**SPECIAL ISSUE: Physiology and Ecology of Halophytes - Plants Living in Salt-Rich Environments**

**OPEN ACCESS – RESEARCH ARTICLE**

# Light-dark $O_2$ dynamics in submerged leaves of C3 and C4 halophytes under increased dissolved $CO_2$: Clues for saltmarsh response to climate change

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**Received: 23 June 2014; Revised: 10 October 2014; Accepted: 22 October 2014**

Published by Oxford University Press on behalf of the Annals of Botany Company.

**ABSTRACT**

Waterlogging and submergence are major constraints to which wetland plants are subjected, with inevitable impacts on their physiology and productivity. Global warming and climate change, as driving forces of sea level rise, tend to increase such submersion periods and also modify the carbonate chemistry of the water column due to the increased concentration of $CO_2$ in the atmosphere. In the present work, the underwater $O_2$ fluxes in the leaves of two abundant Mediterranean halophytes were evaluated at different levels of dissolved $CO_2$. Photosynthetic enhancement due to increased dissolved $CO_2$ was confirmed for both *Halimione portulacoides* and *Spartina maritima*, probably due to high tissue porosity, the formation of leaf gas films and reduction of the oxygenase activity of Rubisco. Enhancement of the photosynthetic rates in *H. portulacoides* and *S. maritima* were concomitant with an increase in energy trapping and transfer, mostly due to the enhancement of the carboxylation reaction of Rubisco, leading to a reduction of the energy costs for carbon fixation. Transposing these findings to the ecosystem, and assuming increased dissolved $CO_2$ concentration scenarios, the halophyte community displays a new ecosystem function, increasing the water column oxygenation and thus reinforcing their role as principal primary producers of the estuarine system.

**Keywords**: Halophytes; PSII photochemistry; rising $CO_2$; underwater photosynthesis.

## INTRODUCTION

Wetlands are among the most productive ecosystems of the planet, retaining about 1/2 to 1/3 of the carbon fixed and providing important ecosystem services to the estuarine system, namely nutrient regeneration, primary production, and shoreline stabilization as well as a habitat for wildlife (Caçador *et al*., 2009). Estuarine wetlands are known for their high productivity, which has been attributed to the high degree of halophyte coverage and diversity, with a specific zonation, resulting from inter-specific relationships between species and competition for specific optimal habitats (Mendelssohn and Morris, 2000). Another key factor defining species expansion, growth and productivity is their exposure to abiotic stresses, both environmental (for example climate driven) and anthropogenic (for example, pollution with heavy metals). If the future of these ecosystems is to be predicted in the face of climate change, it is of great importance to understand plant stress responses and adaptations at the molecular, biochemical levels, cellular and physiological levels (Urano *et al*., 2010; Yamaguchi-Shinozaki and Shinozaki, 2006).

Waterlogging and submergence are two major features to which wetland plants, especially salt marsh halophytes are subjected, with severe impacts on their survival and productivity (Pedersen *et al*., 2010; Bailey-Serres and Voesenek, 2008). Due to tidal flooding, shoots and leaves can become completely submerged, restricting gas exchange and light harvesting (Colmer and Voesenek, 2009). The frequency, duration and depth of tidal flooding can influence the species distribution in salt marshes, this being determined by, for example, their ecophysiological tolerance to flooding (Voesenek *et al*., 2004; Pedersen and Colmer, 2006). Not only does the slow diffusion of $CO_2$ in the aquatic environment limit its uptake by leaves (Smith and Walker, 1980), but also the decreased light availability, due to attenuation down the water column, impairs photosynthesis (Sand-Jensen, 1989; Pedersen *et al*., 2006). Several plant species have developed leaf adaptations to these constraints in order to enhance underwater $CO_2$ uptake by reducing morphologically the boundary-layer and cuticle resistance or by acquiring $HCO_3^-$ directly from the water column (Colmer and Pedersen, 2008). In submerged leaves of halophytes, $CO_2$ limitation becomes severe when concentrations are near air equilibrium, due to the elevated saturation point of underwater photosynthesis (20 to 75-times the water-air equilibrium conditions; Pedersen *et al*., 2010). Taking into account the present projections made by the Intergovernmental Panel for Climate Change (IPCC), it is expected that the increased levels of atmospheric $CO_2$ will lead to an inevitable increase in the dissolved $CO_2$ in the water bodies (IPCC, 2002), in this way altering the availability of $CO_2$ underwater.

*Spartina maritima* (L.) Loisel (Poaceae) and *Halimione portulacoides* (L). Allen (Amaranthaceae) are two of the most abundant and productive halophytic plants present in the Mediterranean estuaries (Duarte *et al*., 2010; Caçador *et al*., 2013). These species typically colonize the lower marsh mudflats and the sides of the network of channels within the marshes, being subjected to tidal flooding twice per day (Duarte *et al*., 2009; Duarte *et al*., 2014a). Nevertheless, these are photosynthetically different species: *H. portulacoides* utilises $C_3$ photosynthesis (Duarte *et al*., 2012; Duarte *et al*., 2014b), while *S. maritima* is a $C_4$ plant (Duarte *et al*., 2013; Duarte *et al*., 2014b). Previous work showed that these species present very different dynamics under atmospheric $CO_2$ enrichment as well as under submersion (Duarte *et al*., 2014a; Duarte *et al*., 2014b).

In the present paper, we report the $O_2$ dynamics both in light and dark of the leaves of the $C_3$ *H. portulacoides* and the $C_4$ *S. maritima* under different dissolved $CO_2$ concentrations. This has provided insights not only on the species tolerance to submersion but also how $CO_2$ can ameliorate the imposed submersion stress and its consequences in the ecosystem services provided, namely in terms of water column oxygenation.

## METHODS

### Plant harvest

Intact turfs of the target species were collected at the end of the growing season (October), one day before the experiments started, from the Tagus estuary (Alcochete, 38°45'38.78''N, 8°56'7.37''W). All the turfs of the same species were of similar height to ensure similar ages. The intact turfs and their rhizosediment were transported to the laboratory of Marine Botany of the Centre of Oceanography, in air-exposed trays and placed in a FytoScope Chamber (Photon-System Instruments, Czech Republic), in a photoperiod (16h / 8h light/dark) at 20 ºC, until the beginning of the experiments. Sediment was supplemented with ¼ Hoagland solution with the salinity adjusted to 20‰ (estuarine salinity) to maintain moisture conditions from the field.

### Underwater Net Photosynthesis and Respiration

The experimental setup was based on that of Colmer and Pedersen (2008) for similar experiments. Fully expanded leaves (n=3) were excised from their base in the stem. In the case of *S. maritima* leaves, samples were sliced into similar rectangular segments (approximately 5 cm) by cutting their extremities. For *H. portulacoides*, intact excised leaves were used, as their approximate length was 5 cm. The segments were immediately placed in 50 mL plastic gas-tight bottles with rubber stopper (3 bottles containing 3 leaf segments each, per treatment). Hoagland solution (¼ strength was used as incubation medium with salinity adjusted to 20‰ (estuarine salinity) with sea salt mixture and supplemented with $KHCO_3$ in order to attain the desired dissolved $CO_2$ levels (0.05, 0.5, 1.0 and 2.0 mM) and the pH adjusted to 6.00 with KOH, according to field measurements (Duarte *et al*., 2014c). These four dissolved $CO_2$ concentrations correspond to 5.0, 50.0, 100 and 200 $\mu$M of $HCO_3^-$ respectively. Three replicate bottles per treatment were maintained in the light (PAR 400 $\mu$mol photons $m^{-2}$ $s^{-1}$ inside the bottle), while the remaining three replicate bottles were darkened. Both groups were maintained at 25 ºC. Bottles containing only incubation medium were placed in the same conditions to confirm that the $O_2$ concentrations were maintained constant in the absence of leaves. Underwater Net Photosynthesis ($P_N$) and Respiration ($R_N$) were measured using a dissolved oxygen electrode (WTW Oxi 330i/SET) after 2

h. All tubes were gently stirred every 30 min to allow a homogenous distribution of oxygen on the incubation medium (Colmer and Pedersen 2008), as confirmed by the constant $O_2$ values verified in the blank tubes (only Hoagland solution at different levels of dissolved $CO_2$ without leaves) during the time course. The lack of headspace prevented the escape of gaseous $CO_2$. Due to differences in the morphology and succulence of the leaves of the two species, the $O_2$ fluxes were expressed on the basis of dry weight. To determine the dry weight (DW) of the leaves, 20 samples per species, from the same intact turfs used in the experiments, were collected and dried at 60 ºC until constant weight and re-weighted. This normalization was adopted instead of using chlorophyll since during stress conditions chlorophyll content can be affected and thus affect the normalization.

In order to achieve a projection of the $O_2$ production/consumption rates in the Tagus estuarine system, the rates determined in this work were combined with the known biomasses and areas colonised by each of the studied species in the Tagus estuary (Caçador *et al*., 2010; Duarte *et al*., 2010; Caçador *et al*., 2013). The computed values were expressed on a daily basis, considering two high tides per day (one in daytime and another during the night) with a maximum period of plant submersion of 3 h (Serôdio and Catarino, 2000; Duarte *et al*., 2014b). The calculations were made for the four dissolved $CO_2$ scenarios tested in the present work and the $O_2$ production/consumption rates obtained experimentally.

**PAM fluorometry**

Modulated chlorophyll fluorescence measurements were made in attached leaves in the field with a FluoroPen FP100 PAM (Photo System Instruments, Czech Republic). For chlorophyll fluorescence measurements leaves from the light incubations were used. All the measurements in the dark-adapted state were made after covering the leaves with aluminium foil for 30 min. The minimal fluorescence ($F_0$) in dark-adapted state was determined using the measuring modulated light, which was sufficiently low (< 0.1 $\mu$mol $m^{-2}$ $s^{-1}$) not to induce any significant variation in fluorescence. The maximal fluorescence level ($F_M$) in the dark-adapted state was measured by a 0.8 s saturating pulse at 8000 $\mu$mol $m^{-2}$ $s^{-1}$. The maximum photochemical efficiency was assessed as $(F_M-F_0)/F_M$. The same parameters were also measured in light –adapted leaves, being $F'_0$ the minimum fluorescence, $F'_M$ the maximum fluorescence and the operational photochemical efficiency. Rapid light curves (RLC) measured in dark-adapted leaves were attained using the pre-programmed LC1 protocol of the FluoroPen, consisting of a sequence of pulses from 0 to 500 μmol $m^{-2}$ $s^{-1}$. During this protocol the $F_0$ and $F_M$ as well as the maximum photochemical efficiency were measured. Each ΦPSII measurement was used to calculate the electron transport rate (ETR) through photosystem II using the following equation: ETR = ΦPSII x PAR x 0.5, where PAR is the actinic photosynthetically active radiation generated by the FluoroPen and 0.5 assumes that the photons absorbed are equally partitioned between PSII and PSI (Genty *et al*., 1989). Without knowledge of the actual amount of light being absorbed, fluorescence measurements can only be used as an approximation for electron transport (Beer *et al*., 1998a, Beer *et al*., 1998b and Runcie and Durako, 2004). Rapid light curves were generated from the calculated ETRs and the irradiances applied during the RLC steps. Each RLC was fitted to a double exponential decay function in order to quantify the characteristic parameters, alpha and $ETR_{max}$ (Platt *et al*., 1980). The initial slope of the RLC ($\alpha$) is a measure of the light harvesting efficiency of photosynthesis and the asymptote of the curve, the maximum rate of photosynthesis ($ETR_{max}$), is a measure of the capacity of the photosystems to utilize the absorbed light energy (Marshall *et al*., 2000). The onset of light saturation ($E_k$) was calculated as the ratio between $ETR_{max}$ and $\alpha$. Excitation light of 650 nm (peak wavelength) from array of three light and emitting diodes was focused on the surface of the leaf to provide a homogenous illumination. Light intensity reaching the leaf was 3000 μmol $m^{-2}$ $s^{-1}$, which was sufficient to generate maximal fluorescence in all individuals. The fluorescence signal is received by the sensor head during recording and is digitized in the control unit using a fast digital converter. The OJIP transient (or Kaustsy curves) depicts the rate of reduction kinetics of various components of PS II. When a dark-adapted leaf is illuminated with the saturating light intensity of 3500 µmol $m^{-2}$ $s^{-1}$ then it exhibits a polyphasic rise in fluorescence (OJIP). Each letter reflects distinct inflection in the induction curve. The level O represents all the open reaction centres at the onset of illumination with no reduction of QA (fluorescence intensity lasts for 10 ms). The rise of transient from O to J indicates the net photochemical reduction of QA (the stable primary electron acceptor of PS II) to $QA^-$ (lasts for 2 ms). The phase from J to I was due to all reduced states of closed RCs such as QA− QB−, QA QB2− and QA− QB H2 (lasts for 2–30 ms). The level P (300 ms) coincides with maximum concentration of QA− QB2 with plastoquinol pool maximally reduced. The phase P also reflects a balance between light incident at the PS II side and the rate of utilization of the chemical (potential) energy and the rate of heat dissipation (Zhu *et al*., 2005). From this analysis several photochemical parameters were attained and here summarized in Table 1.

**Anti-oxidant enzymatic activities**

All enzyme extraction procedures were performed at 4º C. Briefly, fresh leaves were homogenised in sodium phosphate buffer (50 mM, pH 7.6) with Na-EDTA (0.1 mM) at a ratio of 8 ml per 500 mg fresh weight. The homogenate was centrifuged at 8923 rpm for 20 min, at 4 ºC, and the supernatant used for the enzymatic assays. Enzymatic activity measurements were performed at room temperature (18 ºC).

Catalase activity was measured according to the method of Teranishi *et al*. (1974) by monitoring the consumption of $H_2O_2$, and consequent decrease in absorbance at 240 nm. (ε = 39.4 mM-1 cm -1). The reaction mixture contained sodium phosphate buffer (50 mM, pH 7.6), Na-EDTA (0.1 mM) and $H_2O_2$ (100 mM). The reaction was started with the addition of the extract. Ascorbate peroxidase was assayed according to Tiryakioglu *et al*. (2006). The reaction mixture contained sodium phosphate buffer (50 mM, pH 7.0), $H_2O_2$ (12 mM) and L-ascorbate (0.25 mM). The reaction was initiated with the addition of 100 µL of enzyme extract. The activity was recorded as the decrease in absorbance at 290 nm and the amount of ascorbate oxidized was calculated from the molar extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$.

Guaiacol peroxidase was measured by the method of Bergmeyer (1974) with a reaction mixture consisting of sodium phosphate buffer (50 mM, pH 7.0), $H_2O_2$ (2 mM) and guaiacol (20 mM). The reaction was initiated with the addition of 100 µL of enzyme extract. The enzymatic activity was measured by monitoring the increase in absorbance at 470 nm ($\varepsilon$ = 26.6 $mM^{-1}$ $cm^{-1}$).

Superoxide dismutase activity was assayed according to Marklund (1974) by monitoring the reduction of pyrogallol at 325 nm. The reaction mixture contained sodium phosphate buffer (50 mM, pH 7.6), Na-EDTA (0.1 mM), pyrogallol (3 mM) and Mili-Q water. The reaction was started with the addition of 10 µL of enzyme extract. Control assays were done in the absence of substrate in order to evaluate the auto-oxidation of the substrates.

All enzymatic activities were expressed as U $\mu g^{-1}$ of protein, where U, a unit, is the amount of enzyme that catalyses the conversion of 1 μmol of substrate per second. Proteins were determined according with Bradford (1976), using bovine serum albumin (BSA) as the standard protein.

**Statistical Analysis**

In order to compare the results of the different ecophysiological parameters between species at different treatments the Kruskal–Wallis test was employed. Statistical analyses were carried out using the Statistica Software version 10 (Statsoft).

## RESULTS

**Underwater Net Photosynthesis and Respiration**

For *H. portulacoides*, an increase in dissolved $CO_2$ led to an increase in $O_2$ production (photosynthesis) in the light-exposed leaves and a decrease in the $O_2$ consumption (respiration) in the presence of increasing dissolved $CO_2$ concentrations in dark-incubated leaves (Fig. 1). For *S. maritima*, however, increased dissolved $CO_2$ lead to a decrease in $O_2$ consumption rates (respiration) in light-exposed leaves. In fact, underwater photosynthetic $O_2$ production could only be observed under higher dissolved $CO_2$ concentrations. Regarding the dark-adapted leaves of *S. maritima* there was no distinct pattern of variation in $O_2$ consumption rates with change of dissolved $CO_2$ concentration. The highest underwater $O_2$ production (4 μmol $O_2$ $g^{-1}$ DW $h^{-1}$) was recorded in *H. portulacoides* at 0.5 mM dissolved $CO_2$, while *S. maritima* showed the lowest (1.6 μmol $O_2$ $g^{-1}$ DW $h^{-1}$) even at elevated dissolved $CO_2$ concentrations (2 mM). On the other hand, *S. maritima* exhibited the highest respiratory rates ($O_2$ consumption in the dark), independently of the applied dissolved $CO_2$ concentration.



**PAM fluorometry**

**PSII Quantum Efficiencies and Variable Fluorescence:** The maximum PSII quantum efficiencies (dark-adapted leaves) showed no evident differences among the dissolved $CO_2$ treatments for either species tested (Fig. 2). As for the operational PSII quantum efficiencies, there was a tendency for reduction with increasing dissolved $CO_2$ concentrations. The variable fluorescence (Fv) in both dark- and light-adapted leaves of *H. portulacoides* showed a decrease in both with increasing $CO_2$ treatments, presenting a maximum at 0.5 mM dissolved $CO_2$. On the other hand, in *S. maritima* the Fv in dark-adapted leaves showed an evident increase along with increasing dissolved $CO_2$ concentrations. Nevertheless, there was no distinguishing pattern in the variable fluorescence data in light-adapted leaves of *S. maritima*.

**Kautsky Curves and Transient OJIP parameters:** The O-J phase of the Kautsky curves of all samples presented similar patterns, independently of the dissolved $CO_2$ concentrations (Fig. 3). In *H. portulacoides* the more evident differences were observed during the thermal phase (J-I-P), with higher values in the samples exposed to higher dissolved $CO_2$ concentrations. During the photochemical phase (O-J), there was generally an overlap of the Kautsky plots among treatments. For *S. maritima* leaves, the increase in dissolved $CO_2$ lead to an increase in the intensity of both the photochemical and thermal phases, the latter being more pronounced in leaves subjected to the higher concentration of dissolved $CO_2$.

Regarding the OJIP-derived photochemical parameters, as well as the energy flux variables, some differences were evident (Fig. 3 and 4). The maximum quantum yield of primary PSII photochemistry (φPo) was generally maintained both among $CO_2$ treatments and species. On the other hand, in *H. portulacoides*, small fluctuations were observed in the probability of a PSII trapped electron being transferred from QA to QB ($\psi$0) as well as in the electronic transport quantum yield ($\varphi$Eo) across the different applied $CO_2$ treatments. With *S. maritima* both parameters showed an increase in their relative fluorescence with increasing dissolved $CO_2$. The quantum yield of the non-photochemical reactions ($\varphi$Do) decreased only in *S. maritima* with increasing $CO_2$.

If the variation in driving forces at the various concentrations of dissolved $CO_2$ are analysed (Fig. 5), *H. portulacoides* and *S. maritima*, showed a decrease in the driving force for photosynthesis ($DF_{ABS}$) mainly due to a decrease in the trapping ($DF\psi$) of the excitation energy and in the conversion of this excitation energy into electron transport ($DF\varphi$). The $DF_{RC}$ (light energy absorption) was not affected by the increase in dissolved $CO_2$, in both these species. Nevertheless in *H. portulacoides* there was a recovery of the $DF_{ABS}$ at the highest $CO_2$ level, driven by a simultaneous increase in both the $DF\psi$ and $DF\varphi$.

Normalizing the data on a leaf cross-section basis provided new insights (Fig. 6). It was possible to see that neither of the species presented differences in the number of available PSII reaction-centres (RC) for light harvesting (RC/CS), or the absorbed (ABS/CS) or trapped (TR/CS) energy fluxes. On the other hand, the energy fluxes for electron transport (ET/CS) in *S. maritima* showed a marked increase upon $CO_2$ supplementation, while *H. portulacoides* leaves did not show any significant difference in this parameter or in the dissipated energy fluxes (DI/CS). *S. maritima*, on the other hand, showed a marked reduction in the dissipated energy values at above-ambient $CO_2$ concentrations. This analysis also provided insights on the connectivity between PSII antennae and thus the PSII harvesting efficiency (PG). While in *H. portulacoides* the increase of the antennae connectivity only occurred at the highest tested $CO_2$ concentrations, in *S. maritima* this could be observed in all $CO_2$ levels above ambient concentrations.

**Anti-oxidant enzymatic activities**

Anti-oxidant enzymatic defences are presented in (Fig. 7). *H. portulacoides* showed a marked increase in both CAT and SOD activities with the increasing dissolved $CO_2$ supply and in *S.*

*maritima* leaves there was a decreasing trend in APx and GPx activities along with the increasing dissolved $CO_2$. SOD activity increased at the highest dissolved $CO_2$ concentration.

**(De)Oxygenation of the water column**

Considering the biomass values per square meter and the total abundance of each of the analysed species, the underwater oxygen consumption/production rates were calculated per $m^2$ on a daily basis, for four tested scenarios (Table 2). It was possible to calculate that *S. maritima* was responsible for the highest $O_2$ consumptions in the estuarine system. On the other hand, *H. portulacoides* appears as the major contributor for water column oxygenation, with high rates of $O_2$ production and very low rates of respiration during the night-time. Overall at the community level, as represented by these two species, an increase in the dissolved $CO_2$ concentrations in the water column tended to increase the oxygenation of the water column by these halophytes.

## DISCUSSION

Aquatic plants submerged in different coastal ecosystems, are often flooded by water with $CO_2$ concentrations above the air-water equilibrium (Sand-Jensen *et al*., 1992; Keeley, 1998; Ram *et al*., 1999; Pedersen *et al*., 2010; Duarte *et al*., 2014b). The dissolved $CO_2$ concentration is a key factor, as its availability controls underwater net photosynthesis, in both terrestrial and aquatic plants (Colmer and Pedersen, 2008; Sand-Jensen *et al*., 1992). In estuaries populated with halophytes, not only is their growth controlled by dissolved $CO_2$, but also their function as oxygen providers/consumers. The tolerance of these plants to submersion has often been described on a basis of a quiescence response – the lack of shoot elongation conserves the carbohydrates during submersion periods (Bailey-Serres and Voesenek, 2008). At night-time an increased $O_2$ deficit arises as another stressor in submerged conditions, due to the plants' dependency on dissolved $O_2$ entry from flood waters for night respiration (Waters *et al*., 1989; Pedersen *et al*., 2009; Pedersen *et al*., 2006).

In the present study, two of the more abundant halophytic species in the Portuguese estuarine ecosystems showed very different feedbacks to increases in dissolved $CO_2$. The photosynthetic enhancement due to increased dissolved $CO_2$, verified for *H. portulacoides* and *S. maritima*, has already been described for rice and for *Hordeum marinum* (Setter *et al*., 1989; Pedersen *et al*., 2010). These authors suggested enhancement was driven by the presence of gas films on the leaf surface and high tissue porosity, allowing higher rates of gas exchange according to $CO_2$ availability (Pedersen *et al*., 2010). Also, an increase in $[CO_2]/[O_2]$ ratio can improve $CO_2$ use-efficiency, due to a reduction in Rubisco oxygenase activity (Lorimer, 1981; Bowes 1993; Andersen and Pedersen, 2002). Although this enhancement was detected even at low dissolved $CO_2$ concentrations in *H. portulacoides*, for *S. maritima* it was only achieved at high $CO_2$ concentrations. *Spartina maritima* proved to be highly adapted to submersion, in line with a recent study that also demonstrated the plasticity of *S. maritima* as an efficient adaptation to flooding, allowing plants to recover quickly from the stress imposed by this condition (Duarte *et al*., 2014b). Being a $C_4$ species, these results for *S. maritima* point to a reduction of the oxygenase activity of Rubisco as a consequence of the extremely high internal $[CO_2]/[O_2]$; this, together with a high resistance to $CO_2$ entry, restricts underwater photosynthesis (Lorimer, 1981; Bowes 1993; Andersen and Pedersen, 2002). Although *S. maritima* has a high specific leaf area (SLA) compared with *H. portulacoides*, the presence of a thicker cuticle is probably the factor responsible for this reduced diffusion underwater (Mommer *et al*., 2007). In *S. maritima* the $CO_2$ concentration mechanism occurs in the mesophyll cells catalysed by PEPc supplying the bundle sheath with C4 organic acids, independently of the $O_2$ concentration. In sum, $C_4$ plants with their Kranz anatomy rely on the preferential assimilation of $HCO_3^-$ (Raven, 1970; Raven, 1972). Additionally, it should be noted that, under submersion in turbid water, mitochondrial respiration must be included as a possible contributor for the $O_2$ budgets. Nevertheless, under these conditions *S. maritima* $HCO_3^-$-pump allowed an enhancement of the photosynthetic $O_2$ production at $HCO_3^-$ concentrations as low as 200 μM. Thus, the $CO_2$ concentration mechanism in *S. maritima* is apparently highly adapted to underwater conditions, as shown previously (Duarte *et al*., 2014b), needing comparatively low $HCO_3^-$ concentrations to efficiently supply the bundle sheath cells with the required $CO_2$ concentration for restarting the Calvin cycle. On the other hand $C_3$ plants depend on the availability of $CO_2$, and therefore on the leaf gas film formation (Raven, 1970; Raven, 1972). The presence of wax-rich cuticles has been reported in hydrophobic leaves of *H. portulacoides* (Grossi and Raphel, 2003) and can result in an improvement in gas film formation at the leaf surface, thus promoting gas exchange (Setter *et al*., 1989; Wagner *et al*., 2003; Colmer and Pedersen, 2008). Nevertheless, the $C_3$ mechanism of *H. portulacoides*, dependent on the $CO_2$ acquisition, only showed a significant enhancement at 0.5 mM $CO_2$ concentration, making it less efficient than the $C_4$ of *S. maritima*. All these facts indicate that, while underwater, these species are $C_i$-limited at normal inorganic carbon concentration in estuarine water.

Alongside the anatomical differences, the photosynthetic and electronic processes showed marked differences in light harvesting and processing mechanisms. One important fluorescence parameter, normally associated with stress conditions is the variable fluorescence (Fv). The maximum photosynthetic $O_2$ production in *H. portulacoides*, verified at 0.5 mM dissolved $CO_2$, also showed the highest value of F'v, an indicator of low stress conditions (Duarte *et al*., 2012). The same was true, but to a smaller extent, for the operational quantum yield of this species.

Since $CO_2$ and $O_2$ compete for the same Rubisco active sites (Taiz and Zeiger, 2002), favouring the carboxylation process and reduction of the oxygenation capacity of Rubisco also implies a decrease in the energy costs for $CO_2$ fixation and consequent increase in PSII quantum yield for photosynthesis (Furbank, 1998; Taiz and Zeiger, 2002). This was confirmed by analysing the principal driving forces underlying the electronic photosynthetic processes. In *H. portulacoides* and *S. maritima* there was not only an increase in the driving forces related to the trapping of excitation energy, but also in transfer of this energy to the electron transport chain. Both these increases, lead to an enhancement in total driving force for photosynthesis. It was also interesting to note that, in both species, there were no changes in the light energy absorption ability, confirming the above-mentioned hypothesis (increased PSII efficiency induced by higher Rubisco carboxylation rates) and pointing to an increase in photosynthetic activity due to higher $CO_2$

availability. In fact, in *S. maritima* this was related to an increase in the probability of a PSII trapped electron being transferred from QA to QB ($\psi 0$) as well as for the quantum yield of this transport between quinones ($\varphi Eo$).

All these findings are confirmed by the analysis of the energy fluxes on a cross-sectional area basis. Both species maintained their number of available PSII RC constant and thus the absorbed and trapped energy fluxes did not suffer any changes. Differences arise if the integrity of the PSII antennae is observed and are the basis of the increased energy processing efficiency observed in *S. maritima* leaves. This parameter accounts for all the energetic communication pathways between neighbour PSII antennae (Strasser and Stirbet, 2001; Panda *et al*., 2006). On the contrary to what has been observed in other terrestrial plants, there was no loss of connectivity between the antennae of the PSII units during submersion, indicating an improved survival strategy for underwater conditions (Panda *et al*., 2006; Duarte *et al*., 2014b). The increased PSII antennae integrity in *S. maritima* leads to an enhanced efficiency in transporting energy and thus reduction of the dissipated energy. Although this was also noticed in *H. portulacoides* leaves subjected to the highest dissolved $CO_2$ concentrations tested, it did not result in a significant improvement of the energy fluxes use-efficiency, indicating that the increasing dissolved $CO_2$ concentrations does not affect *H. portulacoides* leaves at the energy processing but in deeper processes.

Comparing the data on anti-oxidant enzymatic activities with the data from the oxygen production, *Halimione portulacoides* showed a maximum photosynthetic $O_2$ production at 0.5 mM $CO_2$ decreasing towards higher $CO_2$ concentrations, concomitantly with an increase in catalase activity. This $O_2$ production decrease or increase consumption during daytime conditions as well as increased CAT activity is in agreement with photorespiratory activation. It is known that activation of photorespiration is an important part of the abiotic stress feedback as a means to dissipate the excessive energy trapped (Voss *et al*., 2013). With the activation of this mechanism, $H_2O_2$ is generated and scavenged by the increased CAT activity. In fact, looking at the data from the principal driving forces, the increase in catalase activity appears simultaneously with a decrease in the driving forces for trapping excitation energy and for photosynthesis. In the $C_4$ *S. maritima*, peroxidase activity (APx and GPx) showed a concomitant decrease along with the increase in $O_2$ production. On the other hand, SOD showed two marked peaks driven by distinct and opposite mechanisms. The first peak at 0.05 mM $CO_2$, is concomitant with high respiratory activity and increased non-photochemical activity, indicating a need to dissipate excessive photon energy, due to the Ci limitation, similar to that observed in plants exposed to increased atmospheric $CO_2$ (Geissler *et al*., 2009; Duarte *et al*., 2014a). The second observed SOD peak, observed in individuals exposed 2 mM dissolved $CO_2$, is coincident with the increase in the photosynthetic activity, and consequent superoxide radical production (Taiz and Zeiger, 2002).

Nevertheless, previous studies (Duarte *et al*., 2014a) with $CO_2$-enriched air showed a very different trend, pointing to different stress mechanisms, occurring during submersion. While the $C_3$ *H. portulacoides* showed an enhancement of the photosynthetic rates while exposed to atmospheric $CO_2$ enrichment, the same was not observed for the $C_4$ *S. maritima*. In the present study this grass showed a very significant improvement in its photosynthetic rates upon dissolved $CO_2$ fertilization that was not observed in immersed conditions (Duarte *et al*., 2014b). This points to two interesting aspects: 1) *S. maritima* is probably $C_i$ limited in submersed conditions and 2) $CO_2$ in fact ameliorates the stress imposed by submersion (Duarte *et al*., 2014a).

All these ecophysiological responses have their repercussions for the ecosystems services provided by the salt marshes, namely in terms of $O_2$ and $CO_2$ production for the water column. Considering the average temperature used in these experiments and the biomass production drawn from the literature (Duarte *et al*., 2010; Caçador *et al*., 2009) as well as the species coverage (Caçador *et al*., 2013), a very simple and basic extrapolation was made for the Tagus estuary salt marshes. It was possible to calculate that there is a general trend for increasing water column oxygenation during the daily tidal cycle (two tides, one in the day-time and another during the night-time), driven by plant underwater photosynthesis. This becomes of great importance if we consider salt marshes as one of the most important primary producers in an estuarine system (Caçador *et al*., 2007; Duarte *et al*., 2010; Caçador *et al*., 2013). Oxygen production by the halophytes becomes a key player overcoming the low rates of air-water $O_2$ diffusion, and thus introduces important amounts of oxygen, required for the heterotrophic species (fishes, macro-invertebrates and bacteria). According to this conclusion, a new salt-marsh service arises as a crucial $O_2$ producer for the estuarine aquatic community to accompany the role of these marshes as important carbon-harvesting primary producers.

## CONCLUSIONS

The diurnal tidal flooding imposes on the halophyte community an underwater environment where gas concentrations are very different from the atmospheric ones. The predicted atmospheric $CO_2$ increase will have consequences not only on the atmospheric composition, but also in the carbonate chemistry of estuarine waters. It is important to consider the physiology of each species, and the consequences that their adaptations to altered $CO_2$ concentration have in terms of the ecosystem they inhabit. The presence of leaf gas films, lack of tissue porosity or other morphological traits of the leaf, as well as photochemical differences and biochemical responses to the imposed condition, are very important characteristics that from a holistic point of view have enormous impacts on the estuarine water column chemistry as a habitat for heterotrophic species. Salt marshes will play a crucial role in counterbalancing the effects of climate change, in terms of water column oxygenation and in buffering its acidification by withdrawing excess $CO_2$.

## CONTRIBUTIONS BY AUTHORS

In the present work all the authors were involved according to their area of expertise. Dr. Bernardo Duarte was responsible of the biophysical and photochemical analysis and Dr. Dinis Santos of the anti-oxidant enzymatic assays. Prof. Isabel Caçador and Prof. João Carlos Marques were responsible for the supervision of the work and final corrections and suggestions in the data interpretation. Prof. Helena Silva and Prof. Nomene Sleimi supervised the work and introduced their expertise in terms of halophyte ecology.

**CONFLICTS OF INTEREST**

No conflicts of interest.

**ACKNOWLEDGEMENTS**

The authors would like to thank to the "Fundação para a Ciência e Tecnologia (FCT)" for funding the research in the Centre of Oceanography (CO) throughout the project PEst-OE/MAR/UI0199/2011, the Institute of Marine Research (IMAR) throughout the project PEst-C/MAR/UI0284/2011 and this specific work throughout the ECOSAM project (PTDC/AAC-CLI/104085/2008). B. Duarte investigation was supported by FCT throughout a PhD grant (SFRH/BD/75951/2011).

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**FIGURE LEGENDS**

**Figure 1.** Oxygen production and consumption by the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average ± standard deviation, n=9. Letters indicate significant differences among $CO_2$ treatments at $p < 0.05$).

**Figure 2.** Photosystem II variable fluorescence and quantum efficiencies (operational and maximum) by the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average ± standard deviation, n=9. Letters indicate significant differences among $CO_2$ treatments at $p < 0.05$).

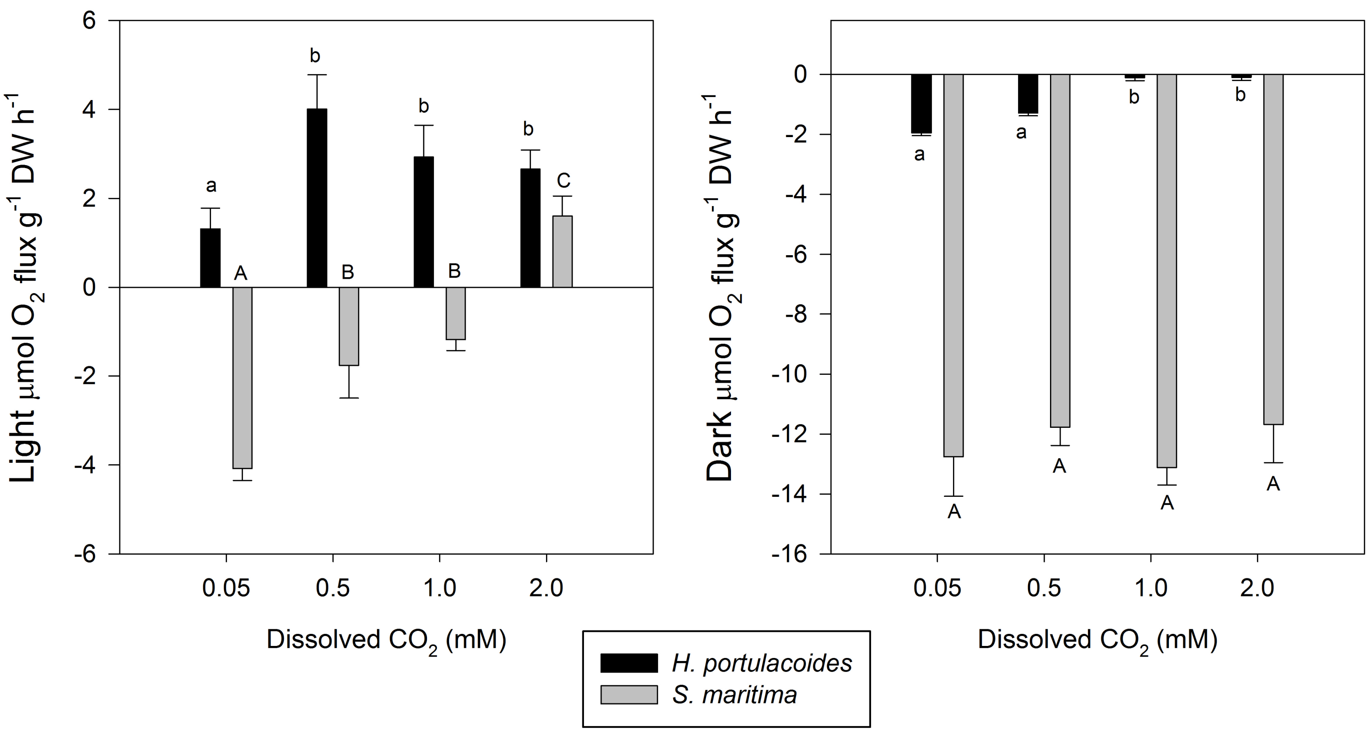
**Figure 3.** Kautsky OJIP curves of the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average, n=9).

**Figure 4.** OJIP-driven photochemical parameters in the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average ± standard deviation, n=9. Letters indicate significant differences among $CO_2$ treatments at $p < 0.05$).

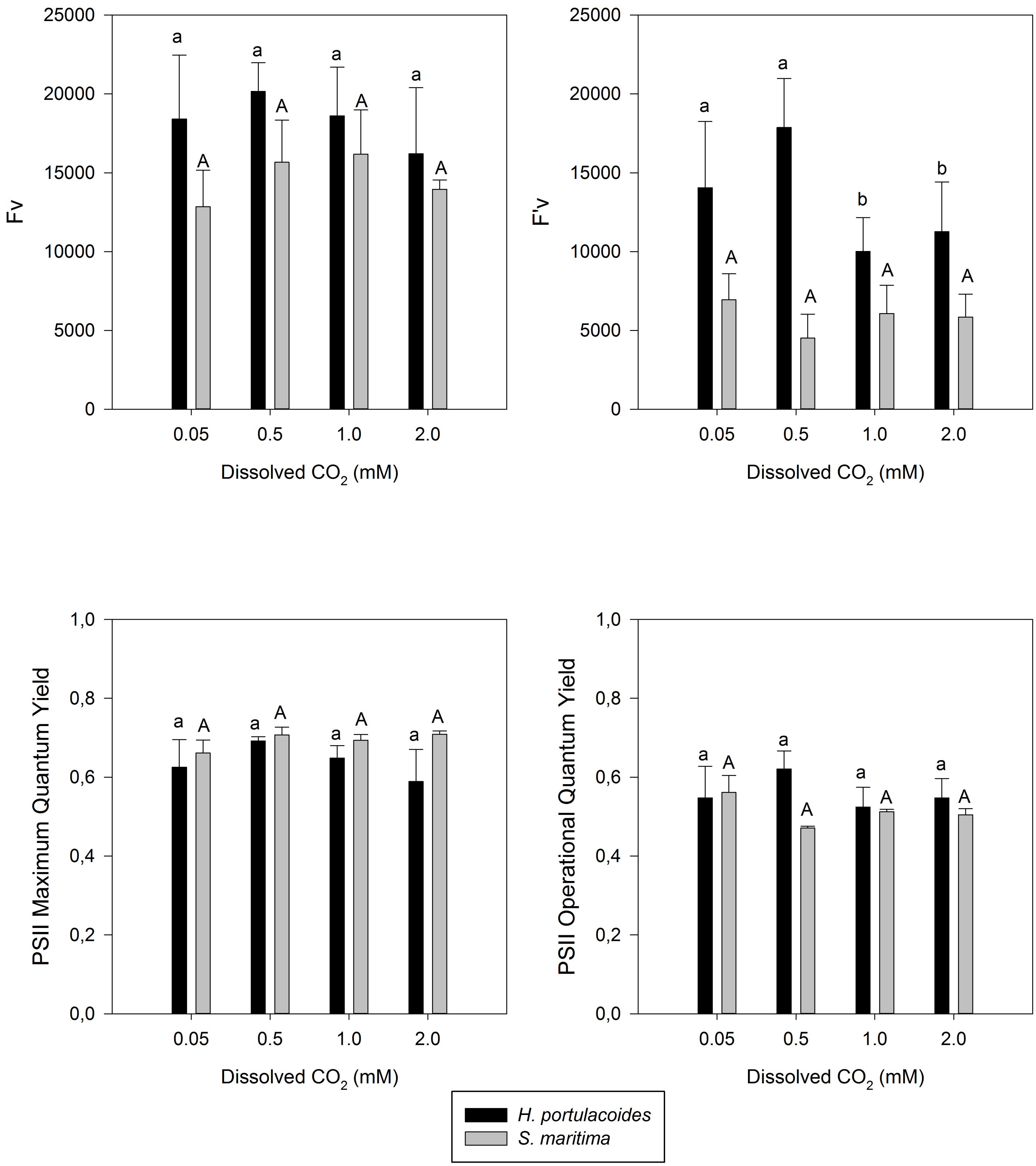
**Figure 5.** Photochemical reactions driving forces in the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average, n=9).

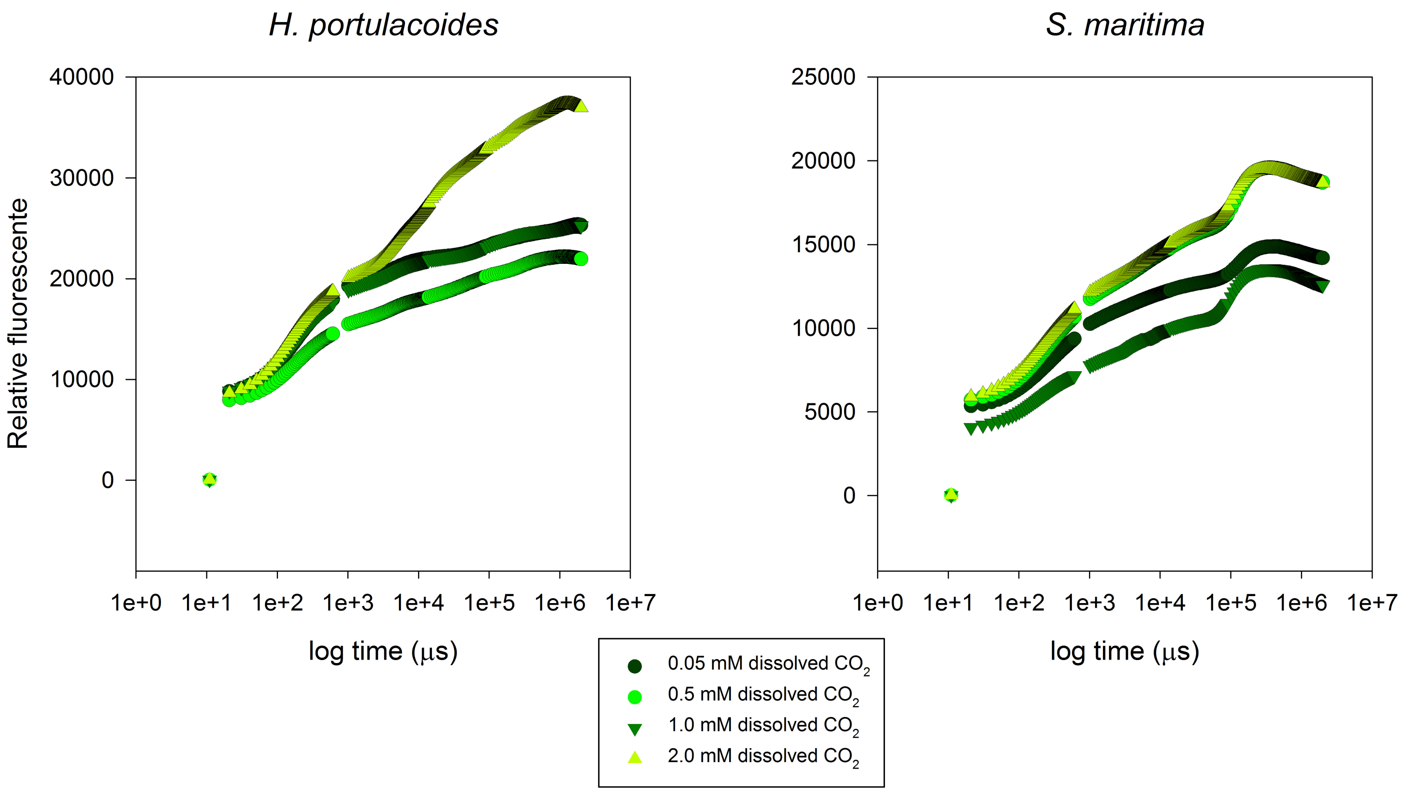
**Figure 6.** PSII antennae connectivity (PG) and energy fluxes on a leaf cross-section basis, in the leaves of the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average ± standard deviation, n=9. Letters indicate significant differences among $CO_2$ treatments at $p < 0.05$).

**Figure 7.** Peroxidase (CAT, APx and GPx) and Superoxide Dismutase (SOD) activity in the leaves of the two tested species in light and dark conditions, at different levels of dissolved $CO_2$ (average ± standard deviation, n=9. Letters indicate significant differences among $CO_2$ treatments at $p < 0.05$).

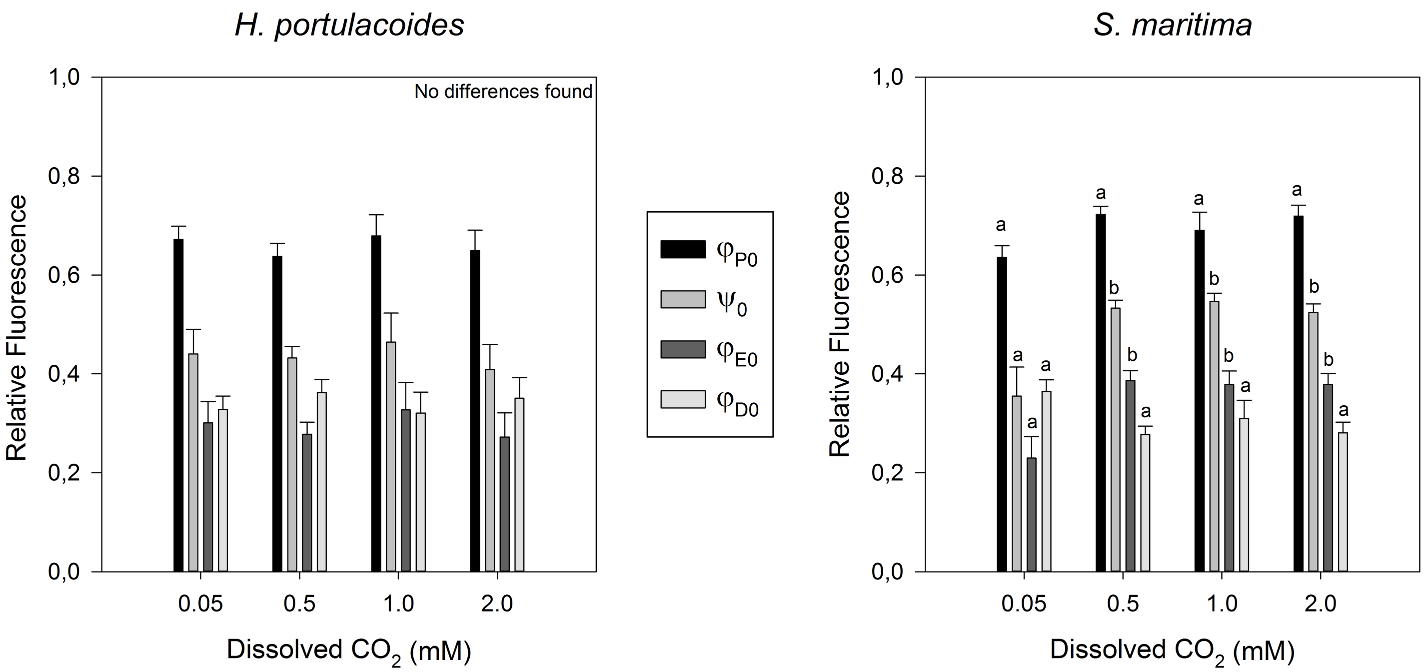


Light μmol $O_2$ flux $g^{-1}$ DW $h^{-1}$
6
4
2
0
-2
-4
-6
a
b
b
b
A
B
B
C
0.05
0.5
1.0
2.0
Dissolved $CO_2$ (mM)
Dark μmol $O_2$ flux $g^{-1}$ DW $h^{-1}$
0
-2
-4
-6
-8
-10
-12
-14
-16
a
a
b
b
A
A
A
A
0.05
0.5
1.0
2.0
Dissolved $CO_2$ (mM)
*H. portulacoides*
*S. maritima*

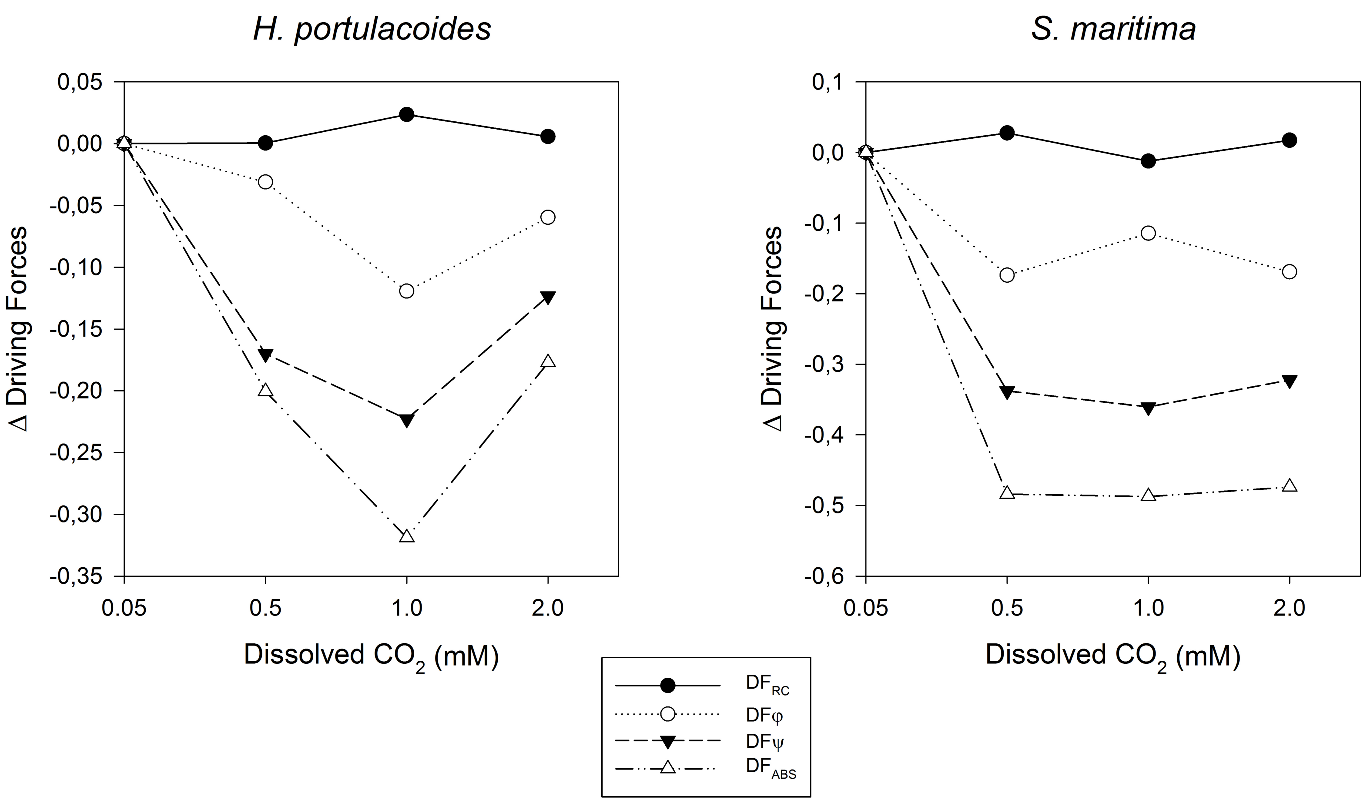

Fv
F'v
PSII Maximum Quantum Yield
PSII Operational Quantum Yield
Dissolved $CO_2$ (mM)
0.05
0.5
1.0
2.0
H. portulacoides
S. maritima

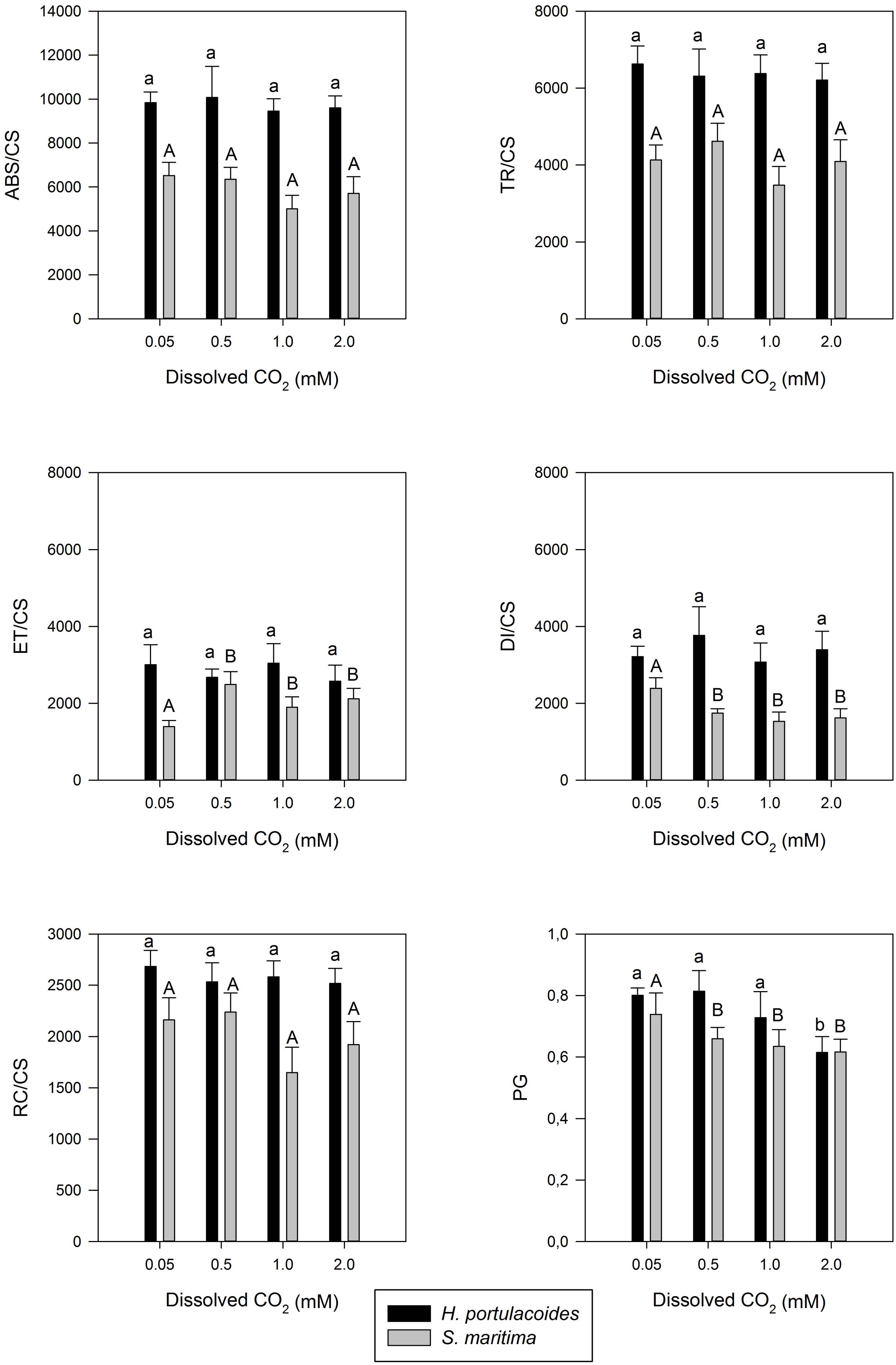


H. portulacoides
S. maritima
Relative fluorescente
40000
30000
20000
10000
0
25000
20000
15000
10000
5000
0
1e+0 1e+1 1e+2 1e+3 1e+4 1e+5 1e+6 1e+7
log time (µs)
0.05 mM dissolved CO2
0.5 mM dissolved CO2
1.0 mM dissolved CO2
2.0 mM dissolved CO2



H. portulacoides
S. maritima
No differences found
Relative Fluorescence
Dissolved CO2 (mM)
0.05
0.5
1.0
2.0
φP0
ψ0
φE0
φD0


H. portulacoides
S. maritima
Δ Driving Forces
Dissolved CO2 (mM)
0.05
0.5
1.0
2.0
DFRC
DFφ
DFψ
DFABS


ABS/CS
TR/CS
ET/CS
DI/CS
RC/CS
PG
0.05
0.5
1.0
2.0
Dissolved $CO_2$ (mM)
a
A
B
b
H. portulacoides
S. maritima

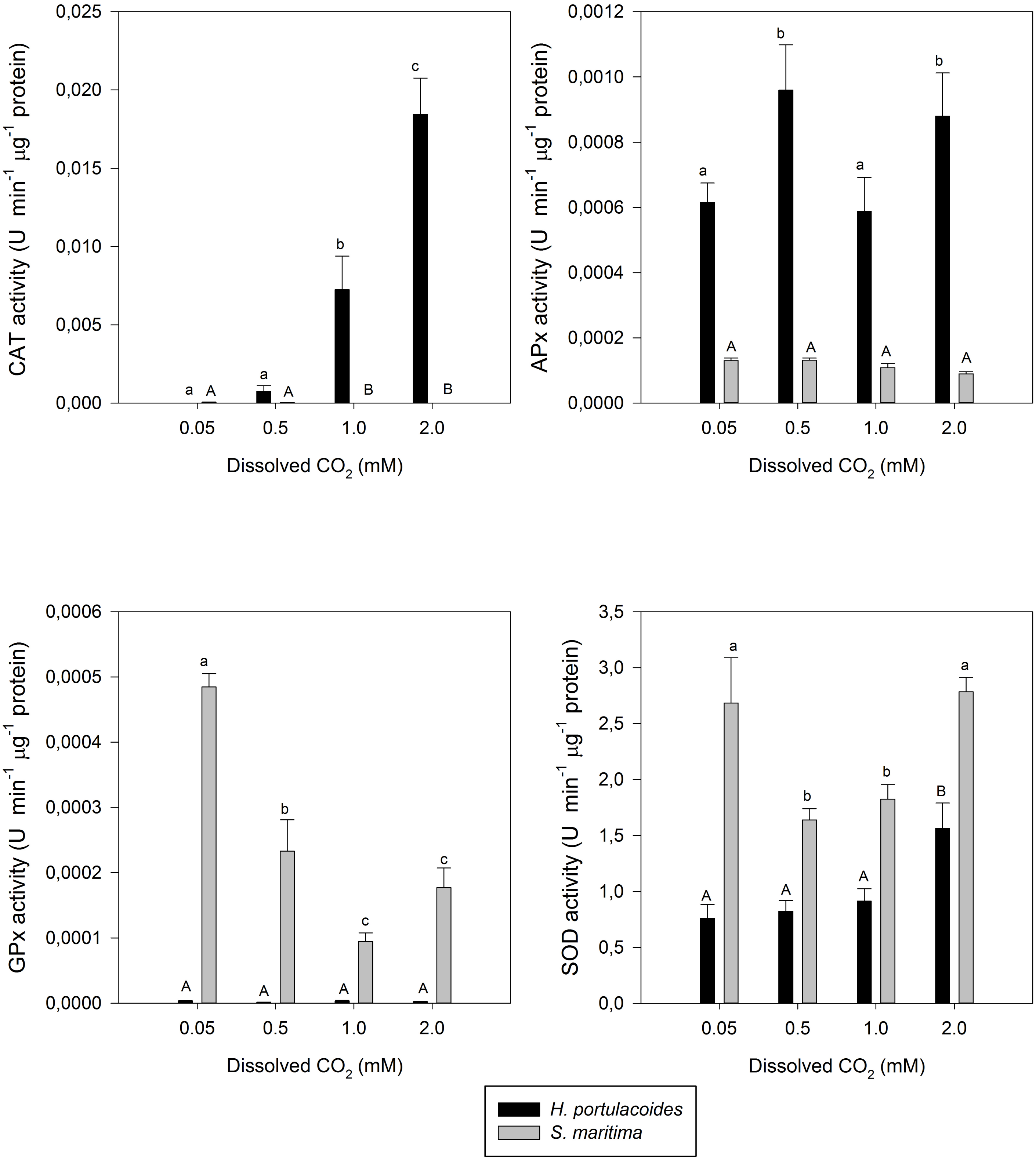

CAT activity (U min-1 µg-1 protein)
APx activity (U min-1 µg-1 protein)
GPx activity (U min-1 µg-1 protein)
SOD activity (U min-1 µg-1 protein)
Dissolved CO2 (mM)
0.05
0.5
1.0
2.0
H. portulacoides
S. maritima

**Table 1.** Summary of Fluorometric analysis parameters and their description.

| | |
|---|---|
| ***Photosystem II Efficiency*** | |
| $F'_0$ and $F_0$ | Basal Fluorescence under weak actinic light in light and dark adapted leaves. |
| $F'_M$ and $F_M$ | Maximum Fluorescence measured after a saturating pulse in light and dark adapted leaves. |
| $F'_v$ and $F_v$ | Variable fluorescence light ($F'_M - F'_0$) and dark ( $F_M - F_0$) adapted leaves. |
| PSII Operational and Maximum Quantum Yield | Light and dark adapted Quantum yield of primary photochemistry, equal to the efficiency by which an absorbed photon trapped by the PSII reaction centre will result in reduction of $Q_A$ to $Q^-_A$. |
| NPQ | |
| ***Rapid Light Curves (RLCs)*** | |
| rETR | Relative electron transport rate at each light intensity (rETR = QY x PAR x 0.5). |
| $\alpha$ | Photosynthetic efficiency, obtained from the initial slope of the RLC. |
| ***OJIP derived parameters*** | |
| $\psi_{P0}$ | Maximum Yield of Primary Photochemistry. |
| $\psi_{E0}$ | Probability that an absorbed photon will move an electron into the ETC. |
| $\psi_{D0}$ | Quantum yield of the non-photochemical reactions. |
| $\varphi_0$ | Probability of a PSII trapped electron to be transported from $Q_A$ to $Q_B$. |
| $P_G$ | The Grouping Probability is a direct measure of the connectivity between the two PSII units (Strasser and Stribet, 2001). |
| ABS/CS | Absorbed energy flux ($F_0$). |
| $TR_0$/CS | Trapped energy flux (ABS/CS x $\psi_{P0}$). |
| $ET_0$/CS | Electron transport energy flux ($\psi_{P0}$ x $\varphi_0$ x ABS/CS). |
| $DI_0$/CS | Dissipated energy flux (ABS/CS – $TR_0$/CS). |
| Diving Force for Photosynthesis (DF ABS) | DF ABS = DF RC + DF $\psi_{P0}$ + DF$\varphi$) |
| Driving Force for Trapping electronic energy (DF $\psi_{P0}$) | DF $\psi_{P0}$ = log ($\psi_{P0}$ / (1 - $\psi_{P0}$) |
| Driving Force for Electron Transport (DF$\varphi$) | DF $\varphi$ = log ($\varphi_0$ / (1 - $\varphi_0$) |
| Driving Force for Energy Absorption (DF RC) | DF RC = log (RC/ABS) |

**Table 2.** $O_2$ (mol) produced (+)/consumed (-) by each halophyte (considering all the coverage area in the Tagus estuary) at the four different considered scenarios per day, during the day and night-time and considering all the estuarine halophyte community (Tagus Estuary), composed by this three species.

| | **Dissolved $CO_2$ concentration (mM)** | | | |
|---|---|---|---|---|
| **Daytime** | **0.05** | **0.5** | **1** | **2** |
| *H. portulacoides* | 3 952 ± 1397 | 12 050 ± 1 397 | 8 816 ± 2 140 | 7 990 ± 1 301 |
| *S. maritima* | -2 039 ± 135 | - 8 79 ± 368 | - 590 ± 124 | 801 ± 224 |
| **Night** | **0.05** | **0.5** | **1** | **2** |
| *H. portulacoides* | - 5 872 ± 196 | - 3 882 ± 193 | - 316 ± 224 | - 297 ± 210 |
| *S. maritima* | -6 379 ± 465 | - 5 885 ± 214 | - 6 557 ± 207 | - 5 840 ± 449 |
| **Daily Budget** | **0.05** | **0.5** | **1** | **2** |
| | -10 338 | 1 404 | 1 353 | 2 654 |