Effects of Dissolved Inorganic Carbon and Oxygen Content on the Photosynthetic Characteristics of two Common Tropical Seagrasses *Halophila ovalis* (R. Br.) Hook. and *Thalassia hemprichii* (Ehrenb.)

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ABSTRACT

We investigated the effects of dissolved inorganic carbon (DIC) and O_2 concentrations on photosynthetic characteristics of two common tropical seagrasses, *Halophila ovalis* and *Thalassia hemprichii*. In the first series of experiments, leaf segments were incubated in 2.2 mM DIC or DIC-free media. Relative electron transport rates (rETR), non-photochemical quenching (NPQ), and maximum quantum yield of photosystem II (F_v/F_m) were measured under saturating irradiance at air-equilibrated O_2 (~300 µM), 3 % of air-equilibrated O_2 (~10 µM), and finally at restored air-equilibrated O_2 (~300 µM). DIC limitation reduced rETR and increased NPQ, while low O_2 reduced rETR and F_v/F_m, indicating that both carbon assimilation and O_2 can act as electron sinks. In the second series of experiments, leaf segments were exposed to irradiance of either 400, 1200, 2000 or 2800 µmol photons·m^{-2}·s^{-1} for 40 min in four different conditions: 1) 2.2 mM DIC, air-equilibrated O_2; 2) DIC-free, air-equilibrated O_2; 3) 2.2 mM DIC, 3 % of air-equilibrated O_2; and 4) DIC-free, 3 % of air-equilibrated O_2. Photoinhibition due to high light exposure was detected; however, no significant effect of DIC or O_2 concentration was observed. This result indicates that alternative electron flow was not a crucial component of photoprotective mechanisms at high irradiance.

Keywords: Chlorophyll, *Halophila ovalis*, Photosynthesis, *Thalassia hemprichii*

INTRODUCTION

Halophila ovalis (R. Br.) Hook. and *Thalassia hemprichii* (Ehrenb.) Asch. are among the most abundant seagrass species commonly found in the intertidal areas along the coast of Thailand as well as in other tropical regions (Kaewsrikhaw and Prathep, 2014; Kaewsrikhaw *et al*., 2016; Buapet *et al*., 2017; Phandee and Buapet, 2018). In such habitats, they are subjected to long hours of high light exposure during low tides in the dry season (Kaewsrikhaw *et al*., 2016; Buapet *et al*., 2017). Additionally, a periodic limitation of dissolved inorganic carbon (DIC) as well as high O_2 content brought about by the intensive photosynthetic activity of the primary producer community have been widely observed in shallow coastal habitats (Mvungi *et al*., 2012; Buapet *et al*., 2013a). In such conditions, particularly under high light intensity, non-CO_2 assimilatory electron flow via photorespiration (through the consumption of ATP and NADPH) and direct photoreduction of O_2 (also known as the Mehler-peroxidase reaction) can maintain electron flow, and thus facilitate the dissipation of excess light energy (Buapet and Bjök, 2016). Although these processes have been extensively studied in C3 terrestrial plants, their roles in seagrasses are poorly understood. Photorespiratory activity has been reported in some species such as *Cymodocea rotundata* Ehrenberg and Hemprich ex Ascherson,

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Halophila ovata Gaudichaud, *Ruppia maritima* L., and *Zostera marina* L. (Black *et al*., 1976; Downton *et al*., 1976; Buapet *et al*., 2013b; Buapet and Björk, 2016), and a few studies have mentioned the possibility of the Mehler-peroxidase reaction occurring in seagrasses (Mass *et al.*, 2010; Silva *et al*., 2013; Buapet and Björk, 2016). Previous studies in algae and terrestrial plants have yielded contrasting results with regards to a photoprotective function of alternative electron flow to O_2. While many studies have provided some support for this idea (Heber *et al*., 1996; Kozaki and Takeba, 1996; Park *et al*., 1996; Jiang *et al*., 2006; Oja *et al*., 2011; Roberty *et al*., 2014), others have concluded that they did not play significant roles in protecting the photosynthetic process (Brestic *et al*., 1995; Ruuska *et al*., 2000; Nogués and Alegre, 2002; Driever and Baker, 2011). Previous research has sought to assess the tolerance of seagrasses to high light intensity in the upper intertidal zone, particularly *Halophila ovalis* (Ralph, 1999; Beer and Björk, 2000; Beer *et al*., 2006; Phandee and Buapet, 2018; Buapet *et al*., 2020), as well as their mechanisms of tolerance, including the possible role of energy dissipation via non-photochemical quenching (Ralph, 1999; Phandee and Buapet, 2018; Buapet *et al*., 2020). However, any photoprotective role by alternative electron flow to O_2 in this group of plants remains unexplored.

This study investigates how changes in DIC and O_2 content in seawater affect electron transport rates (ETR), maximum photochemical efficiency of photosystem II (PSII) (F_v/F_m), and non-photochemical quenching (NPQ) in *Halophila ovalis* and *Thalassia hemprichii*. The aim is to evaluate the contribution of O_2 as electron sink and its photoprotective function under DIC-limiting conditions and/or high light intensities. Our previous studies revealed relatively high tolerance to high light intensities and oxidative stress in these two seagrass species, but suggested different underlying tolerance mechanisms (Buapet *et al*., 2017; Phandee and Buapet, 2018; Buapet *et al*., 2020). The results obtained in this study will further improve our understanding of the photoprotective mechanisms of these two seagrasses and provide a baseline for future ecophysiological investigation in tropical intertidal seagrasses.

MATERIALS AND METHODS

Healthy shoots of *Halophila ovalis* and *Thalassia hemprichii* were collected from Trang Province, Thailand (7.24349°N, 99.61599°E). The specimens were immediately transported to the Division of Biological Science, Prince of Songkla University, Hat Yai campus. They were maintained in natural seawater (salinity 30 psu) under natural light (irradiance 0-650 μmol photons$\cdot m^{-2}\cdot s^{-1}$) and ambient temperature (25-30 ºC) prior to the experiments. Fresh plant specimens were collected weekly. The experiments were conducted using artificial seawater prepared according to Beer and Rehnberg (1997), and the dissolved inorganic carbon (DIC) content was adjusted by adding $NaHCO_3$. The salinity of the artificial seawater was 30 psu. Fresh artificial seawater was prepared each morning before starting the experiments. Saturating irradiance for electron transport rates (ETR) of the two seagrasses was determined beforehand by generating rapid light response curves of ETR (Mini-PAM, Walz, Heinz Walz GmbH, Effeltrich, Germany, data not shown).

ETR, NPQ and F_v/F_m under saturating irradiance (400 µmol photons$\cdot m^{-2}\cdot s^{-1}$)

The experiment consisted of two treatments with different initial DIC levels: 1) artificial seawater with 2.2 mM DIC, representing the condition in which carbon assimilation was the main electron acceptor, and 2) artificial seawater with no DIC source added, representing the condition in which O_2 was the main electron acceptor. Whole leaves of *Halophila ovalis* or leaf segments of 2 cm length of *Thalassia hemprichii* were fixed in three closed incubation chambers (5 mL in volume). A magnetic stirrer was used to prevent gas accumulation at the boundary layer. Light was provided by LED-spotlights at saturating irradiance (400 µmol photons $\cdot m^{-2}\cdot s^{-1}$). The room temperature was kept constant at ca. 25 °C, and chambers were surrounded by a layer of water to prevent warming from the light source (temperature within the chambers was 26-27 °C). Photosynthetic characteristics were investigated using a pulse amplitude modulated fluorometry technique (Mini-PAM, Walz, Heinz Walz GmbH, Effeltrich, Germany). Measurements were recorded at three stages of the experiment: 1) after the leaves were incubated at air-equilibrated O_2 level for 20 min; 2) after the O_2 level was lowered to ~10 µM (~3 % of air equilibrium) by purging with N_2 gas and an additional 20 min of saturating irradiance was provided; and 3) after the O_2 level was restored and leaves were incubated for another 20 min. First, leaf segments were dark-adapted for 12-15 min to allow the assessment of the maximum quantum yield of PSII ($F_v/F_m = (F_m–F_0)/F_m$). Any leaves with initial F_v/F_m below 0.7 were removed from the experiments. Then, the leaves were exposed to an irradiance of 400 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (saturating irradiance for electron transport, determined from the rapid light curves) for 20 min, and the quantum yield of electron transport through PSII ($\Phi_{PSII} = (F_m'–F')/F_m'$) was recorded. Final F_v/F_m of the exposed samples was subsequently determined after another round of dark adaptation. Low O_2 condition (approximately 10 µM O_2) was then applied by replacing and bubbling the seawater with nitrogen gas (N_2). The same steps as previously described were taken, and Φ_{PSII} and F_v/F_m were measured. Finally, the O_2 concentration was restored by replacing the seawater with fresh seawater, and Φ_{PSII} and F_v/F_m were determined as previously described. The relative electron transport rates (rETR) at each stage were calculated as rETR = Φ_{PSII}×PAR, where PAR is the photosynthetically active radiation. Non-photochemical quenching (NPQ) was quantified as $(F_m'–F')/F_m'$.

F_v/F_m after prolonged exposure to high irradiance

This experiment consisted of four treatments with different initial levels of DIC and O_2: 1) artificial seawater with 2.2 mM DIC and air-equilibrated O_2 levels, representing well- equilibrated seawater in which both carbon assimilation and oxygen can act as electron acceptors; 2) artificial seawater with 2.2 mM DIC and low O_2, representing the condition in which carbon assimilation is the only electron acceptor; 3) artificial seawater without DIC source and with air-equilibrated O_2 levels, representing the condition in which O_2 is the main electron acceptor; and 4) DIC-free artificial seawater and low O_2, representing the condition in which both carbon assimilation and O_2 are unavailable as electron acceptors. A low O_2 level was achieved by purging the seawater constantly with nitrogen gas, thus keeping the O_2 concentration at approximately 10 µM. The same settings were used as in the previous experiment. First, the leaf segments were dark-adapted for 12-15 min before the initial F_v/F_m was determined. Then, an irradiance of either 400, 1200, 2000 or 2800 µmol photons$\cdot m^{-2} \cdot s^{-1}$ was provided for 40 min before dark adaptation and F_v/F_m was re-measured. A decrease in the fluorescence parameter F_v/F_m was used as an indication of photoinhibitory damage of PSII.

Statistical analysis

The effects of O_2 level (300 µM, 10 µM and 300 µM (restored)) and DIC (2.2 mM or DIC-free) on relative electron transport rate (rETR), maximum quantum yield (F_v/F_m) and non-photochemical quenching (NPQ) were analyzed using repeated-measures ANOVA (O_2 content as the within-group factor and DIC concentration as the categorical factor). Fisher's least significant difference (LSD) post-hoc test was adopted to analyze differences in photosynthetic parameters across treatments.

Linear regression was adopted to analyze the functional relationship between light intensity and F_v/F_m. Furthermore, analysis of covariance (ANCOVA) was used to examine differences between the linear relationships. Cochran's test was used to test the ANOVA's assumption of homogeneity of variances.

RESULTS

Both the DIC and O_2 levels affected the rETR of *Halophila ovalis* (Figure 1a) and *Thalassia hemprichii* (Figure 1b) (repeated ANOVA, $p<0.05$), with a significant interaction between the two factors in *H. ovalis* (repeated ANOVA, $p<0.05$). The rETR of the seagrasses incubated in seawater without an added DIC source was significantly lower than for those with 2.2 mM DIC. Lowering the O_2 concentration caused a decrease in rETR at both DIC levels. Recovery of the initial rETR was observed after the O2 concentration was restored (LSD test, $p<0.05$).

In *H. ovalis*, F_v/F_m was affected by O_2 concentration (repeated ANOVA, $p<0.001$) but not DIC level. At both DIC levels, lowering the O_2 concentration resulted in a decrease in F_v/F_m (LSD test, $p<0.05$). However, the F_v/F_m failed to recover once the O_2 levels were restored (Figure 2a). In *T. hemprichii*, a significant interaction between DIC and O_2 levels was observed. With 2.2 mM DIC, lowering the O_2 concentration resulted in a decrease in F_v/F_m, which recovered once the O_2 was restored (LSD test, $p<0.05$). In the seawater with no DIC source added, lowering the O_2 concentration caused a reduction in F_v/F_m, but it did not recover once the O_2 was restored (Figure 2b).

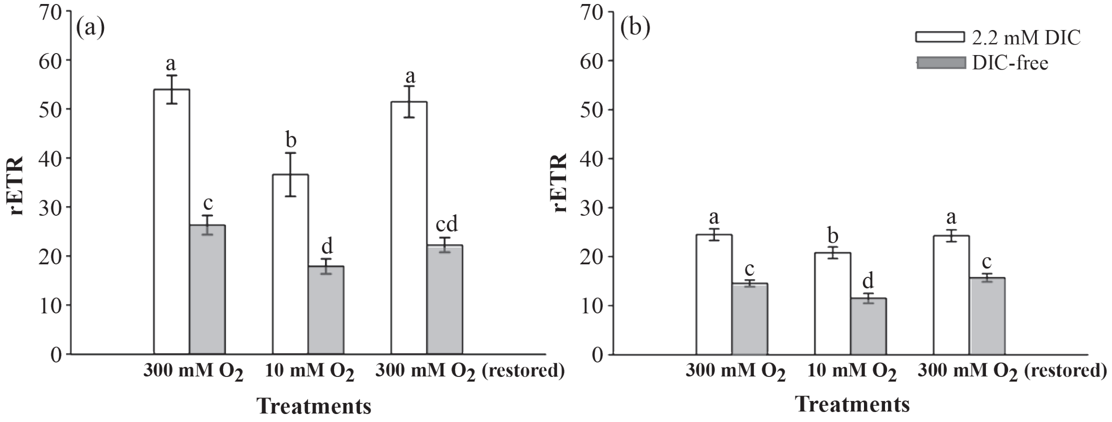

Figure 1. Relative electron transport rate (rETR) after 20 min treatment of (a) *Halophila ovalis* and (b) *Thalassia hemprichii* at different dissolved inorganic carbon (DIC) and O_2 levels. Irradiance was 400 µmol photons·m^{-2}·s^{-1}. Error bars represent SD (n = 8-10). Different letters above the bars represent significant difference ($p<0.05$).

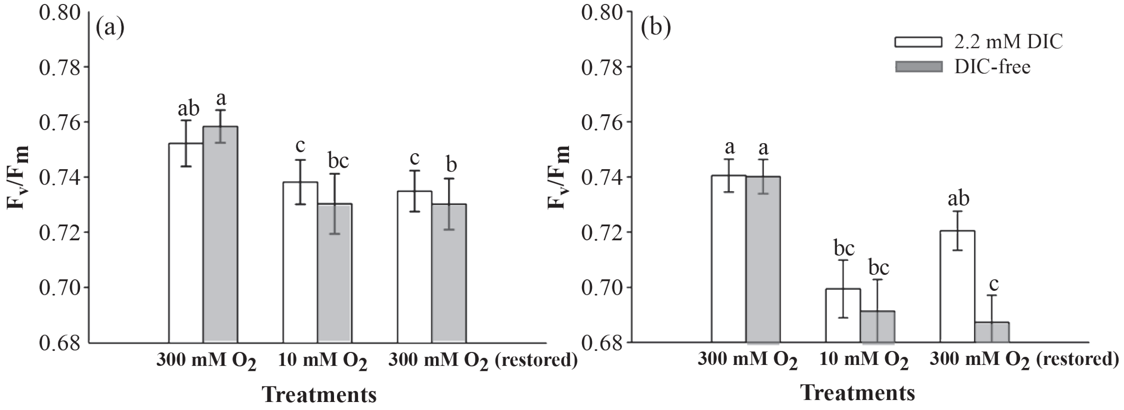

Figure 2. Maximum quantum yield (F_v/F_m) after 20 min treatment of (a) *Halophila ovalis* and (b) *Thalassia hemprichii* at different dissolved inorganic carbon (DIC) and O_2 levels. Irradiance was 400 µmol photons·m^{-2}·s^{-1}. Error bars represent SD (n = 8-10). Different letters above the bars represent significant difference ($p<0.05$).

Significant effects of DIC level were detected on the non-photochemical quenching (NPQ) of *H. ovalis* (repeated ANOVA, $p<0.05$, Table 1), while O_2 level did not affect the NPQ of either species (Figure 3a). The leaves of *H. ovalis* incubated in seawater with no added DIC source exhibited a significantly higher NPQ than those measured in seawater with 2.2 mM DIC. There was no significant effect of either DIC or O_2 level detected in *T. hemprichii* (Figure 3b).

Figure 4 displays the maximum quantum yield (F_v/F_m) of the seagrass leaves exposed to a range of low to high light intensities in different combinations of DIC and O_2 levels. In all treatments, F_v/F_m exhibited a negative correlation with irradiance (regression analysis, $p< 0.001$). However, the analysis of covariance revealed no significant effects of treatments on the functional relationships between irradiance and F_v/F_m.

Table 1. Summary of ANOVA of chlorophyll fluorescence parameters of *Halophila ovalis* and *Thalassia hemprichii* with different dissolved inorganic carbon (DIC) and O_2 levels.

Species	**Variable**	**Factor**	**df**	**MS**	**p**
Halophila ovalis	rETR	DIC level	1	9519.55	<0.001
		O_2 level	2	897.39	<0.001
		DIC level×O_2 level	2	161.33	<0.05
		Error	36	33.60	
	Fv/Fm	DIC level	1	0.00007	0.846838
		O_2 level	2	0.00321	<0.001
		DIC level×O_2 level	2	0.00027	0.240566
		Error	36	0.00018	
	NPQ	DIC level	1	1.9081	<0.05
		O_2 level	2	0.1904	0.178380
		DIC level×O_2 level	2	0.0292	0.759564
		Error	36	0.1052	
Thalassia hemprichii	rETR	DIC level	1	1029.34	<0.001
		O_2 level	2	70.04	<0.001
		DIC level×O_2 level	2	1.87	0.428135
		Error	28	2.14	
	Fv/Fm	DIC level	1	0.00231	0.189823
		O_2 level	2	0.00912	<0.001
		DIC level×O_2 level	2	0.00118	<0.05
		Error	28	0.00032	
	NPQ	DIC level	1	0.0745	0.655520
		O_2 level	2	0.1163	0.473960
		DIC level×O_2 level	2	0.4980	0.052399
		Error	28	0.1517	

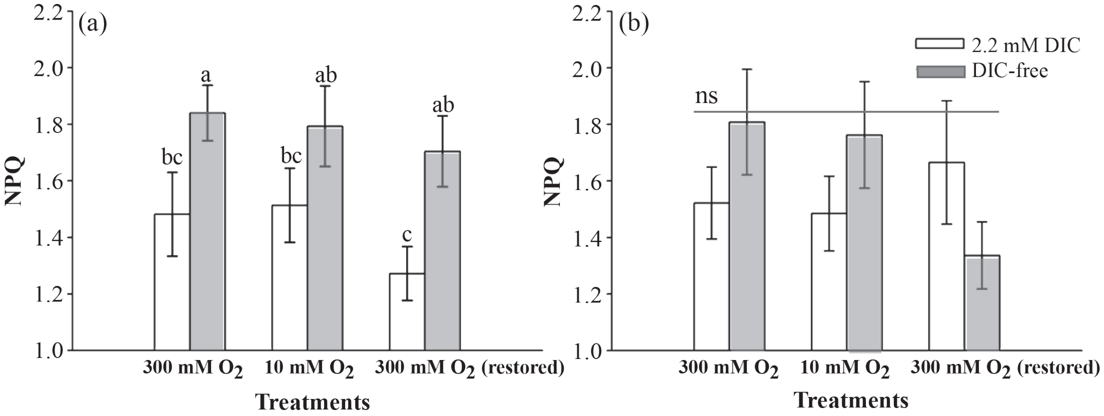

Figure 3. Non-photochemical quenching (NPQ) after 20 min treatment of (a) *Halophila ovalis* and (b) *Thalassia hemprichii* at different dissolved inorganic carbon (DIC) and O_2 levels. Irradiance was 400 µmol photons $\cdot m^{-2}\cdot s^{-1}$. Error bars show SD (n = 8-10). Different letters above the bars represent significant difference ($p<0.05$). ns indicates no statistical significance.

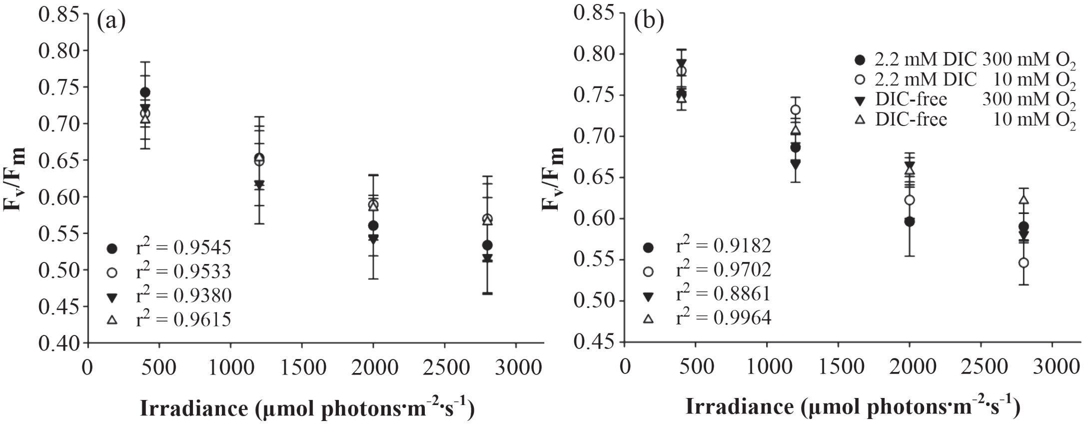

Figure 4. Maximum quantum yield (F_v/F_m) of (a) *Halophila ovalis* and (b) *Thalassia hemprichii* exposed to different irradiances and different DIC and O_2 levels for 40 min. Error bars show SD (n = 5-8).

DISCUSSION

CO_2 is normally the principal sink for photosynthetically-derived electrons. Therefore, it is expected that depleting the dissolved inorganic carbon (DIC) would result in a suppression of rETR. However, it is demonstrated here that carbon assimilation was not the only electron acceptor for *Halophila ovalis* and *Thalassia hemprichii*, as rETR was still significantly maintained in DIC-free seawater. It is possible that non-assimilatory electron flow to O_2 facilitated the remaining rETR (although the internal recycling of CO_2 could also contribute to such rETR). The significant decrease in rETR when the O_2 concentration was reduced to 10 µM (~3 % of air equilibrium) and the recovery of rETR after the O_2 level was restored provide further evidence that O_2 supports an alternative electron flow in *H. ovalis* and *T. hemprichii*. Suppressing alternative electron flow to O_2 by depleting O_2 is thus a plausible explanation for such a decrease in electron transport rates. From the results, we can roughly estimate that *H. ovalis* relied more on O_2 as alternative electron acceptor than did *T. hemprichii*, as exhibited by the relatively larger reduction in rETR when O_2 was reduced. The Mehler-peroxidase reaction is generally saturated at a much lower O_2 concentration than for photorespiration due to the higher affinity for O_2 (Laisk and Edwards, 1998; Badger *et al*., 2000). Therefore, it is likely that the low O_2 level used in this experiment primarily affected photorespiration, whereas the Mehler-peroxidase reaction might still operate during the experiments. This explains the retained rETR in the DIC-free, low O_2 condition. Similar results were also observed in our previous study on a temperate seagrass, *Zostera marina*, in which ETR responses were assessed using light response curves (Buapet and Björk, 2016). Only a small decrease in F_v/F_m (between 0.75 and 0.7) was observed when the concentrations of DIC and O_2 were lowered in *T. hemprichii*, suggesting that short-term changes in DIC and O_2 induced minor photodamage in *T. hemprichii* and no photodamage in *H. ovalis* under saturating irradiance. Additionally, it can be inferred that alternative electron transport to O_2 plays a minor role in photoprotection in *H. ovalis* and *T. hemprichii* under saturating irradiance, as suppressing it (by lowering O_2) led to a further but minor decrease in F_v/F_m. Moreover, only low DIC was shown to modulate non-photochemical quenching (NPQ) in *H. ovalis*. A rise in NPQ observed at low DIC implies that plants were actively engaged in the process of getting rid of excess energy from the light harvesting antenna. This suggests that when carbon assimilation is restricted, the contribution of electron flow to O_2 might be insufficient to relieve the photooxidative pressure.

The second experiment revealed that changes in DIC and O_2 composition did not aggravate photoinhibition under high irradiance. It is possible that the electron flow supported by O_2 in *H. ovalis* and *T. hemprichii*, although substantial, was not sufficient to mitigate photoinhibition under the strong irradiances used. Moreover, when alternative electron flow to O_2 was suppressed, excess light energy could be dissipated by other means, thus compensating for the loss of an alternative electron acceptor. The process most likely to be responsible for this is non-photochemical quenching (NPQ). NPQ can relieve the excitation pressure at an earlier stage in photosynthesis by dissipating large fractions of excess absorbed light energy already at the level of the light harvesting antenna. This might make it the preferred mechanism at high irradiance levels. High capacity for NPQ in intertidal seagrasses has been reported, and it has been proposed that such capacity involved the activity of the xanthophyll cycle and chloroplast relocation (Ralph *et al*., 2002; Ralph and Gademann, 2005; Buapet *et al*., 2017; Phandee and Buapet, 2018; Buapet *et al*., 2020). In addition to NPQ, the Mehler reaction, plastoquinol terminal oxidase (PTOX) and the cyclic electron flow around PSII and PSI have been suggested to take part in excess energy dissipation (Heber, 2002; Shinopoulos and Brudvig, 2012; Laureau *et al*., 2013).

The main conclusion that can be drawn from the present study is that low DIC and low O_2 significantly affect photosynthetic electron transport. Thus, plant photosynthetic efficiency may be influenced by a periodic fluctuation of DIC and O_2 in natural settings. When carbon assimilation is restricted, O_2 can act as an alternative electron acceptor in *H. ovalis* and *T. hemprichii*. However, such alternative electron flow was not a crucial component of the photoprotective mechanisms at high irradiances. In this case, down-regulation of electron transport via non-photochemical quenching might assume a more prominent role, as previously suggested by Brestic *et al*. (1995) and Nogués and Alegre (2002). However, plant responses to stress in natural settings involve various regulatory mechanisms interacting with one another. While hypoxia might not directly promote photoinhibition, it may negatively affect plant productivity and energy homeostasis by reducing cellular respiration, making the plants more sensitive to other environmental changes (Rasmusson *et al*., 2017).

CONCLUSION

Low DIC and low O_2 significantly affect photosynthetic electron transport. When carbon assimilation is restricted, O_2 can act as an alternative electron acceptor in *Halophila ovalis* and *Thalassia hemprichii*. However, such alternative electron flow is not a crucial component of the photoprotective mechanisms at high irradiances.

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