a, the ETR was significantly affected by both DIC and O_2 levels (repeated-measures ANOVA, $p < 0.001$). A significant interaction was detected between O_2 and DIC levels (repeated-measures ANOVA, $p < 0.01$). Lowering O_2 level did not affect ETR when DIC was provided and there was a slight increase of ETR when O_2 was restored (Fig. 1a, solid bars). However, an inhibitory effect of O_2 depletion was observed in DIC-free treatment (post hoc pair-wise comparisons LSD test $p < 0.001$, Fig. 1a, open bars). The ETR recovered once the O_2 level was restored.

Non-photochemical quenching (NPQ) was significantly affected by both DIC and O_2 levels (repeated-measures ANOVA, $p < 0.05$). Similar to ETR, lowering O_2 did not affect NPQ when DIC was provided, whereas NPQ decreased slightly when O_2 was restored (Fig. 1b, solid bars). There was a significant increase in NPQ when O_2 was lowered in DIC-free treatment (post hoc pair-wise comparisons LSD test $p < 0.05$, Fig. 1b, open bars). However, it returned to the initial level once O_2 level was restored.

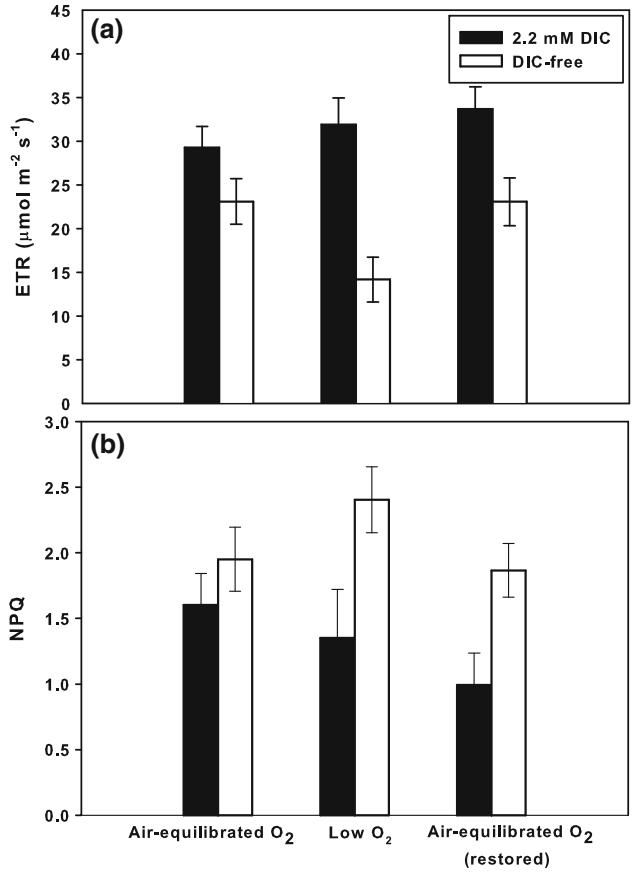

Fig. 1 **a** Electron transport rates (ETRs) and **b** non-photochemical quenching (NPQ) after 20-min incubation at saturating irradiance (400 μmol photons m^{-2} s^{-1}) in different O_2 (air-equilibrated levels, 3 % of air equilibrium and restored air-equilibrated levels) and dissolved inorganic carbon (DIC) levels; *error bars* indicate SE, $n = 8$–10

Effect of DIC, O_2 availability, and glycolaldehyde on the electron transport rates (ETRs) and non-photochemical quenching (NPQ) as the functions of irradiance

As shown in Fig. 2a, the response of ETR to irradiance under control conditions (i.e. 2.2 mM DIC in the presence of O_2) exhibited the classical saturation curve (see the photosynthetic parameters in Table 1). When carbon assimilation and photorespiration were inhibited by glycolaldehyde, a significant negative effect on ETR was observed. The initial slope of the light response curve ($\propto$) and the minimum saturating irradiance (E_k) were significantly lower, while the ETR_{max} decreased to approximately 19 % of that of the untreated segments (Kruskal–Wallis multiple comparison test, $p < 0.05$, Table 1).

The ETR was not affected by removing O_2 and supplying saturating CO_2 (N_2 + 1 % CO_2, Fig. 2b). However, strong inhibition was observed in glycolaldehyde-treated leaves, in which neither CO_2 nor O_2 could act as an

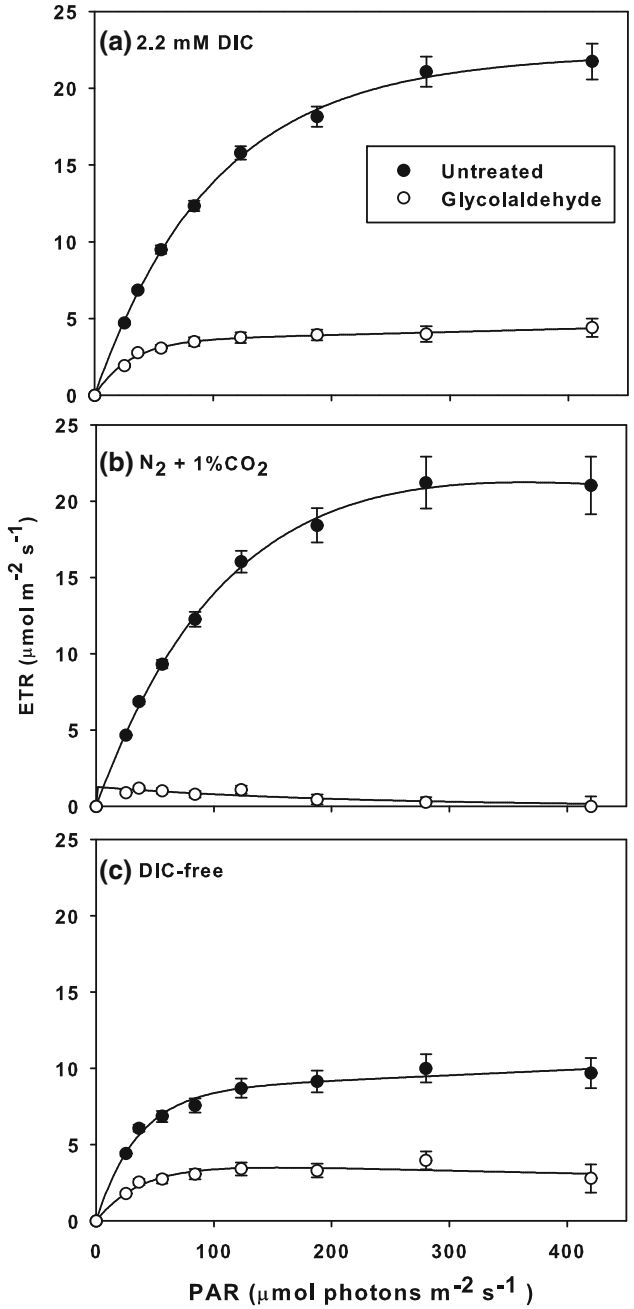

Fig. 2 Electron transport rates (ETRs) as a function of irradiance (PAR) measured in *Zostera marina* leaf segments untreated (*solid circles*) and pre-treated with glycolaldehyde (*open circles*) in **a** seawater containing 2.2 mM DIC and air-equilibrated levels of O_2, **b** seawater bubbled with N_2 + 1 % CO_2 until the O_2 concentration reached 0–5 μM, and **c** DIC-free seawater; *error bars* indicate SE, $n = 10$–18

electron sink. The degree of suppression was much greater than that in controls, in which O_2 was still available. Here, the ETR reached saturation at a very low irradiance (i.e. 15 μmol photons m^{-2} s^{-1}) and ETR_{max} was reduced to approximately 6 % of that of the untreated samples. At

Table 1 Photosynthetic parameters derived from irradiance response curves under different incubation conditions (mean ± SE, n = 10–18)

Parameters	2.2 mM DIC, 300 μM O_2		N_2 + 1 % CO_2, 0–5 μM O_2		DIC-free, 300 μM O_2	
	Untreated	Glycolaldehyde-treated	Untreated	Glycolaldehyde-treated	Untreated	Glycolaldehyde-treated
ETR_{max} (μmol m^{-2} s^{-1})	20.66 ± 1.30	3.98 ± 0.40	21.07 ± 2.03	1.34 ± 0.28	8.82 ± 0.87	3.73 ± 0.36
α	0.22 ± 0.01	0.13 ± 0.01	0.22 ± 0.01	0.19 ± 0.07	0.26 ± 0.01	0.15 ± 0.04
E_k (μmol photons m^{-2} s^{-1})	95.05 ± 6.73	35.97 ± 5.35	96.08 ± 10.02	14.72 ± 3.79	35.95 ± 4.06	43.18 ± 6.79
NPQ_{max}	0.85 ± 0.17	2.03 ± 0.24	0.45 ± 0.10	1.42 ± 0.19	1.10 ± 0.08	1.89 ± 0.34
α	0.003 ± 0.001	0.27 ± 0.07	0.003 ± 0.001	0.30 ± 0.11	0.01 ± 0.00	0.25 ± 0.04
E_k (μmol photons m^{-2} s^{-1})	441.42 ± 101.74	15.98 ± 5.50	273.53 ± 61.56	11.01 ± 2.80	135.93 ± 20.28	11.16 ± 5.01

higher irradiance, above 180 μmol m^{-2} s^{-1}, the ETR became fully inhibited (Fig. 2b; Table 1).

Carbon depletion significantly affected the ETR (Fig. 2c), reducing ETR_{max} by half relative to that under the previous two conditions (Fig. 2 a, b untreated) and lowering E_k significantly ($p < 0.05$; Table 1). A further decrease was observed when photorespiration was inhibited by glycolaldehyde pre-treatment: the maximum ETR was lowered by more than 50 %, while α significantly decreased ($p < 0.05$; Fig. 2c; Table 1).

Under control conditions, NPQ gradually increased with higher irradiance and reached saturation at approximately 440 μmol photons m^{-2} s^{-1} (Fig. 3a; Table 1). Blocking the RuBP regeneration with glycolaldehyde pre-treatment resulted in a sharp rise in NPQ, as the initial slope of the light response curve (∝) increased greatly and the asymptotic maximum of NPQ (NPQ_{max}) increased more than twofold, while the minimum saturating irradiance (E_k) was reduced to 16 μmol photons m^{-2} s^{-1} (Kruskal–Wallis multiple comparison tests, $p < 0.05$; Table 1).

Saturated CO_2 and depleted O_2 (N_2 + 1 % CO_2) conditions did not cause any change in the NPQ response to light (Fig. 3b). A steep increase in NPQ was also observed in glycolaldehyde-treated leaves. However, in these leaves, in which neither CO_2 nor O_2 could act as an electron sink, NPQ_{max} was slightly lower than that in controls in which O_2 was still available.

As shown in Fig. 3c, carbon depletion caused a small increase in NPQ_{max} relative to that under carbon-saturating conditions (Table 1). As in the other two experimental conditions, NPQ also formed a sharply rising curve when photorespiration was inhibited by glycolaldehyde pre-treatment ($p < 0.05$; Table 1). Here, the NPQ_{max} was similar to the value obtained in the control.

Superoxide production at different light intensities and dissolved inorganic carbon (DIC) concentrations

Dark blue formazan precipitation in the NBT-infiltrated leaves indicates varying superoxide production. In the synthetic seawater containing 2.2 mM DIC, the staining intensity increased with increasing light intensity ($p < 0.05$, regression analysis; Fig. 4a). When the leaves were exposed to a light intensity of 1500 μmol m^{-2} s^{-1}, the staining intensity indicated an increasing trend as DIC became more limiting, although not to a statistically significant extent ($p = 0.06$, regression analysis; Fig. 4b). No staining occurred in the control leaves.

Discussion

Zostera marina maintained notable electron transport rate (ETR) in DIC-free medium, at air-equilibrated level of O_2. If carbon fixation was the only electron acceptor, a complete inhibition of ETR would be expected when DIC supply was removed. Here it was likely that the remaining electron transport rate was largely supported by a non-assimilatory electron flow to O_2 (although this could also be the effect of internal recycling of CO_2). It became more evident that O_2 acts as an alternative electron acceptor in *Z. marina* when a significant decrease in ETR was observed as the O_2 concentration was reduced to 3 % of air-equilibrated level and that the restoration of O_2 caused a recovery of ETR. However, the effect of low O_2 on ETR was observed only when leaves were incubated in DIC-free seawater, suggesting that O_2 did not play a significant role as an electron acceptor when DIC supply was sufficient. Similar to ETR, the effect of low O_2 on non-photochemical quenching (NPQ) was only detected in DIC-free conditions. An increase in NPQ at low O_2 indicates that more effort was put into dissipating excess energy as heat from the light-harvesting antenna when electron transport to carbon assimilation and O_2 was restricted. Incubation of the leaf segment in DIC-free seawater did not affect NPQ under air-equilibrated level of O_2, implying that electron flow to O_2 might be able to sustain photon utilisation when carbon fixation was limited.

The irradiance response curves of ETR combined with the pre-incubation in the RuBP regeneration inhibitor,

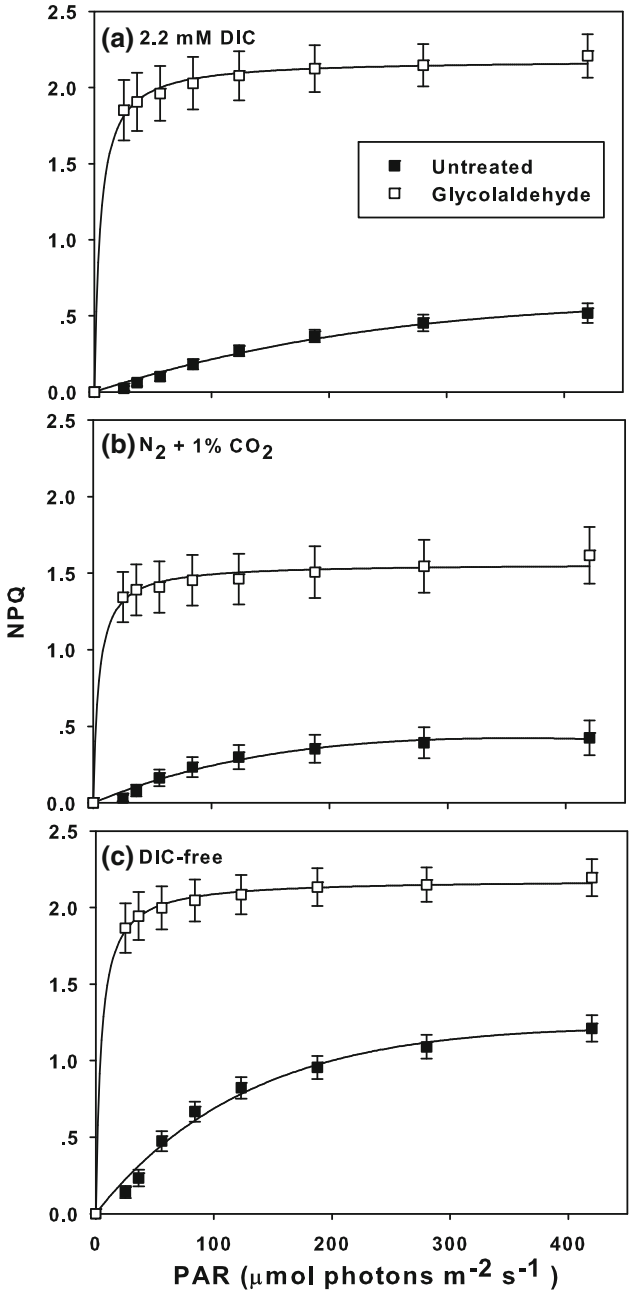

Fig. 3 Non-photochemical quenching (NPQ) as a function of irradiance (PAR) measured in *Zostera marina* leaf segments untreated (*solid squares*) and pre-treated with glycolaldehyde (*open squares*) in **a** seawater containing 2.2 mM DIC and air-equilibrated levels of O_2, **b** seawater bubbled with N_2 + 1 % CO_2 until the O_2 concentration reached 0–5 µM, and **c** DIC-free seawater; *error bars* indicate SE, n = 10–18

glycolaldehyde, provide further evidence of whether electron flux to O_2 is linked to photorespiration or the Mehler reaction. Although a partial recovery of photosynthesis in leaves after treatment with glycolaldehyde has been reported, the recovery is slow (Wiese et al. 1998), and would not have affected the ETR response to different light intensities and the F_v/F_m in our study since these were assessed within a short period of time (15 min). Similar to the results discussed earlier, when no carbon source was available (i.e. in DIC-free synthetic seawater), O_2 served as an alternative electron acceptor and as such maintained substantial ETR, although not as high as could be supported by CO_2 fixation. As in previous works (Badger et al. 2000; Noctor et al. 2002; Ort and Baker 2002; Driever and Baker 2011), the photorespiratory sink was found to provide the predominant support for such electron transport indicated by the large decline of the ETR when plants in DIC-free seawater were pre-treated with glycolaldehyde (which inhibits both photorespiration and the re-fixation of CO_2). Nevertheless, a certain ETR was maintained in glycolaldehyde-treated leaves and became completely suppressed only when O_2 was also removed (glycolaldehyde in N_2 + 1 % CO_2, no O_2). This suggests the presence of other O_2-dependent alternative electron flows, likely via direct O_2 photoreduction at PSI (e.g. the Mehler reaction), as evidenced by the superoxide staining results. As the Mehler reaction reduces O_2 from PSI for the later formation of superoxide (Asada 1999), the quantification of superoxide can provide an estimate of the reaction (Driever and Baker 2011). In the present study, superoxide production was demonstrated to be dependent on the level of photosynthetic activity, i.e. formazan accumulation increased with increasing light intensity and decreasing DIC. Plastid terminal oxidase (PTOX) can also mediate electron flows from the reduced plastoquinone (PQH2) to O_2, making it another possible pathway for O_2-sensitive electron flow that could contribute to what is here regarded as the Mehler reaction (McDonald et al. 2011; Laureau et al. 2013; Shirao et al. 2013). The molecular mechanisms of PTOX are not yet fully understood. Although it has been proposed to serve as a safety dissipation for excess excitation, a study of the kinetics of PTOX (Trouillard et al. 2012) revealed that the rates of electron transport to PTOX were much lower than those of linear photosynthetic electron pathway and that the alternative oxidation of PQH2 by photosystem II was faster than that by O_2 (Laisk et al. 2015). It has been proposed that PTOX catalyses a four-electron reduction of O_2 and primarily generates water (Stepien and Johnson 2009). On the contrary, the study by Heyno et al. (2009) showed that overexpression of PTOX induced oxidative stress in tobacco, and recent work by Yu et al. (2014) demonstrated that the formation of superoxide can occur when electron donor is liposomal $DPQH_2$. Nevertheless, our results suggest that *Zostera marina* has a low potential magnitude of the Mehler reaction, as this reaction supported slow rates of linear electron flow even under the most conductive conditions (i.e. DIC-limiting non-photorespiratory conditions). This stands in contrast to the results of studies of other marine photosynthetic

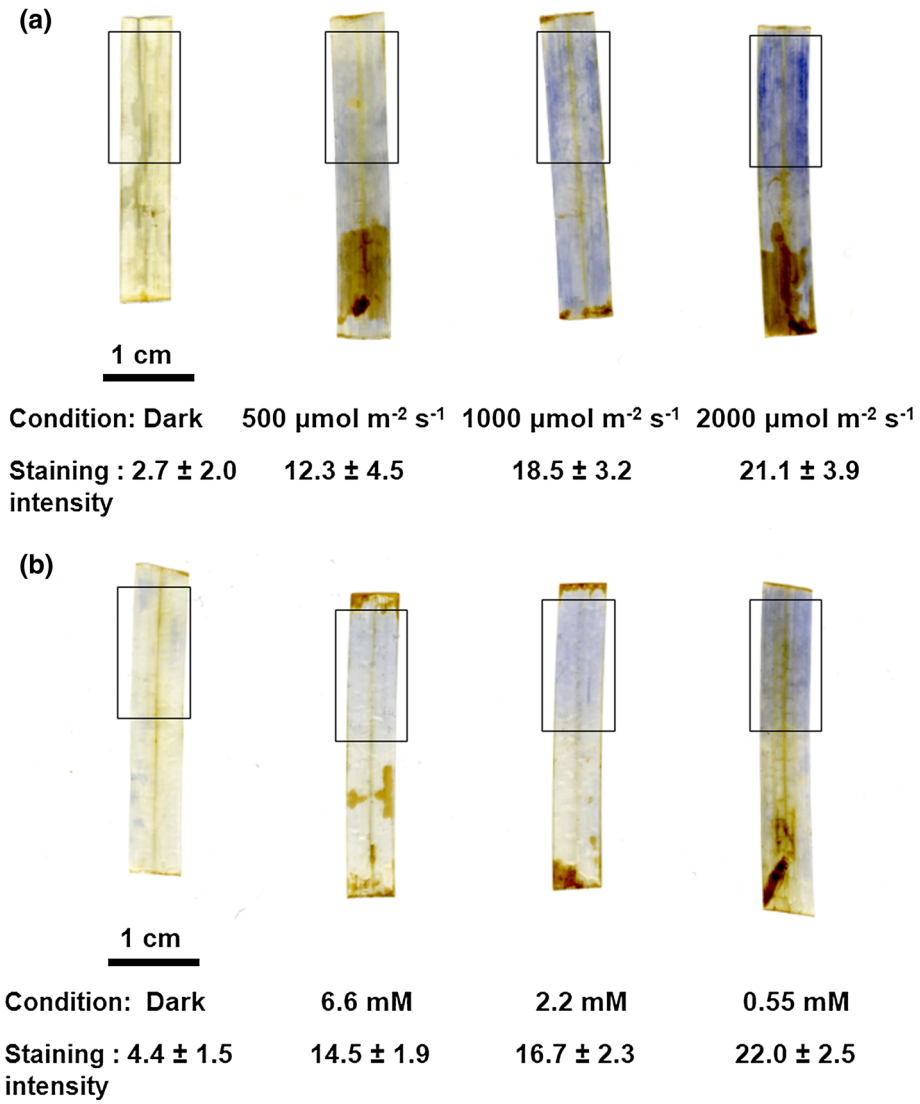

Fig. 4 *Zostera marina* leaves stained with nitroblue tetrazolium (NBT). *Dark blue formazan accumulation* indicates the presence of superoxide. The area of each leaf exposed to light is indicated by the *rectangles*. **a** After NBT infiltration, leaves were exposed to light intensities of 0, 500, 1000, or 2000 µmol photons m^{-2} s^{-1} and 2.2 mM DIC for 40 min. **b** After NBT infiltration, leaves were incubated in DIC concentrations of 6.6, 2.2, or 0.55 mM at an irradiance of 1500 µmol m^{-2} s^{-1} for 40 min. The staining intensity was assessed using ImageJ; *error bars* indicate SE, $n = 4$

organisms such as cyanobacteria (Badger et al. 2000) and diatoms (Waring et al. 2010), in which the Mehler reaction was found to be a major alternative electron sink (up to 50 %). Recent work by Roach et al. (2015) showed that the Mehler reaction assessed by the production of H_2O_2 was instead induced by high rates of linear electron transport promoted by high CO_2 availability under high light intensity in *Chlamydomonas reinhardtii*. This was not the case in our studies as we observed higher superoxide production (NBT staining) when DIC availability was lowered. Our results are consistent with the results of studies of a number of terrestrial C3 plant species, such as tobacco, barley, and French beans (Badger et al. 2000; Ruuska et al. 2000; Clarke and Johnson 2001; Driever and Baker 2011). This indicates that the potential to use O_2 as an alternative electron acceptor and the partitioning of electron flux between photorespiration and the Mehler reaction in *Z. marina* resemble those of terrestrial C3 plants. In addition, the results confirm earlier suggestions that alternative electron flows can cause the discrepancies between ETR and gross photosynthetic rates determined by gas exchange observed in seagrasses (Silva and Santos 2004; Silva et al. 2009). As carbon limitation and exposure to high irradiance are quite common in near-shore habitats, primary production values obtained via chlorophyll fluorescence techniques could be overestimated if ETR sustained by electron transport to alternative electron sinks is not taken into account.

Regarding the dissipation of the excess light energy by non-photochemical quenching (*NPQ*), our results indicate that both CO_2 and O_2 can act as electron acceptors for the formation of NPQ in untreated plants. NPQ increased greatly when no electron flow could be sustained by carbon assimilation and photorespiration (pre-treatment with

glycolaldehyde). The formation of NPQ depends on the pH gradient generated across the thylakoid membranes (Kramer et al. 2003). When proton consumption (via the use of ATP) by both carbon assimilation and photorespiration is absent, the electron flow supported by the non-ATP-consuming Mehler reaction, although making a minor contribution, can generate a sufficient trans-thylakoid pH gradient to form a large NPQ (Oja et al. 2011). Nevertheless, NPQ decreased only slightly when carbon assimilation and photorespiration were inhibited in the absence of O_2, indicating that the Mehler reaction was not a crucial component of NPQ formation in *Z. marina*. This finding stands in contrast to those of other studies on terrestrial higher plants and bryophytes (Schreiber and Neubauer 1990; Lovelock and Winter 1996; Proctor and Smirnoff 2011), in which decreases in NPQ and in zeaxanthin formation have been reported when the Mehler reaction was suppressed. The remaining NPQ in the absence of O_2-dependent electron flow could be achieved by modulating the conductivity of the ATP synthase to proton efflux (Kanazawa and Kramer 2002; Avenson et al. 2005; Oja et al. 2011). This allows sufficient build-up of trans-thylakoid pH gradient when the linear electron flow and the proton flux to the lumen are small (Kanazawa and Kramer 2002; Avenson et al. 2005; Oja et al. 2011). Moreover, another alternative electron flux, i.e. cyclic electron flows around PSI, could also generate the pH gradient necessary for NPQ activation (Heber 2002; Makino et al. 2002; Golding and Johnson 2003; Munekage et al. 2004). Further experiments in which the NPQ components, i.e. pH-dependent quenching qE, state transition, and dynamic photoinhibition, are assessed when carbon assimilation and photorespiration are suppressed might help clarify the relationship between alternative electron transport to O_2 and NPQ formation in *Z. marina*.

In conclusion, we have demonstrated that when light is in excess of what is utilised for the assimilation of available carbon in *Z. marina*, photosynthetically driven electrons can alternately be transferred to O_2 via photorespiration and/or the Mehler reaction. Photorespiration provides the major alternative sink for electrons, allowing an excess photon use that might be able to alleviate excitation pressure on PSII during energy imbalance. The Mehler reaction, on the other hand, supports electron flows only slightly and does not provide crucial photoprotection via non-photochemical quenching induction in *Z. marina* as previously demonstrated in other plant species.

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