Close but distant: Emersion promotes ecophysiological differentiation between two rhodophytes within an estuarine intertidal zone

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Abstract

Environmental factors associated with emersion determine, among others, the spatial distribution of intertidal seaweeds. This study investigates the emersion tolerance of *Bostrychia scorpioides* and *Catenella caespitosa*, in relation to their zonation pattern in a soft-bottom intertidal zone. The two species are vertically segregated, *B. scorpioides* occupying the uppermost horizon and *C. caespitosa* the lowest one, but they coexist at intermediate tidal parts. Three sets of laboratory-controlled experiments were carried out at ecologically meaningful conditions to achieve the following specific objectives: 1) to identify differences in their water loss and retention; 2) to assess the interactive effect of emersion and temperature (E × T) and emersion and salinity (E × S) on their growth, % DW and elemental composition; 3) to analyse the effect of short-term (3 h) and mid-term (1-6 d) desiccation periods on their photosynthetic performance and its recovery following reimmersion. Our main findings demonstrated that combination of high levels of emersion and temperature (8 h $\cdot$ d^{-1}, 25°C) had a synergistic negative effect on the growth of both species leading to growth imbalance, especially in *Catenella caespitosa*, the lowermost intertidal species. Both species could grow under continuous emersion if thalli do not completely dehydrate, but only *B. scorpioides* maintained similar growth rate and C:N ratio as under continuous submersion, evidencing its higher emersion tolerance. In contrast, the combination of emersion and salinity had an antagonistic outcome, since the influence of salinity decreased as daily emersion period increased. After 3 d of continuous air exposure, both species recuperated 50% of their net photosynthetic rate at saturating irradiance, but after 6 d *B. scorpioides* was able to photosynthetically recover to a greater extent than *C. caespitosa* (20% *vs*. 8% recovery), showing a better physiological performance of recovery following reimmersion. We demonstrated that the uppermost growing species (*B. scorpioides*) possessed higher emersion tolerance and recovery abilities of physiological performance following

reimmersion. The different effect of emersion on species growth and mid-term abilities to recover their physiological fitness clearly supported the observed zonation pattern.

Keywords: *Bostrychia*; *Catenella*; emersion; estuary; intertidal zonation; recovery.

Abbreviations: ASW, artificial sea water; E_{PAR}, irradiance; ETR, electron transport rate; NPR, net photosynthetic rate; NSW, natural sea water; PAM, pulse amplitude modulated; P_{sat}, net photosynthetic rate at saturating irradiance; RGR, relative growth rate; RWC, relative water content.

1. Introduction

Emersion tolerance represents one of the main vertical structuring factors for intertidal seaweeds (Davison and Pearson, 1996; Dring and Brown, 1982; Schonbeck and Norton, 1978). Species inhabiting the upper intertidal zones tend to present higher resistance mechanisms to desiccation, temperature, hyperosmotic conditions and excess irradiance than those occupying lower tidal levels (Davison and Pearson, 1996; Hurd et al., 2014). However, towards the upper intertidal zone, the energetic costs of emersion tolerance should be balanced by the potential benefits in a way that survival is ensured (Hunt and Denny, 2008; Johnson et al., 1998).

Diverse adaptative features and physiological acclimation mechanisms allow intertidal seaweeds to tolerate desiccation, depending upon the extent of the previous emersion period (Dring and Brown, 1982; Lalegerie et al., 2020). On the one hand, some functional forms and growth patterns can minimize water losses (Collado-Vides et al., 1997; Hunt and Denny, 2008; Littler and Littler, 1980). On the other hand, many species are able to fully recover or even enhance their physiological performance following submersion, particularly those occupying upper tidal levels (Dring and Brown, 1982; Ji et al., 2005; Kim et al., 2008; Thomas and Turpin, 1980), based on physiological and biochemical mechanisms that help maintaining cell integrity and reduce emersion-induced oxidative stress (Karsten, 2012; Parages et al., 2014).

Emersion, temperature and salinity act as environmental stressors for intertidal algae, although the species-specific response depends on the severity, duration, frequency of exposure and how the levels of these variables are combined over the tidal cycle (Hurd et al. 2014). In their natural environment, the interplay among emersion variables is mediated by daily and seasonal changes in tidal regime (i.e. timing of the tides) (Williams and Dethier, 2005) and meteorological conditions (Lamote et al., 2012). Combinations of environmental

variables can have antagonistic or synergistic effects on algal ecophysiology, and can affect each physiological process differently (i.e. growth, photosynthesis) (Brown, 1987; Lapointe et al., 1981). For example, temperature and severe emersion (above certain water loss) can act synergistically in intertidal algae (Fernández et al., 2015; Martínez et al., 2012; Matta and Chapman, 1995). On the other hand, antagonistic effects have been reported, as the thalli growing under canopies or forming dense tufts retain humidity that can ameliorate thermal stress (Fernández et al., 2015; Umanzor et al., 2019). Nevertheless, some studies pointed out that desiccation can also play a protective role from excessive thermal stress in some intertidal algae (Hunt and Denny, 2008). Intertidal algae are well known to tolerate a wide range of salinities during emersion (i.e. 10-100, Hurd et al., 2014), being both variables intertwined (Karsten, 2012). Although comparisons of desiccation responses have been studied between brackish and marine ecotypes of several intertidal algae (e.g. Gylle et al., 2009), to our knowledge, the direct effect of combining desiccation and salinity has not been approached at experimental level.

The present study focuses on the intertidal zonation of two red seaweeds from a temperate estuary (Palmones Estuary, Algeciras Bay, southern Spain): *Bostrychia scorpioides* (Hudson) Montagne ex Kützing (Rhodophyta, Ceramiales) and *Catenella caespitosa* (Withering) L. M. Irvine (Rhodophyta, Gigartinales), an algal association reported in many other temperate saltmarshes as *Bostrychetum* (Chapman, 1938). Zonation in soft-bottom coastal ecosystems has been studied to a lesser extent, but the upper limits of intertidal algae in these areas are as well restricted by physical variables as for marine rocky shores (i.e. emersion, temperature, salinity, nutrients) (Lewis, 1964; Melville and Pulkownik, 2007). In the Palmones Estuary, these epiphytic species grow entangled and attached to the lower parts of the lignified stems of the halophytes *Sarcocornia perennis* (Mill.) A. J. Scott and *Atriplex portulacoides* (L.) Aellen. *Bostrychia scorpioides* grows towards the upper intertidal zone (1.10-1.30 m above lowest

astronomical tide (LAT)) and *C. caespitosa* occupies the lower zone (1.00-1.20 m above LAT), whereas both species coexist and overlap at intermediate tidal levels (1.10-1.20) (Sánchez de Pedro et al., 2013), a distribution pattern similar to the reported in other locations (Melville et al., 2005; Pedroche et al., 1995). Despite their vertical zonation stretching a narrow soft-bottom intertidal fringe, small-scale gradients in abiotic conditions and nutrient availability are apparent (Sánchez de Pedro et al., 2016, 2014, 2013).

Both species are mostly emerged during a semi-diurnal tidal cycle, with mean submersions times of 2.5 h (*B. scorpioides*) and 5 h (*C. caespitosa*) (Sánchez de Pedro et al., 2013). From lower to upper intertidal levels temperature ranges from 12ºC (water) to 17.5ºC (air) in winter and 22ºC (water) to 30.2ºC (air) in summer (Sánchez de Pedro, 2017). Salinity ranges from 15 to 37, mostly controlled by irregular flooding events in the river basin catchment (Avilés and Niell, 2005), but a progressive salinization has been noted in the estuary (Clavero et al., 1997; Ruiz-Nieto, 2014).

Previous investigations on these two rhodophytes have addressed specific questions in relation to their carbon acquisition mechanisms (Mercado and Niell, 1999, 2000; Ruiz-Nieto et al., 2014), nutrient uptake (Sánchez de Pedro et al., 2013), light responses (Sánchez de Pedro et al., 2014), and *in situ* intraspecific phenotypic variability in relation to their physiology and biochemical composition (Sánchez de Pedro et al., 2016). According to these studies, growth of *B. scorpioides* was limited by light at the lower parts of the zonation, where *C. caespitosa* is the dominant species. In contrast, the upper intertidal limits for *C. caespitosa* were not limited by light. The absence of *C. caespitosa* at the uppermost intertidal parts was partly attributed to its higher dependency on external nutrient supply (Sánchez de Pedro et al., 2013), but it is unknown to which extent emersion related variables can influence their intertidal zonation.

Based on the observed zonation pattern and the previous ecophysiological knowledge, we

hypothesized that the uppermost growing species in the estuarine intertidal (*B. scorpioides*) should possess higher emersion tolerance and recovery abilities of physiological performance following reimmersion. If both species possessed similar ranges of physiological tolerance to emersion they would coexist at the upper intertidal zone, where longer emersion occurs. Since species of these genera are emersion-tolerant and possess the ability to take up atmospheric CO_2 (Mercado and Niell, 2000; Ruiz-Nieto et al., 2014), we further hypothesized that emersion would not be limiting their growth if thalli remained hydrated, a situation that they frequently encounter at low tide when abiotic conditions are mild (e.g. low temperature, high air relative humidity). For this purpose, three sets of laboratory-controlled experiments were carried out at ecologically meaningful conditions by following three specific objectives: 1) to identify differences in their water loss and retention, given the differences in their branching pattern and surface area (Sánchez de Pedro, 2017); 2) to assess the interactive effect of emersion and temperature (E × T) and emersion and salinity (E × S) on their growth, % DW and elemental composition, and infer the nature of such interactions (i.e. synergistic, antagonistic); and 3) to analyse the effect of short-term (3 h) and mid-term (1-6 d) desiccation periods on their photosynthetic performance and its recovery following reimmersion.

2. Material and methods

2.1. Collection site and culture acclimation conditions

Bostrychia scorpioides and *Catenella caespitosa* specimens were collected from the intertidal zone of a channel in the Palmones Estuary (36º10'13.4" N -5º26'27.6" W), at low tide, between January-May 2015. The Palmones River Estuary (Algeciras Bay, southern Spain) is a shallow, temperate and partially mixed estuary located at the end of a small catchment Mediterranean basin with a tidal regime Atlantic-influenced.

Thalli were removed from the intermediate tidal elevation where both species coexist in their vertical distribution (1.10-1.20 m above the lowest astronomical tide), as described in Sánchez

de Pedro et al. (2013). Algal material was transported in a cooler to the laboratory under dry conditions, coinciding with their emersion period. Once in the laboratory, thalli were rinsed in natural non-filtered seawater to remove sediment and epiphytes.

Fresh material was acclimated to laboratory conditions for 3-5 days prior to the experiments. Algae were cultured at 15ºC, in Perspex cylinders filled with filtered natural seawater (NSW) (Whatman GF/C, Maidstone, UK), with a salinity of 36 and pH of 8.2. Cylinders containing each species were exposed at an irradiance of 45 µmol photons · m^{-2} · s^{-1} of white light (cool daylight, F-18W/54-765 Sylvania) with a 12:12 h L:D photoperiod. A spherical sensor connected to a radiometer (LI-250A Quantum, Radiometer, Photometer, LI-COR® Biosciences) was used to adjust all the irradiances in the study.

2.2. Experimental design

2.2.1. Experiment set 1: Water loss rates

In the first experiment, rates of desiccation or water loss were determined, as it is an essential measurement in comparative studies on desiccation tolerance (Black and Pritchard, 2002). To assess the algal water status in terms of cellular hydration, changes in the relative water content (RWC) were followed for 3 h, at two temperatures (15, 25ºC) and two initial hydration conditions (blotted, hydrated) (Table 1). Fresh weight of algal biomass (FW) was obtained after blotting surface water on paper towel until reaching constant weight. About 200 mg of FW were placed in pre-weighed Petri dishes, at 45 µmol photons · m^{-2} · s^{-1}, in a walk-in cold room at 15 or 25ºC and 54 ± 1% relative humidity. Thalli for the blotted treatment were directly left to dry naturally, while those for the hydrated treatment were spiked with 200 µL of artificial seawater (ASW) and then exposed to air, providing RWC above 100%. Weight measurements (W_t) were taken at fixed times (0, 0.25, 0.5, 0.75, 1, 1.5, 2 and 3 h) to determine the relative water content (RWC) of each individual sample over time. Dry weight (DW) was estimated from the percentage dry weight values calculated for both species by Sánchez de

Pedro et al. (2013).

Relative water content was calculated as follows (Barrs and Weatherley, 1962):

(1) $RWC(\%) = \frac{W_t - \mathrm{DW}}{\mathrm{FW} - \mathrm{DW}}$

where W_t is the weight at each time interval t after emersion; FW is the fresh weight; and DW is the dry weight. The rate of water loss from plant tissues decreases exponentially over time (under constant temperature and relative humidity) (Black and Pritchard, 2002).

The curves of water loss were fitted to the one-phase exponential equation, modified from Tompsett and Pritchard (1998):

(2) $RWC(t) = \left(RWC_i - RWC_f\right) \times e^{(-K \times t)} + RWC_f$

where RWC_i and RWC_f are the initial and final relative water content (%) (at 0 and 3 h, respectively); K is the rate of water loss, which can be used as an expression of dehydration rate ($\% \cdot h^{-1}$); and t is time of drying.

2.3. Experiment set 2: Growth responses to emersion

To assess the effect of emersion on the growth of *B. scorpioides* and *C. caespitosa* in combination to temperature or salinity and infer the nature of the interactive effects between these variables, two independent growth experiments were carried out (Table 1): 1) a first experiment that combined daily emersion periods with temperature (Exp. 2.1 E × T); 2) and a second experiment that combined daily emersion periods and salinity (Exp 2.2 E × S).

For both experiments four daily emersion periods were chosen, based on different conditions these species encounter in the estuary: 0 h (continuously submerged), 3 h (moderate desiccation), 8 h (severe desiccation) and 24 h (hydrated+emerged, H+E). Moderate desiccation (3 h) corresponded with the emersion time at which 90% water loss occurred at 15ºC after blotting the thalli (tested in this study). Severe desiccation (8 h) corresponded to the emersion time experienced by algal specimens located at the uppermost parts of the intertidal zone over a semi-diurnal tidal cycle (Sánchez de Pedro et al., 2013). The hydrated+emerged

treatment (24 h emersion period) represented the conditions under which thalli keep hydrated (RWC > 100%) for several days, due to the water retention during short emersion periods at lower intertidal positions or under conditions of low air temperature and high atmospheric relative humidity. For the moderate and severe desiccation treatments, thalli were blotted dry once a day, placed on Petri dishes and exposed to air for 3 and 8 h, respectively. In the hydrated+emerged treatment, thalli were placed over a filter paper and sprinkled with 200 µL nutrient enriched ASW twice a day.

In the E × T experiment, the four emersion treatments were combined with two temperatures (15, 25ºC) at a constant salinity of 36 (Table 1). Temperature levels were close to the mean values occurring at the lower (15ºC) and upper (25ºC) intertidal parts in their natural habitat during the period of study.

In the E × S experiment, three out of the four described emersion treatments (0, 3, 8 h) were combined with four levels of salinity (10, 20, 36, 45) at a constant temperature (15ºC) (Table 1). Salinity levels were slightly beyond the natural range of values commonly found in the estuary (15-37), since a previous study suggested that physiological responses of these species within the natural range of salinity were similar (Ruiz-Nieto, 2014).

For both growth experiments, about 200 mg FW of algal biomass (corresponding to 4-5 complete thalli) were grown for 10 days in independent Erlenmeyer flasks, filled with 50 mL of nutrient enriched ASW with a pH of 8.2. Experimental salinity levels were obtained by diluting ASW at salinity 36 with distilled water for salinity levels of 10 and 20 and adding NaCl to reach a salinity level of 45 as in Ruiz-Nieto (2014). Culture medium was prepared at the corresponding experimental salinity levels and then spiked with concentrated nutrient solutions to achieve final nutrient concentrations of 40 µM NH_4^+, 5 µM NO_3^-, 2 µM PO_4^{3-}. To prevent nutrient limitation culture medium was daily renewed, and adequate aeration (1 L · min^{-1}) was provided to prevent the formation of boundary layer around thalli. Algae were

grown at an irradiance of 45 µmol photons · m^{-2} · s^{-1}, corresponding to the mean irradiance values at the intermediate parts of the macroalgal intertidal zonation within the sampling period (Sánchez de Pedro et al., 2014). The photoperiod was 12:12 h light:dark (L:D). Biomass was weighed every 3-4 days by blotting thalli for FW determination, and relative growth rate (RGR) was estimated using the equation:

(3) $$RGR\ (\% \cdot\ d^{-1}) = \frac{[\ln(final\ biomass) - \ln(initial\ biomass)]}{elapsed\ time} \times 100$$

In addition, to determine changes in algal composition derived from the variables tested in the growth experiments, we analysed the percentage dry weight (% DW) and elemental composition (internal C, N). Total C and N contents were determined from dry algal material as described in Sánchez de Pedro et al. (2013) and measured in a Perkin Elmer 2400 series CHN elemental auto-analyser (Perkin Elmer Analytical Instruments, Waltham, Massachusetts, USA) following the DOI method (Kristensen and Andersen, 1987). Algal elemental composition (total C, N) was expressed as percentage DW, and tissue C:N as molar ratio.

2.4. Experiment set 3: Desiccation and recovery effect on photosynthesis

Desiccation tolerance and recovery were compared between the two studied species at two ecologically meaningful time-scales in two independent experiments (Table 1): 1) a short-term experiment to test the effect of moderate levels of tidal emersion on photosynthesis (3 h-emersion period) and their following recovery (daily scale); and 2) a mid-term recovery experiment following 1, 3 or 6 d of continuous emersion, which naturally occur during neap tides in the estuary. The short-term responses are representative of the physiological performance over a daily tidal cycle, while the long-term ones relate to long desiccation periods when they can be emerged for several days in the field. Additionally, since the photosynthetic recovery of some intertidal seaweeds can occur within the first 10 minutes after reimmersion (Schagerl and Möstl, 2011), we assessed the fast recovery capacity (within

1 h) of *B. scorpioides* and *C. caespitosa* after 3 h emersion in an independent and complementary experiment. Photosynthetic performance was evaluated by means of two methods described in section 2.5: 1) chlorophyll fluorescence measurements using pulse-amplitud-modulated (PAM) fluorometry (Diving-PAM, Walz GmbH, Effeltrich, Germany) to assess the state of PSII; and 2) net photosynthetic rates by oxygen evolution.

2.4.1. Short-term recovery

For the fast and short-term recovery, about 200 mg FW of algal material were placed on Petri dishes and left air exposed for 3 h at 15ºC, 45 µmol photons · m^{-2} · s^{-1}, and at 54 ± 1% relative humidity. In the short-term recovery (experiment 3.1), relative water content and photosynthetic responses (F_v/F_m and rapid light curves (PAM) and P_{sat} (O_2 evolution), see section 2.5) were measured at 1, 2, 3 h of air exposure, and at 0.5, 1, 2, 3 and 24 h following reimmersion during the recovery period (Table 1).

Independently, we assessed fast recovery of PSII by continuously monitoring changes in maximum quantum yield (F_v/F_m) during 1 h of recovery following the same emersion period (3 h of air exposure), for which twenty thalli of each species were used. Replicates were delayed into darkness to obtain Fv/Fm values. After each pulse, thalli returned to darkness and were not measured again up to 15 minutes later.

Recovery conditions consisted in filling the Petri dishes with nutrient enriched ASW (pH 8.2, salinity 36, 40 µM NH_4^+, 5 µM NO_3^-, 2 µM PO_4^{3-}), at the same experimental conditions than during the desiccation period (15ºC, 45 µmol photons · m^{-2} · s^{-1}, and at 54 ± 1 % relative humidity).

2.4.2. Mid-term recovery

In the mid-term desiccation and recovery experiment about 200 mg FW of algal material were placed on Petri dishes and left air exposed for 1, 3 or 6 days. Photosynthetic ability to recover from continuous desiccation periods was measured at 0.5, 1, 2, 3 and 24 h after reimmersion,

following the same methodology and experimental conditions than in the short-term experiment. Due to longer emersion periods, thalli RWC was below 5% and was not monitored.

2.5. Photosynthetic measurements

2.5.1. PAM fluorometry

To assess the state of PSII by pulse-amplitude-modulated (PAM) fluorometry algal thalli were placed directly on the tip of the fluorometer optic fiber using the supplied sample clip. Rapid light curves (RLC) were determined as the fluorescence response to nine increasing actinic irradiances over the range of 0 to 1250 µmol photons · m^{-2} · s^{-1}, using a Diving-PAM (Heinz Walz, Effeltrich, Germany). Electron transport rates (ETR) as an estimate of gross photosynthesis were calculated as:

(4) $\mathrm{ETR} = \Phi_{\mathrm{PAR}} \times \mathrm{E}_{\mathrm{PAR}} \times 0.5 \times \mathrm{AF}$

where φ_{PSII} is the effective quantum yield of PSII (δF/Fm') at each E_{PAR} (the incident PAR irradiance); 0.5 is the estimated fraction of photons absorbed by the photosynthetic pigments associated with PSII; and AF, the absorption factor, calculated by measuring the fraction of the incident PAR absorbed by thalli, as A = 1 - T, where T is the transmitance and assuming no significant reflectance. The transmittance was determined by comparing readings from the irradiance with a quantum flat-head PAR sensor (Licor LI-189) connected to a radiometer (LICOR 1800, LI-COR Biosciences), with and without the thallus placed on its surface, with a halogen lamp irradiating perpendicularly at a fixed distance. AF values were obtained for *B. scorpioides* (0.70 ± 0.05) and *C. caespitosa* (0.76 ± 0.04) (n = 30).

Non-linear least square fittings were calculated from the ETR-PAR relationships, using the photosynthetic model equation of Webb et al. (1974):

(5) $ETR = ETR_m[1 - \exp(\frac{-\alpha \times E_{PAR}}{ETR_m})]$

where ETR is the electron transport rate at irradiance E_{PAR}, ETR_m is the maximum electron transport rate, α is the photosynthetic efficiency. When data did not fit the saturation model, ETR_m was calculated as the mean of ETR values obtained above saturating irradiance for photosynthesis (230 µmol photons · m^{-2} · s^{-1}, Sánchez de Pedro et al. 2014).

The maximum quantum yield of PSII (F_v/F_m) was estimated as:

(6) $\frac{F_v}{F_m} = \frac{F_m - F_0}{m}$

where F_0 is the initial fluorescence after 15 min of dark-acclimation and F_m is the fluorescence following saturating pulse of actinic light (Schreiber et al., 1986). This parameter reflects the physiological fitness in photosynthetic organisms and has been extensively used as an stress indicator in macroalgae under emersion conditions (Lamote et al., 2012; Schagerl and Möstl, 2011).

2.5.2. O_2 evolution

Net photosynthetic rates at saturating irradiance for photosynthesis (P_{sat}) were determined by measuring oxygen production using a Clark-type Oxygen electrode in a 2.5 ml DW1/AD chamber (Oxygraph systems, Liquid-Phase Oxygen Electrode Chamber, Hansatech Instruments), thermostated by a F25-ME Refrigerated/Heating Circulator (JULABO USA, Inc.) connected to the water jacket of the electrode chamber. A known weight of alga (about 25 mg) was placed in the reaction vessel containing 1.5 mL of filtered NSW. Respiration was measured in darkness before switching on the light for 20 minutes and afterwards oxygen evolution was recorded at 230 µmol photons · m^{-2} · s^{-1} for 5-10 min. This irradiance corresponded with the actual values under the canopy cover in the field at the uppermost parts of the zonation and saturating for both species (Sánchez de Pedro et al., 2014). Light was provided by a halogen lamp (KL 1500 compact Schott). Experimental irradiance was obtained using neutral plastic density filters, which were quantified inside the chamber with a spherical sensor as described in section 2.1. Oxygen evolution was expressed on a FW basis.

2.6. Statistical analysis

The results were shown as the mean ± SD of n measurements of independent replicates, specified for each experiment in table 1. Percentage data used for ANOVA analyses were arcsine square-root transformed.

In experiment 1 (water loss and retention, Table 1), a three-way Model III ANOVA was used to detect differences in the desiccation rates (K), with temperature, initial hydration conditions and species as fixed factors.

In experiment set 2 (growth responses to emersion), we applied three-way Model III ANOVAs to analyse the effect of emersion and temperature (Exp. 2.1 E ×T) or emersion and salinity (Exp. 2.1 E ×T) on the relative growth rates, %DW and elemental composition (Table 1).

In experiment set 3 (desiccation tolerance and recovery experiments), a model selection approach has been adopted to make ecological inferences from the data, given its possibility to confront multiple hypotheses and generate a confidence set of models. Corrected Akaike's index (AIC_c) was used to test the fitting model more likely to have generated the relationships between ETR and time and F_v/F_m and time in the recovery experiments (i.e. linear, saturation, sigmoidal) (Table 1). Due to the time-course design following emersion and after reimmersion (recovery periods) we followed a repeated measures (RM) ANOVA design.

In the short-term experiment (Exp. 3.1), a one-way ANOVA was applied to test the effect of desiccation and recovery on the RWC and photosynthetic responses (F_v/F_m, ETR_m, P_{sat}), with species as fixed factor and recovery time as within factor (Table 1).

In the mid-term experiment (Exp. 3.2), one-way RM ANOVAs were performed to each species independently to determine the effect of emersion period (1, 3, 6 days) on F_v/F_m, ETR_m, and P_{sat} with days of continuous emersion as fixed factor and recovery time as within factor (Table 1).

All the data used in the ANOVA analyses were tested for normality (Kolmogorov- Smirnov test) and subjected to Levene's test (factorial ANOVA) or Mauchley Sphericity test (RM ANOVA) to verify the homogeneity of variance or data sphericity, respectively. When homoscedasticity assumption was not met in the factorial designs (exp. 1, 2) we applied non-parametric Kruskal-Wallis ANOVA on Ranks (H, K-W), with pairwise multiple comparisons for significant results. For repeated measures designs (exp. 3), we applied Greenhouse-Geisser corrected degrees of freedom to obtain adjusted *P*- values when data sphericity assumption was not met.

Tukey's HSD was used for *post-hoc* pairwise comparisons of means when significant results. Interspecific differences were analysed by *t*-test in experiment 3. The significance level was set at $\alpha = 0.05$ for all statistical analyses. Graphs were plotted in GraphPad Prism (v. 8.01 for windows, GraphPad Software Inc., San Diego, CA, USA) and statistical analyses were performed in STATISTICA 7.0 (StatSoft Inc., Tulsa, OK, USA) and SPSS Statistics 25 (IBM, Armonk, NY, USA).

3. Results

3.1. Experiment 1: Water loss rates

Significant interactive effects among initial hydration conditions, temperature and species were detected during the 3 h exposure to emersion (Fig. 1, Table 2). In the blotted treatment, *Bostrychia scorpioides* lost water faster than *Catenella caespitosa* at both temperatures, according to the desiccation rate (K) value (Table 2). Water loss in *B. scorpioides* occurred 1.5 times faster at 15 than 25ºC, while in *C. caespitosa* it increased by 1.3-fold with temperature (Table 2). After 1 h desiccation *B. scorpioides* and *C. caespitosa* experienced water losses of 75% and 44.3% at 15ºC, and 63% and 50% at 25ºC, respectively. Nevertheless, after 3 h emersion both species reached 95% of water loss regardless of temperature (Fig. 1). Thalli subjected to the hydrated treatment experienced lower water loss than those from the blotted

one (Table 2). Despite initial RWC was higher in *B. scorpioides* (249%) than in *C. caespitosa* (229%), both species experienced similar water loss within each temperature level (Fig 1). For initially hydrated specimens, water loss of *B. scorpioides* was not affected by temperature levels, while *C. caespitosa* experienced faster water loss at 25ºC, reaching final RWC values of 70% at 15ºC and 40% at 25ºC after 3 h of air exposure (Fig. 1).

3.2. Experiment 2: Growth, %DW and elemental composition responses to emersion

Growth rates of the two rhodophytes from this study declined at increasing daily emersion time, showing maximum growth under continuous submersion conditions. Growth rates data were homoscedastic in experiment 2.1 (Emersion×Temperature, Levene's test, F=1.10, P=0.374) and 2.2 (Emersion×Salinity, Levene's test, F=1.61, P=0.166), and three-way ANOVAs were applied. In both experiments, emersion had a significant effect and explained 46% and 75% of total variance, while temperature and salinity explained 14% and 6%, respectively (Table 2). Specific results and interactions are described in the following sections.

3.2.1. Emersion × Temperature experiment

Increasing daily emersion periods from 0 to 8 h had a greater negative effect on the RGR of both species at 25ºC than at 15ºC (Fig. 2, Table 3). Three-way ANOVA indicated significant interactions among all factors, but it only explained 2% of the total variance of the data (Table 3). At 15ºC, only severe emersion (8 h $\cdot$ d^{-1}) had a significant effect on the RGR of *B. scorpioides*, with minimum values of 0.16 ± 0.48 % $\cdot$ d^{-1} (Fig. 2). For this species we did not detect significant differences in RGR among the submersion treatment, moderate emersion (3 h) or H+E treatment, with RGR ranging between 2.3-2.8 % $\cdot$ d^{-1} (Fig. 2). At 25ºC, RGR of *B. scorpioides* decreased when thalli were exposed to moderate (3 h) and severe emersion (8 h), while those exposed to the H+E treatment presented similar RGR to submersion (0 h).

406 Temperature only had a differential effect on the RGR of *B. scorpioides* under moderate
407 emersion (Fig. 2). Growth responses of *C. caespitosa* were more sensitive to increasing
408 emersion than those of *B. scorpioides* (Fig. 2). Maximum growth rates of 4% · d^{-1} were
409 obtained under continuous submersion at 15ºC, but when *C. caespitosa* was exposed to
410 moderate or H+E treatment RGR was 75% lower than the continuously submerged treatment.
411 Thalli of this species exposed to severe emersion experienced growth impairment (-0.6 ±
412 0.8% · d^{-1}, Fig. 2).
413 At 25ºC, RGR of *C. caespitosa* under continuous submersion declined by 40% in comparison
414 to 15ºC, with a RGR of 2.4 ± 0.48 % · d^{-1}. At warmer temperature, moderate and severe
415 emersion had a profound negative effect on the RGR of *C. caespitosa,* reaching negative
416 RGRs ranging between -4.2 and -2.6 % · d^{-1}. On the other hand, RGR of *C. caespitosa* from
417 the H+E treatment did not vary with temperature but were 67% lower than under continuous
418 submersion (Fig. 2).
419 When comparing the growth rates of both species, *C. caespitosa* exhibited higher values than
420 *B. scorpioides* when continuously submerged, irrespective of the temperature level. In
421 contrast, RGR of *B. scorpioides* was higher than that of *C. caespitosa* under any emersion
422 treatment, except at 15ºC and at 8 h (Fig. 2). This fact is supported by the significant
423 interaction detected between species and emersion, which explained 17% of the total variance
424 of the data (Table 2).
425 Emersion and temperature had significant effects on the DW percentage and elemental
426 composition (Kruskal-Wallis ANOVA on ranks, $P < 0.001$), but pairwise comparisons
427 showed slight differences among the groups analyzed (Tables S1, S2).
428 Percentage DW of *B. scorpioides* did not experience significant changes with emersion or
429 temperature, while *C. caespitosa* presented higher values at 25ºC. A significant increase in %
430 DW was detected at 3 and 8 h of daily emersion, but they did not differ from the initial values

(Table S1). Total carbon significantly increased in both species after 10 days of culture under continuous submersion, but only at 15ºC. No other variation in C content of the algae respect to emersion treatments or at 25ºC were observed (Table S1). Pairwise comparison for total nitrogen only revealed a lower value for *C. caespitosa* at 15ºC- 0h, coinciding with its maximum RGR (Fig. 2, Table S1). In contrast, algae exposed to the H+E treatment, that had similar RGR than under moderate emersion in *C. caespitosa*, were able to keep their total N without experiencing dilution by growth (Table S1). C:N ratio was significantly higher at 15ºC than at 25ºC, under continuous submersion for *C. caespitosa* (Table S1).

3.2.2. Emersion × Salinity experiment

Three-way ANOVA revealed significant interactions between species and emersion and emersion and salinity, which accounted for 13% and 1% of total variance, respectively (Fig. 3, Table 3). Interaction between species and emersion was based on the higher emersion tolerance of *B. scorpioides* in comparison to *C. caespitosa*, regardless of the salinity level (SP×E, $P < 0.001$; Rank order of main factors: C 0 h > (B 0 h = B 3 h) > C 3 h > B 8 h > C 8 h) (Fig. 3, Table 3). Figure 3 shows the rank order of the factor levels, where *C. caespitosa* reached the highest RGR under continuous submersion, followed by the RGR of *B. scorpioides* at 0 and 3 h. *Catenella caespitosa* experienced a marked decline in its RGR with emersion, with growth rates significantly lower than *B. scorpioides* (Fig. 3). At 8 h of daily emersion and the highest salinity level (45), RGR of *scorpioides* and *C. caespitosa* declined by 52% and 25% respect to the maximum values under continuous submersion.

Salinity lost its effect on growth as emersion increased, which was supported by the significant interaction detected between both variables (Fig. 3, Table 3). Under submersion conditions (0 h) RGR of both species was maximized at salinities of 10 and 20.

When algae were exposed to moderate emersion (3 h), growth progressively declined with salinity (Fig. 3). In contrast, at 8 h of daily emersion, negative growth rates were obtained for both species and salinity effect was not significant (Fig. 3). At this maximum emersion level assayed *B. scorpioides* and *C. caespitosa* had mean RGRs of -0.02 ± 0.49 and -0.58 ± 0.64 % $\cdot$ d^{-1}, respectively (Fig. 3).

The combined action of salinity and emersion did not show a marked effect on the percentage dry weight of *B. scorpioides* and *C. caespitosa,* although significant differences were detected among some groups (Table S2, Table S3). At 0 h, DW was higher at 20 for *B. scorpioides* and 45 for both species, whereas at 8 h of daily emersion salinity did not affect their percentage DW (Tables S2, S3). Elemental composition of *B. scorpioides* and *C. caespitosa* showed significant variations among treatments (Tables S2, S3). Submerged thalli tended to have higher total C content and lowest total N content, thus experiencing greater nutritional limitations associated to their higher growth (Fig. 3, Table S3). However, within each emersion treatment, elemental composition was unaffected by salinity (Table S3).

3.3. Experiment set 3: Desiccation and recovery effect on photosynthesis

3.3.1. Short-term desiccation (3 h) and recovery

Recovery within the first 60 minutes was assessed in a preliminary experiment by continuous measurements of F_v/F_m (Fig. S1 A). A remarkably fast recovery of F_v/F_m was found for both species, since F_v/F_m of *B. scorpioides* and *C. caespitosa* recovered by 50% of their initial values after 17 s and 70 s, respectively (Table S4). F_v/F_m of *B. scorpioides* and *C. caespitosa* fully recovered within 1 and 5 minutes following reimmersion, respectively (Fig. S1 B).

In the short-term experiment, algae were air exposed for 3 h at 15ºC, and changes in RWC, F_v/F_m, ETR_m and P_{sat} were monitored (Fig. 4). One-way RM ANOVA indicated that the time

481 of recovery explained most of the total variation of the data (43-93%) (Table S5). After 1 h
482 exposed to air, specimens from both species had already lost 40-50% of their tissue water
483 content (Fig. 4, Table S5). One hour later, water loss was about 80%, while at the end of the
484 experiment RWC of the thalli was around 10% for both species.
485 Maximum quantum yield of PSII (F_v/F_m) decreased during the experiment (Fig. 4, S5). When
486 RWC was below 40% (between 2 and 3 h air exposure), *B. scorpioides* had significantly lower
487 F_v/F_m compared to *C. caespitosa* and declined more abruptly with desiccation time (Fig. 4).
488 *Bostrychia scorpioides* reached final F_v/F_m values below 0.2, whereas minimum ones for *C.*
489 *caespitosa* were between 0.2-0.4 (Fig. 4). Maximum electron transport rate (ETR_m) values
490 gradually decreased with desiccation time, but significant differences were only detected
491 after 2 h of emersion (Fig. 4). At that sampling time, ETR_m was reduced by 95% for *B.*
492 *scorpioides* and 68% for *C. caespitosa* compared to initial values (Fig. 4). After 3 h
493 desiccation ETR_m dropped to zero for both species, and data fitted better to a linear than to the
494 saturation model (Fig. S2). P_{sat} decreased gradually with emersion time for both species, but
495 significant changes were only found between initial and final values. After 3 h air exposure,
496 P_{sat} was reduced between 70-85% for both species, reaching similar P_{sat} values.
497 After 3 h emersion algae were resubmerged and both species fully recovered the initial F_v/F_m,
498 ETR_m and P_{sat} values within the first 30 minutes (Fig. 4). Interspecific differences were only
499 found 30 minutes after reimmersion for ETR_m, when *B. scorpioides* presented higher values
500 than *C. caespitosa* (Fig. 4, Table S5). Recovery measurements taken 24 h after reimmersion
501 were not significantly different from the values obtained at the different recovery times along
502 the experiment.

503 **3.3.2. Mid-term desiccation (1, 3, 6 days continuous exposure) and recovery**

504 The ability to recover photosynthetic activity of *B. scorpioides* and *C. caespitosa* was tested after
505 1, 3 and 6 days of continuous emersion, following changes in P_{sat}, F_v/F_m and ETR_m at 0.5, 1,

2, 3 and 24 hours upon reimmersion (Fig. 5). One-way repeated measurements ANOVA showed significant interactions among emersion treatments and the recovery time (Table S6). After 1 day air exposure F_v/F_m increased from 0.4 to 0.6 within the first 30 minutes in both species but it did not vary significantly between 1 and 24 h of reimmersion. Likewise, ETR_m values of both species recovered within the first hour of the recovery experiment (Fig. 5). On the other hand, both species regained initial P_{sat} values after 24 h (Fig. 5). At the beginning of the recovery, P_{sat} was 9 and 18% of the initial values in *B. scorpioides* and *C. caespitosa*, respectively. For *B. scorpioides*, P_{sat} gradually recovered, reaching 71% of initial values after 3 h, whereas P_{sat} of *C. caespitosa* recovered by 50% after 2 h of reimmersion and then remained constant (Fig. 5).

When algae were emerged for 3 days, F_v/F_m of *B. scorpioides* increased from 0.36 to 0.54 within the first 30 minutes of recovery and it did not change significantly during the rest of the experiment (Fig. 5, Table S6). ETR_m reached its maximum 2 h after reimmersion, when values were above initial ones, but returned to 100% recovery at 24 h. Maximum quantum yield and ETR_m of *C. caespitosa* exposed to 3 d emersion followed a similar recovery than after one day exposure, but ETR_m values were significantly higher after 3 d of emersion (Fig. 5). On the other hand, a lag phase of 1 h was detected in the recovery of P_{sat} in *B. scorpioides*, which recovered by 35% between 2-3 h and by 93% 24 h later (Fig. 5). By contrast, *C. caespitosa* recovered 63% of its initial P_{sat} within the first 30 minutes and presented a plateau over 3 h.

Despite *C. caespitosa* showed a faster initial recovery in P_{sat} than *B. scorpioides* following reimmersion, P_{sat} of *C. caespitosa* declined to 20% of initial values after 24 h (Fig. 5).

After 6 days continuously emerged, F_v/F_m of *B. scorpioides* was between 0.27-0.35 within the first 3 h after reimmersion, but it reached a final value of 0.53 ± 0.08 at 24 h (74% recovery, Fig. 5). ETR_m of *B. scorpioides* regained 42% of the control values within the first hour but

reached significantly higher values at 24 h (Fig. 5). On the other hand, F_v/F_m and ETR_m of *C. caespitosa* progressively increased from 0 to 2 hours, followed by a plateau between 2-24 h (Fig. 5). *Bostrychia scorpioides* regained 20% of its submerged control P_{sat} 30 min after reimmersion and maintained that rate for the rest of the experiment. By contrast, *C. caespitosa* was only able to recover 8% of submerged control values (Fig. 5).

4. Discussion

The different effect of emersion on the growth and mid-term abilities to recover physiological fitness of the two estuarine rhodophytes clearly support the observed zonation pattern. Our major findings confirm the initial hypothesis that the uppermost intertidal species, *Bostrychia scorpioides* had higher emersion tolerance than *Catenella caespitosa,* demonstrated by lower losses in its growth potential under high levels of emersion and temperature and its greater capacity to recover initial net photosynthetic rates after 6 d of continuous emersion. This ability would be also supported by its high independence on external nutrient supply and higher light affinity (Sánchez de Pedro et al., 2014, 2013). On the contrary, the lowermost growing species, *C. caespitosa*, exhibited higher sensitivity to increasing emersion and temperature, experiencing greater growth imbalance, which together with its ability to grow at low irradiances and its dependence on external nutrient supply, explains its vertical distribution (Sánchez de Pedro et al., 2014, 2013).

4.1. Water loss and retention

Water loss in the field can differ from that in the laboratory depending on ambient humidity and temperature. For example, between consecutive high tides in winter desiccation can be less acute and algal thalli desiccate slowly without reaching complete dehydration (Johnson et al., 1974; Nitschke et al., 2012), while in summer, low tides at mid-day can lead to severe emersion conditions (Hurd et al., 2014). In our study we used small tufts of thalli instead of single thalli, but it still did not mimic the actual disposition of the algae in the field. Thalli are

attached to the stems of the salt-marsh halophytes, floating during high tides and prostrated forming clumps during low tide. As indicated by Hunt and Deny (2008) for another intertidal rhodophyte, external thalli would be exposed to higher emersion stress than those that keep understory. In fact, intraspecific physiological differences were detected in the field for both species (Sánchez de Pedro et al., 2016), which suggests that small-scale gradients can increase the variability of emersion responses in comparison to those obtained by the present study. Water retention by intertidal algae is also influenced by their thallus morphology and growth patterns (Collado-Vides et al., 1997; Littler and Littler, 1980) and by crowding among individuals forming tufts (Hunt and Denny, 2008; Schagerl and Möstl, 2011). In this study, no clear differences in water loss were observed between treatments (blotted *vs*. hydrated), which can be attributed to their similar functional forms. Despite *B. scorpioides* possesses a greater SA:V ratio than *C. caespitosa* (6.0±2.8 *vs*. 10.6±1.6 cm^{-1}, Sánchez de Pedro et al., 2013), the more branched thallus and thorn-like apical parts of the former species (Collado-Vides et al., 1997) may contribute to their similar capacity to retain surface water. Likewise, the greater water content (Sánchez de Pedro et al., 2013) and a hollow thallus of *C. caespitosa* respect to the compact internal morphology of *B. scorpioides* (Van Reine, 1983; Van Reine and Sluiman, 1980) may also explain the similar response.

4.2. Interactive effects among emersion stressors

Combination of emersion and temperature levels had a synergistic deleterious effect on growth of both species. When temperature increased from 15 to 25ºC, growth of both species was more negatively affected by desiccation, particularly in *C. caespitosa* given the more pronounced growth imbalance experienced at severe emersion. In this study, growth responses were tested under continuous temperature levels, but temperature regime in the intertidal habitat is more complex (Hurd et al., 2014). For instance, there is a clear temperature gradient across the intertidal zone of Palmones Estuary, due to the buffering effect of the canopy vegetation.

581 This implies that the sensitivity of *B. scorpioides* and *C. caespitosa* to desiccation will tend to
582 increase towards the upper parts of the intertidal zone, especially that of *C. caespitosa*. The
583 combined action of desiccation and temperature negatively affects the physiology and growth
584 potential of photosynthetic organisms (Dring and Brown, 1982; Fernández et al., 2015).
585 Nevertheless, antagonistic interaction with other variables can ameliorate these effects (e.g.
586 high humidity) (Guenther and Martone, 2014; Martínez et al., 2012), as also noted in *B.*
587 *scorpioides* under the hydrated but emerged conditions.
588 Our study added evidence to the fact that emersion does not necessarily impose a limitation to
589 growth of intertidal algae if desiccation is prevented (Dring and Brown, 1982; Nitschke et al.,
590 2012). Unexpectedly, the dilution effect in growth observed for all specimens subjected to
591 continuous submersion was less apparent in those exposed to hydrated and emerged
592 conditions. Even though nutrients were less available due to the lack of continuous
593 submersion, both species were able to maintain their physiological fitness under these
594 conditions. Nonetheless, the results suggested that hydrated and emerged conditions would
595 benefit growth of *B. scorpioides* to a greater extent than *C. caespitosa*, which would be an
596 efficient mechanism to maximize its growth while emerged, but not desiccated, given the
597 longer emersion periods that species experiences in the field (Sánchez de Pedro et al., 2013).
598 In this sense, Ji and Tanaka (2002) found a positive relationship between water retention and
599 the capacity to maintain photosynthesis under slight desiccation in some intertidal seaweeds.
600 However, since studied species had similar water retention and photosynthetic responses,
601 such differences in their growth at hydrated+emerged conditions might be due to different
602 CO_2 uptake abilities in air. In fact, emersion can promote CO_2 uptake from air in these
603 rhodophytes (Mercado and Niell, 2000; Ruiz-Nieto, 2014). For *Bostrychia scorpioides*, these
604 authors reported a greater C uptake in air than in seawater, as well as a higher external
605 carbonic anhydrase activity compared to that of *C. caespitosa* (Ruiz-Nieto, 2014). The high

606 rates of C assimilation in seaweeds from the high intertidal zone when emerged but not
607 dehydrated have previously been observed (Gao et al., 1999; Johnson et al., 1974; Zhou et al.,
608 2014), due to the greater accessibility of CO_2 in air for photosynthesis (Madsen and Maberly,
609 1990) and the presence of enzymatic mechanisms that enhance the CO_2 flux to the thalli
610 (Mercado and Niell, 2000; Surif and Raven, 1990).
611 Salinity and desiccation can be regarded as comparable abiotic stressors because both induce
612 cellular osmotic changes, but they affect physiological processes in different ways (Karsten,
613 2012; Kumar et al., 2014). Water loss induces transient changes in salinity in the surface water
614 layer of intertidal seaweeds, which can occur at shorter timescales than daily or seasonal
615 changes in salinity. In Palmones Estuary, under conditions of high evaporation in summer
616 months, the formation of salt precipitates can be observed in the thallus surface of *B.*
617 *scorpioides* and *C. caespitosa* (Ruiz-Nieto, 2014). According to that, additive or synergistic
618 effects between saline and desiccation stress might have been expected; however, the
619 combination of both variables had an antagonistic effect on algal growth. Salinity exerted a
620 major control on growth rates of these species, but it loses importance with longer emersion
621 periods. For instance, both species maximized their growth rates at salinities 10-20 under
622 continuous submersion, which was consistent with the optimum found for estuarine algae
623 (Kamer and Fong, 2000; Karsten and Kirst, 1989a), but as daily emersion increased, growth
624 became independent of this variable. Based on our results, we suggest that *B. scorpioides* may
625 optimize its growth at low salinities and at a higher tidal position, while *C. caespitosa* would
626 be primarily limited by desiccation. This would be in accordance with Almodóvar and Biebl
627 (1962), who suggested that tolerance to changes in desiccation and salinity affect the vertical
628 distribution of intertidal macroalgae. Evidence for this was found in some *Bostrychia* species
629 from Australian mangroves, which grew at a higher vertical location in the intertidal zone when
630 salinity was lower (Davey and Woelkerling, 1985). In this sense, Karsten and Kirst (1989b)

proposed that high desiccation may limit the capacity of *B. scorpioides* to regulate turgor pressure at high salinity. The interactive effect between both factors on their osmotic acclimation processes requires further investigation.

4.3. Desiccation tolerance and recovery

Despite both macroalgae are desiccation tolerant species, our study showed that their growth potential is clearly determined by their tolerance to prolonged emersion periods, adding evidence of this pattern for soft-bottom intertidal shores. The highly resilient and similar responses of both species to short-term desiccation indicated that the emersion times occurring at their actual intertidal position are not stressful for them, or that they possess efficient adaptation mechanisms to cope with. Stressful conditions for a given species should be defined based on its response rather than the value of the variable (Davison and Pearson, 1996). At the intertidal zone in Palmones Estuary these species can be exposed for more than 8 h, even at the tidal positions dominated by *C. caespitosa* (e.g. between spring tides, Sánchez de Pedro et al., 2016). The ability of both species to recover physiological performance upon reimmersion over a tidal cycle meets the necessary trait to inhabit the intertidal environment, which is supported by this and previous studies (Brown, 1987; Ji et al., 2005; Smith and Berry, 1986). This result also suggests that desiccation will be a lethal stress for these species only if it exceeds their long-term threshold of tolerance (e.g. less frequent or shorter reimmersion, interaction with other abiotic stressors).

The intertidal rhodophytes from this study sustained net photosynthetic activity upon 50-60% water loss, similar to the responses found in several seaweeds from the upper intertidal zone (Madsen and Maberly, 1990; Zhou et al., 2014). However they did not exhibit the initial increase in NPR after slight desiccation observed in some other intertidal species (Mann and Steinke, 1988; Peña-Salamanca et al., 1999). Slight water loss can increase photosynthesis (Brinkhuis et al., 1976; Gao et al., 1999), but at further dehydration it declines due to the

656 interruption of the electron transport between photosystems (Bewley, 1979; Ji and Tanaka,
657 2002). This fact would explain why photosynthetic responses measured by fluorescence
658 methods dropped faster than those obtained by oxygen evolution. At RWC below 50%, ETR_m
659 and P_{sat} decreased similarly in both species, although F_v/F_m declined faster in *B. scorpioides*
660 than in *C. caespitosa*. Recovery of physiological activity depends on the extent of the previous
661 emersion period (Dring and Brown, 1982), as observed in our study. We found out that both
662 species recovered F_v/F_m remarkably fast, which is an advantageous physiological mechanism for
663 macroalgae inhabiting the upper intertidal zone (Dring and Brown, 1982; Schagerl and Möstl,
664 2011). In addition, both species can withstand RWCs below 10% at least for 3 days, since
665 they recovered initial photosynthetic rates within 1-3 h of reimmersion. Only when both
666 species were emerged for six days, they were unable to fully recover after 24 h of reimmersion,
667 although *B. scorpioides* did it to a greater extent that *C. caespitosa*. Nevertheless, the F_v/F_m
668 values reached after 24 h reimmersion indicate that their thylakoid membranes were not
669 severely damaged (values above 0.5), which suggests that at least after 6 d of continuous
670 emersion they may fully recover their initial P_{sat} after a longer recovery period. This suggests
671 that they may also thrive longer emersion periods, especially *B. scorpioides*. In fact, these
672 species may be continuously emerged up to two weeks in their natural habitat, although as for
673 other intertidal communities, the severity of the emersion stressors will vary upon seasonality
674 and meteorological conditions (Dethier and Williams, 2009; Schagerl and Möstl, 2011).
675

676 **5. Conclusions**

677 This study demonstrates that emersion tolerance represents a main structuring factor for the
678 intertidal zonation of *Bostrychia scorpioides* and *Catenella caespitosa* in Palmones Estuary,
679 adding evidence to the previous studies in other marine intertidal communities (Chapman,
680 1938; Dring and Brown, 1982; Phillips et al., 1996). From the perspective of the interspecific

competition, *C. caespitosa* will be the dominant species under conditions close to continuous submersion, as those experienced in the lower part of the intertidal zone, where this species is restricted to. However, as emersion increases, the competitive advantage in terms of primary production clearly shifts towards *B. scorpioides*, which thrives high up in the intertidal zone due to its better ability to maintain growth rates at harsher abiotic conditions. Biotic variables (e.g. competition, facilitation and herbivory pressure) may also modulate their zonation pattern, which should be addressed in future field and experimental studies. Our findings also highlight the importance of assessing mid-term responses to emersion, since short-term responses may not be helpful to discern the actual abilities to tolerate extended periods of emersion and in turn, not relate to the tidal position of seaweeds. The results of this work could be used to hypothesize the future performance of the populations of these species towards estuarine salinization and global warming.

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Table 1. Summary of the experimental design, indicating experimental conditions, treatment levels for emersion and statistical analysis applied.

Experiment set	Sub-experiment	Duration	Treatment/ Emersion	Temperature	Salinity	Control variables	Statistical design	n
Exp.1: Water loss and retention	-	3h	Initial thalli hydration (Blotted, hydrated)	15, 25	36	RWC	Factorial 3-way ANOVA (SP × Initial thalli hydration × T)	4-12
Exp.2: Growth responses to emersion	2.1 E × T	10 d	0, 3, 8 h · d^{-1} H+E (24 h · d^{-1})	15, 25	36	RGR, % DW, Elemental composition	Factorial 3-way ANOVA (SP × E × T)	4-7
	2.2 E × S	10 d	0, 3, 8 h · d^{-1}	15	10, 20, 36, 45	RGR, % DW, Elemental composition	Factorial 3-way ANOVA (SP × E × T)	4
Exp.3: Desiccation and recovery effect on photosynthesis	3.1 Short-term	24 h	3 h emersion + 0, 0.5, 1, 2, 3, 24 h recovery	15	36	RWC, F_v/F_m, ETR_m, P_{sat}	Repeated measures 1W RM-ANOVA (SP × t)	3
	3.2 Mid-term	6 days	1, 3, 6 d of continuous emersion + 0, 0.5, 1, 2, 3, 24 h recovery	15	36	F_v/F_m, ETR_m, P_{sat}	Repeated measures Within SP: 1W RM-ANOVA (E × t) Between SP: *t*-test	4

Emersion (E) in h · d^{-1}, Temperature (T) in°C, Salinity (S). Relative growth rate (RGR) in % d^{-1}. Relative water content (RWC) in %. Percentage thalli dry weight (% DW). Species (SP) are *B. scorpioides* and *C. caespitosa*. Time (t). Maximum quantum yield (F_v/F_m), Maximum electron transport rate (ETR_m), Net photosynthetic rate at saturating irradiance (P_{sat}), sample size per condition (n). In experiment 1, treatments were Blotted (t0 = FW) and Hydrated (t0 = FW+200µL ASW). In experiment 2.1, the hydrated thalli (RWC 100%) under continuous emersion (24 h· d^{-1}) treatment is indicated as H+E. In experiment set 3, recovery conditions following desiccation periods were carried out at 15°C and salinity 36. For all experiments, irradiance was set at 45 µmol photons · m^{-2} · s^{-1}, and relative humidity in the culture chamber at 54 ± 1%.

Table 2. Kinetic parameters of the relative water content curves from experiment 1 (water loss), measured in *B. scorpioides* and *C. caespitosa* at two temperatures (15, 25º C) and two initial hydration conditions (blotted, hydrated).

Treatment	Blotted				Hydrated				Kruskal-Wallis ANOVA	
Species	*B. scorpioides*		*C. caespitosa*		*B. scorpioides*		*C. caespitosa*			
Temperature	15ºC	25ºC	15ºC	25ºC	15ºC	25ºC	15ºC	25ºC	$H_{(52,7)}$	*P*
RWC_i	99.4 ± 0.57 [a]	102 ± 0.54 [b]	101 ± 0.69 [b]	103 ± 0.66 [c]	249 ± 10.9 [d]	244 ± 15 [d]	229 ± 11.2 [d]	229 ± 8.43 [d]	47.5	<0.001
RWC_f	1.28 ± 1.18 [a]	0.00 ± 0.00 [b]	2.57 ± 2.67 [a]	0.00 ± 0.00 [b]	0.00 ± 0.00 [b]	21.75 ± 9.98 [c]	6.46 ± 12.91 [b]	23.09 ± 3.52 [c]	37.1	<0.001
K	2.04 ± 0.24 [a]	1.36 ± 0.06 [b]	0.83 ± 0.08 [c]	1.08 ± 0.09 [d]	0.42 ± 0.04 [e]	0.72 ± 0.11 [c]	0.38 ± 0.04 [e]	0.83 ± 0.10 [c]	47.7	<0.001
r^2	0.997	0.997	0.996	0.990	0.996	0.992	0.992	0.995		

RWC_i and RWC_f are the relative water contents at the beginning and at the end of the experiment (%). K represents the desiccation rate ($\% \cdot h^{-1}$). r^2 is the coefficient of determination. Values are mean ± SD for parameters (n = 4-12). Same letters indicate homogeneous groups of data within each variable obtained from non-parametric Kruskal-Wallis ANOVA ($H_{N,df}$, $P < 0.05$) and pairwise comparisons.

Table 3. Three-way ANOVAs for the relative growth rates (RGR) of *B. scorpioides* and *C. caespitosa* from experiment 2.1 (Emersion × Temperature) and experiment 2.2. (Emersion × Salinity). Bold font highlights the statistical significance for each factor.

Experiment	Source of variation	Percentage of total variation (%)	F	P
2.1. Emersion × Temperature	SP	8	90	< **0.0001**
	T	14	152	< **0.0001**
	E	46	170	< **0.0001**
	SP × T	4	46	< **0.0001**
	SP × E	17	63	< **0.0001**
	T × E	5	17	< **0.0001**
	SP × T × E	2	6	**0.0008**
2.1. Emersion × Salinity	SP	0	0	0.7302
	S	6	42	< **0.0001**
	E	75	760	< **0.0001**
	SP × S	0	2	0.106
	SP × E	13	131	< **0.0001**
	S × E	1	5	**0.0004**
	SP × S × E	1	2	0.0789

Factors are species (SP), temperature (T), emersion hours per day (E) and salinity (S)

APPENDIX A. SUPPLEMENTARY MATERIAL

FIGURE S1. Fast recovery of maximum quantum yield (F_v/F_m) of *B. scorpioides* and *C. caespitosa* during 1 h (A) and 5 min (B) of reimmersion after being exposed to 3 h emersion at 15ºC.

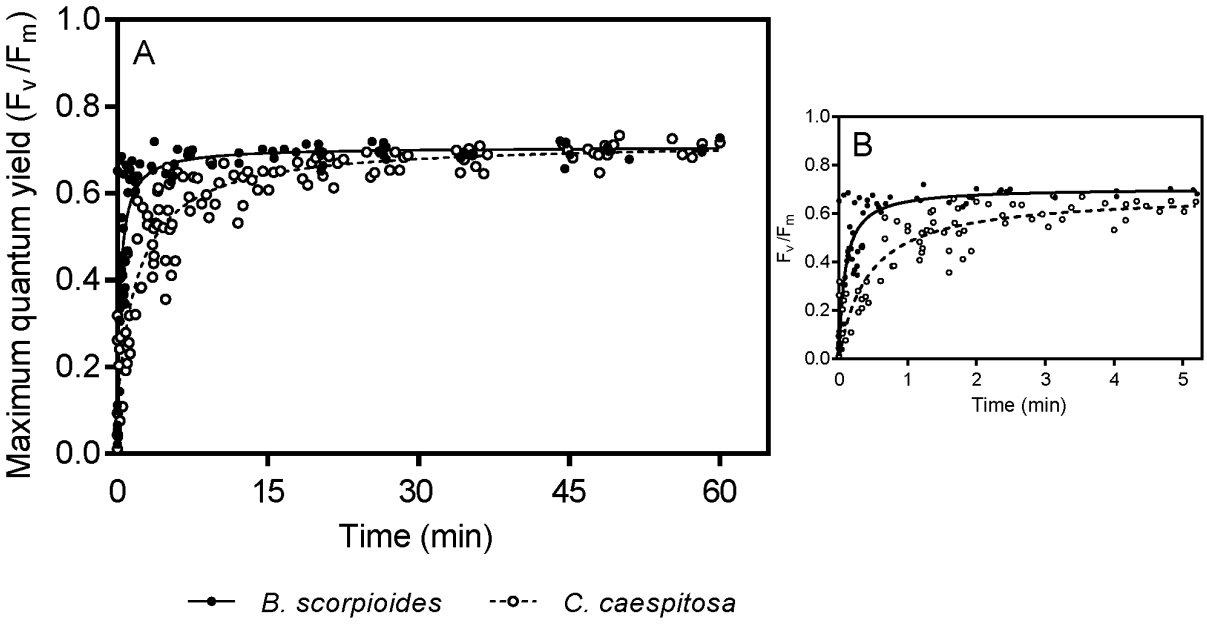


FIGURE S2. ETR-irradiance (ETR-E) curves of *B. scorpioides* and *C. caespitosa* measured during 3 hours of air exposure and during 3 hours following reimmersion (experiment 3.1), at 15ºC, salinity 36 and 45 µmol photons · m^{-2} · s^{-1}. Values are mean ± SD (n = 4).

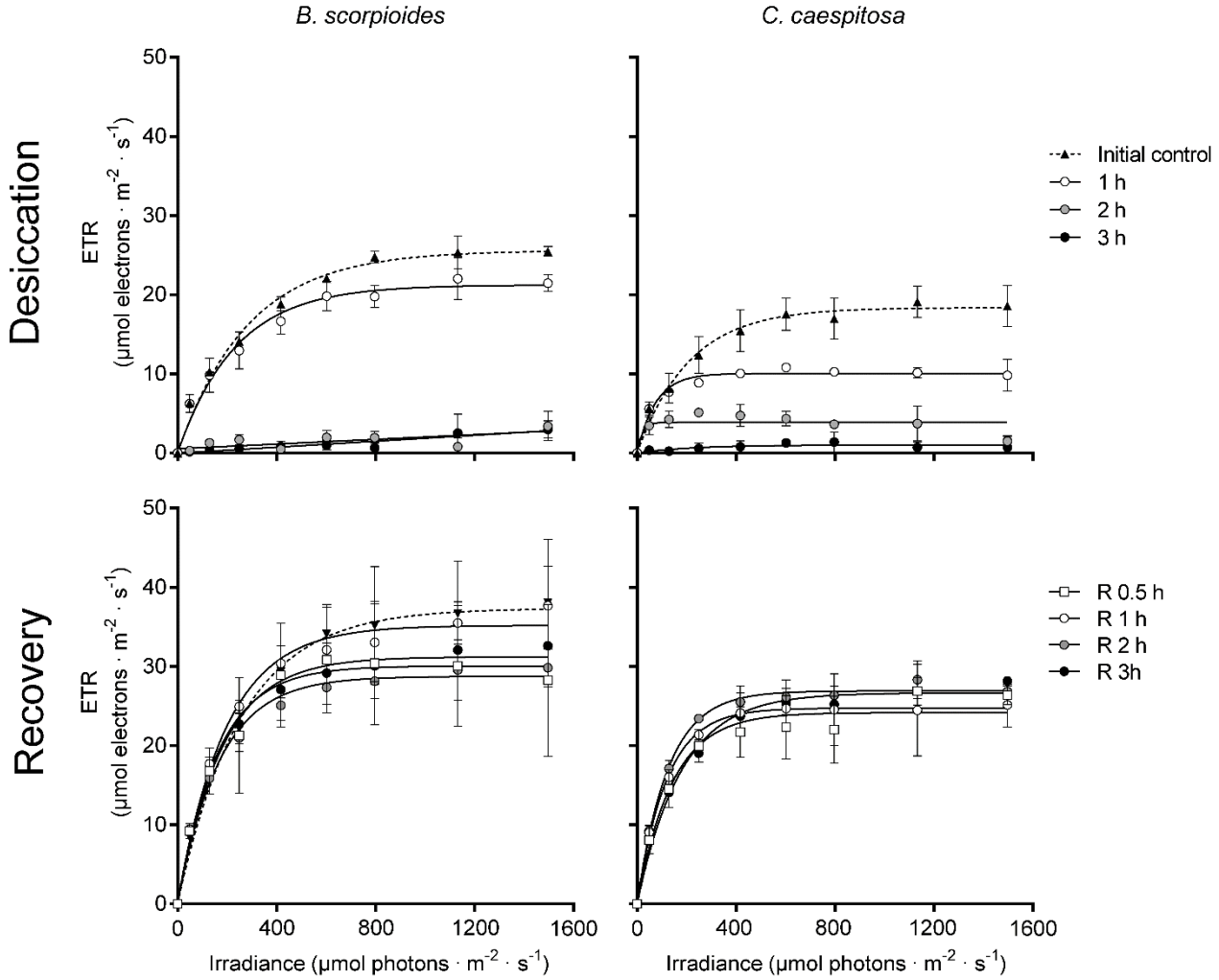


FIGURE S3. Recovery of ETR curves of *B. scorpioides* and *C. caespitosa* after 1, 3 and 6 days of continuous emersion (experiment 3.2), measured during 3 hours following reimmersion and at 24 h, at 15ºC, salinity 36 and 45 µmol photons · m^{-2} · s^{-1}. Values are mean ± SD (n = 4).

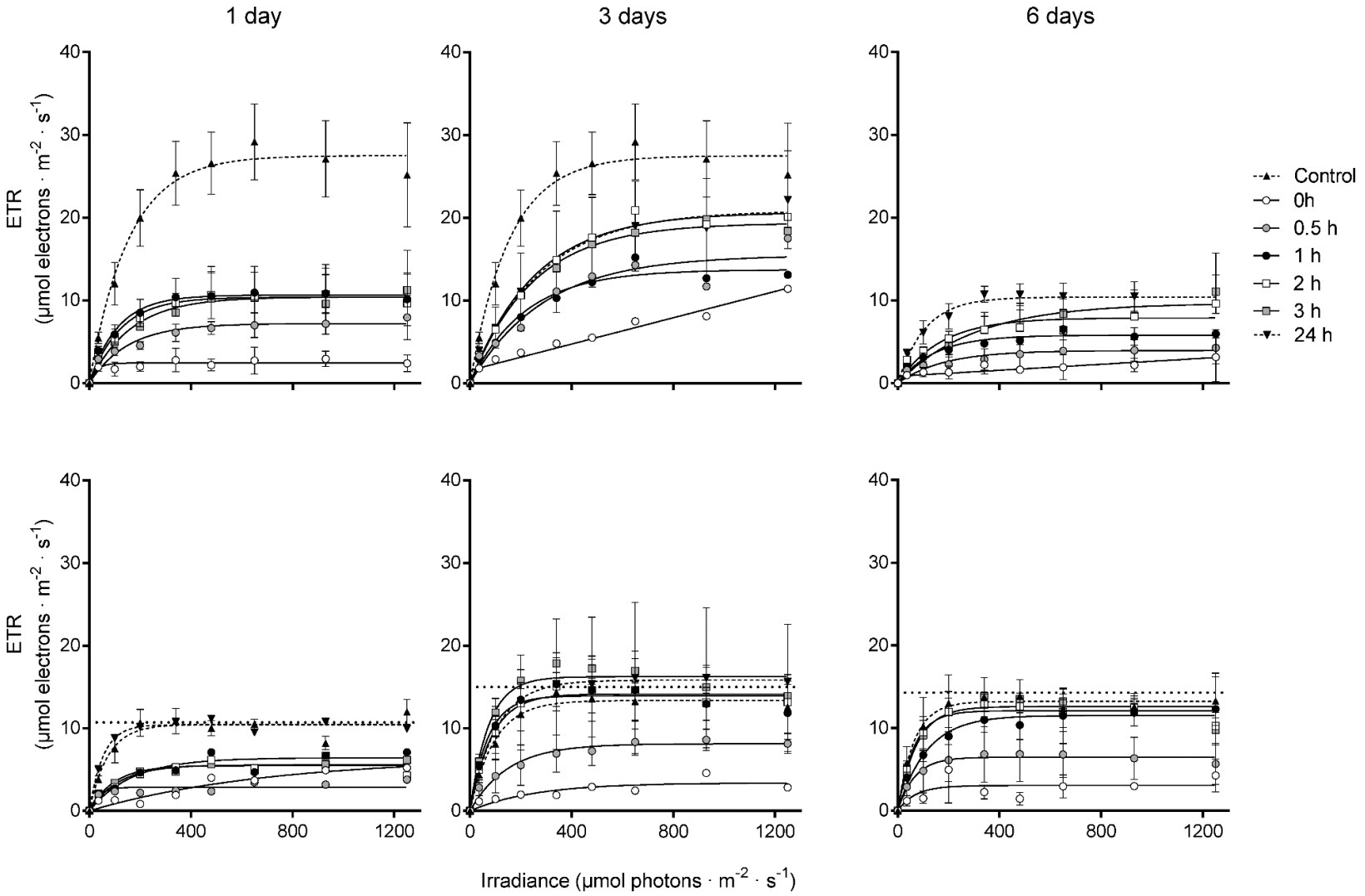


TABLE S1. Percentage dry weight (%DW) and elemental composition (% DW and molar ratio) of *B. scorpioides* (B) and *C. caespitosa* (C) (experiment 2.1, Emersion × Temperature), cultured for 10 days at two temperatures (15, 25ºC) and four emersion treatments (0, 3, 8 h of daily diurnal emersion and H+E treatment).

Temperature			15ºC				25ºC			
Emersion		Initial	0h	3h	8h	H+E	0h	3h	8h	H+E
Variable	Species									
%DW	B	29.3±0.4	32.6±3.3^{f}	29.3±0.5^{ef}	27.4±2.4^{def}	31.4±1.6^{ef}	31.5±1.4^{f}	31.0±0.6^{ef}	29.4±0.8^{cdef}	29.5±0.9^{def}
	C	18.2±0.3	16.2±2.1^{a}	19.3±0.6^{bcde}	17.9±1.5^{bcd}	17.6±0.6^{ab}	21.5±0.4^{cdef}	21.7±0.7^{cde}	19.9±0.3^{bcd}	20.1±0.5^{bc}
%C	B	32.7±1.1	35.3±1.2^{e}	31.9±0.5^{bcd}	33.1±1.2^{bcd}	32.9±0.3^{d}	32.8±0.9^{ab}	31.5±0.1^{ab}	31.2±0.4^{ab}	32.7±0.8^{abcd}
	C	28.7±2.9	30.1±0.7^{bc}	28.4±0.2^{ab}	29.6±0.9^{ab}	29.4±0.3^{ab}	28.2±0.0^{a}	27.8±1.0^{ab}	28.7±0.6^{ab}	28.2±0.1^{ab}
%N	B	4.5±0.2	3.9±0.1^{b}	3.6±0.2^{b}	4.7±0.2^{b}	4.1±0.2^{b}	4.5±0.2^{b}	3.9±0.1^{b}	4.2±0.2^{b}	4.5±0.2^{b}
	C	4.6±0.7	3.2±0.1^{a}	3.6±0.1^{b}	4.5±0.1^{b}	4.3±0.1^{b}	4.2±0.1^{ab}	3.7±0.3^{b}	4.3±0.1^{b}	4.2±0.1^{b}
C:N	B	8.4±0.3	10.6±0.5^{cd}	10.4±0.4^{bcd}	8.3±0.2^{ab}	9.4±0.3^{bc}	8.6±0.2^{abc}	9.4±0.2^{bcd}	8.7±0.3^{ab}	8.4±0.3^{ab}
	C	7.3±0.3	10.9±0.6^{d}	9.3±0.1^{ab}	7.7±0.1^{ab}	8.0±0.3^{ab}	7.9±0.1^{ab}	8.8±0.3^{ab}	7.7±0.1^{a}	7.9±0.1^{ab}

Same letters indicate homogeneous groups of data within each variable obtained from non-parametric Kruskal-Wallis ANOVA ($P < 0.05$) and pairwise comparisons (see Table S2). Values are mean ± SD (n = 4). H+E: Hydrated+Emerged.

TABLE S2. Non-parametric Kruskal-Wallis ANOVA for the percentage dry weight (%DW) and elemental composition of *B. scorpioides* and *C. caespitosa* for experiments 2.1 (Emersion × Temperature) and 2.2. (Emersion × Salinity), after 10 days of culture. Bold font highlights the statistical significance for each factor.

Experiment	Variable	$H_{(70, 15)}$	*P*
2.1. Emersion × Temperature	%DW	49.2	< **0.0001**
	%C	47.3	< **0.0001**
	%N	35.9	**0.002**
	C:N	44.8	< **0.0001**
2.2. Emersion × Salinity	%DW	49.2	< **0.0001**
	%C	47.3	< **0.0001**
	%N	35.9	**0.002**
	C:N	44.8	< **0.0001**

Kruskal-Wallis ANOVA H (N, df).

TABLE S3. Percentage dry weight (%DW) and elemental composition (% DW and molar ratio) of *B. scorpioides* (B) and *C. caespitosa* (C) (experiment 2.2, Emersion × Salinity), cultured for 10 days at four salinities (10, 20, 36, 45) and three emersion treatments (0, 3, 8 h of daily diurnal emersion).

Emersion		0 h				3 h				8 h			
Salinity		10	20	36	45	10	20	36	45	10	20	36	45
Variables	Species												
%DW	B	30.3±1.3^{abcd}	37.7±1.6^{a}	32.6±3.3^{abc}	36.5±2.3^{ab}	23.8±1.1^{cdef}	25.5±0.2^{cdef}	29.3±0.5^{abcde}	29.5±0.5^{abcde}	27.9±3.0^{bcdef}	26.1±2.7^{cdef}	27.4±2.4^{abcdef}	28.4±2.1^{bcdef}
	C	16.4±1.9^{fg}	15.9±0.4^{g}	16.2±2.1^{fg}	21.9±1.7^{def}	15.0±0.7^{efg}	16.9±0.5^{defg}	19.3±0.6^{defg}	20.4±0.3^{def}	17.3±2.7^{defg}	16.1±3.7^{defg}	17.9±1.5^{efg}	17.8±2.3^{def}
%C	B	34.5±0.4^{ab}	34.7±0.4^{a}	35.3±1.2^{a}	35.9±0.2^{a}	34.3±0.5^{bcd}	33.7±0.7^{bcd}	31.9±0.5^{bcd}	32.1±0.3^{bcd}	32.5±0.9^{bcd}	32.8±1.3^{bcd}	33.1±1.2^{bcd}	32.5±1.2^{bcd}
	C	33.1±0.3^{bc}	32.6±0.2^{bcd}	30.1±0.7^{cd}	31.6±0.7^{bcd}	31.6±0.1^{bcd}	30.0±0.3^{cd}	28.4±0.2^{cd}	27.2±0.1^{d}	30.4±1.9^{cd}	30.9±2.3^{bcd}	29.6±0.9^{cd}	29.1±1.3^{cd}
%N	B	3.9±0.3^{cdef}	3.6±0.1^{bcde}	3.9±0.1^{def}	3.9±0.2^{defg}	4.3±0.0^{fg}	4.0±0.1^{efg}	3.6±0.2^{defg}	3.8±0.1^{defg}	4.6±0.3^{g}	4.8±0.2^{g}	4.7±0.2^{g}	4.6±0.3^{g}
	C	3.5±0.1^{abcd}	3.3±0.2^{ab}	3.2±0.1^{a}	3.4±0.1^{abc}	4.2±0.1^{efg}	3.7±0.1^{defg}	3.6±0.1^{bcdef}	3.4±0.0^{bcdef}	4.6±0.3^{fg}	4.6±0.4^{g}	4.5±0.1^{fg}	4.4±0.2^{fg}
C:N	B	10.4±0.7^{abcd}	11.3±0.3^{ab}	10.6±0.5^{abc}	10.8±0.5^{abc}	9.4±0.1^{de}	9.8±0.1^{cde}	10.4±0.4^{bcde}	9.9±0.3^{de}	8.2±0.3^{e}	8.0±0.1^{e}	8.3±0.2^{e}	8.2±0.2^{e}
	C	10.9±0.4^{abc}	11.6±0.5^{a}	10.9±0.6^{ab}	10.9±0.3^{ab}	8.8±0.2^{de}	9.4±0.2^{de}	9.3±0.1^{de}	9.3±0.1^{de}	7.8±0.1^{e}	7.8±0.1^{e}	7.7±0.1^{e}	7.8±0.1^{e}

Same letters indicate homogeneous groups of data within each variable obtained from non-parametric Kruskal-Wallis ANOVA ($P < 0.05$) and pairwise comparisons. Values are mean ± SD (n = 4).

TABLE S4. Kinetic parameters derived from the fast recovery of F_v/F_m following reimmersion in the short-term emersion experiment (3 h air exposure, exp. 3.1).

Experiment	Recovery	
Variables	F_v/F_m *vs.* time (s)	
Best fit model	Saturation (Michaelis-Menten)	
Species	*B. scorpioides*	*C. caespitosa*
$F_v/F_{m\ max}$	0.71 ± 0.03	0.68 ± 0.03
K_s	17 s	70 s
h	-	-
r^2	0.67	0.78
n	62	71

K_s represents the time (s) at which half maximum value of F_v/F_m is reached. h is the Hill-Slope coefficient from the sigmoidal function, which describes the steepness of the curve. Values are mean ± SE for n = 20.

TABLE S5. Statistical analyses from experiment 3.1 (short-term desiccation and recovery experiment). Non-parametric Kruskal-Wallis ANOVA of the relative water content (RWC) and one-way repeated measurements ANOVA of F_v/F_m, ETR_m and P_{sat} of *B. scorpioides* and *C. caespitosa*.

Variable	Source of variation	Percentage of total variation (%)	$H_{(17, 54)}$	F	*P*
RWC	-	-	52.67	-	< **0.0001**
F_v/F_m	SP	1	-	48.53	**0.002**
	t	93		483.6	< **0.0001**
	SP×t	5		26.38	< **0.0001**
ETR_m	SP	7	-	16.2	**0.016**
	t	82		82.6	< **0.0001**
	SP×t	5		5.43	< **0.0001**
P_{sat}	SP	5	-	4.07	0.113
	t	43		5.90	< **0.0001**
	SP×t	19		2.62	**0.025**

Factors are species (SP) and recovery time (t).

TABLE S6. One-way repeated measurements ANOVA of P_{sat}, F_v/F_m and ETR_m of *B. scorpioides* and *C. caespitosa* from experiment 3.2 (mid-term desiccation and recovery experiment), after exposure to 1, 3 and 6 days of continuous emersion.

Variable	Species	Source of variation	Percentage of total variation (%)	F	*P*
F_v/F_m	*B. scorpioides*	E	44	70.8	**< 0.0001**
		t	40	54.7	**< 0.0001***
		E×t	6	4.19	**0.016***
	C. caespitosa	E	19	29.2	**0.0001**
		t	64	71.0	**< 0.0001**
		E×t	6	3.08	**0.005**
ETR_m	*B. scorpioides*	E	34	75.3	**< 0.0001**
		t	39	26.0	**< 0.0001***
		E×t	11	3.53	**0.027***
	C. caespitosa	E	38	68.4	**< 0.0001**
		t	21	8.10	**0.002***
		E×t	16	3.09	**0.030***
P_{sat}	*B. scorpioides*	E	24	72.9	**< 0.0001**
		t	53	94.3	**< 0.0001***
		E×t	16	14.7	**< 0.0001***
	C. caespitosa	E	35	16.3	**0.001**
		t	14	14.4	**0.0001***
		E×t	33	16.6	**< 0.0001***

Factors are days of continuous emersion (E) and recovery time (t). * Adjusted *P*-values from Greenhouse- Geisser correction. Bold fonts highlights significant effects for each factor.

Figure captions

Figure 1. Changes in relative water content (RWC) of *B. scorpioides* and *C. caespitosa* at two temperatures (15, 25ºC) and two emersion treatments (blotted, hydrated), from experiment set 1. Dashed horizontal line indicates 100% RWC. Values are mean ± SD (n = 4-12).

Figure 2. Growth rates of *B. scorpioides* and *C. caespitosa* at two temperatures (15, 25ºC) and four emersion treatments (0 h: Continuous submersion; 3, 8 h: 3 and 8 h emersion per day; H+E: 24 h emerged but hydrated twice a day), from experiment 2.1. (E × T). Same letters indicate homogeneous groups from the 3-way ANOVA and Tukey's *post-hoc* analysis ($P < 0.05$). Values are means ± SD (n = 4-7).

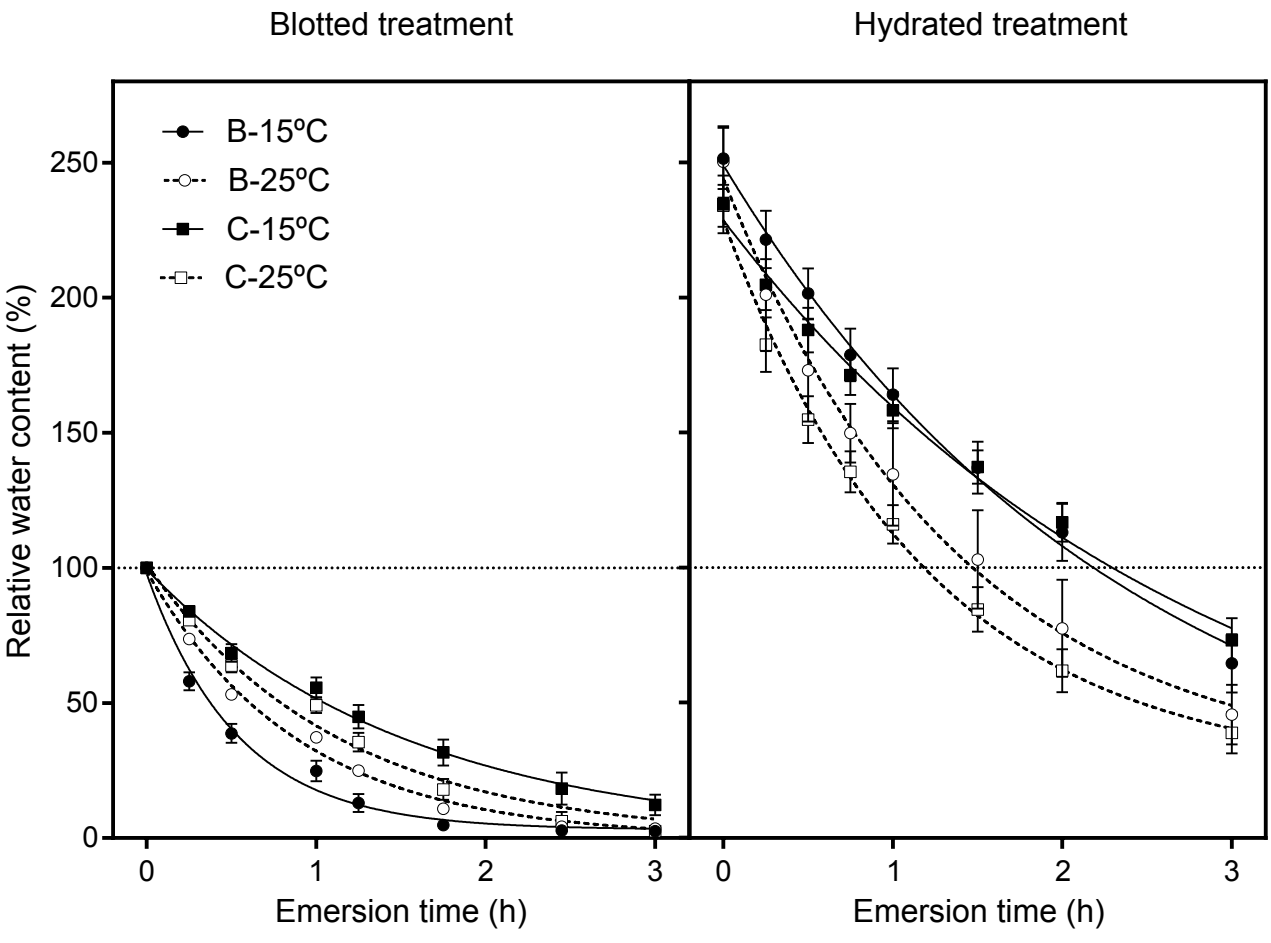
Figure 3. Growth rates of *B. scorpioides* and *C. caespitosa* after 10 days of culture at four salinities (10, 20, 36, 45) and three levels of emersion (0 h: Continuous submersion; 3 h, 8 h: 3 and 8 h emersion per day), from experiment 2.2 (E × S). Tukey's post-hoc differences are indicated for the interaction between salinity and emersion levels ($P < 0.05$). Rank order of the main factor levels for the significant interaction between species and emersion treatment is indicated in the text (section 3.2.2). B: *B. scorpioides*; C: *C. caespitosa*. Values are mean ± SD (n = 4).

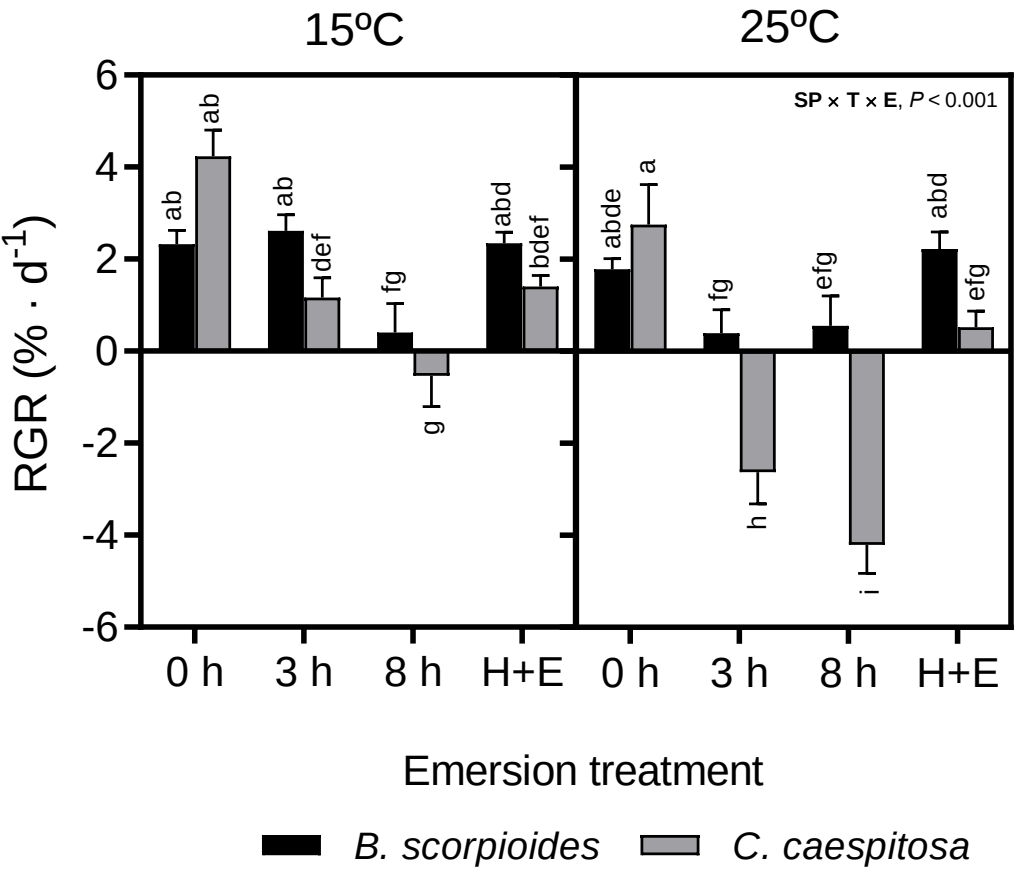
Figure 4. Time course of the RWC, F_v/F_m, ETR_m and P_{sat} of *B. scorpioides* and *C. caespitosa* during 3 h emersion and following reimmersion at 15ºC, from experiment 3.1 (short-term recovery). Grey areas indicate emersion treatments. Same letters indicate homogeneous groups from the 1-way RM ANOVA and Tukey's *post-hoc* analysis ($P < 0.05$). Values are mean ± SD (n = 3). n. s. non-significant changes.

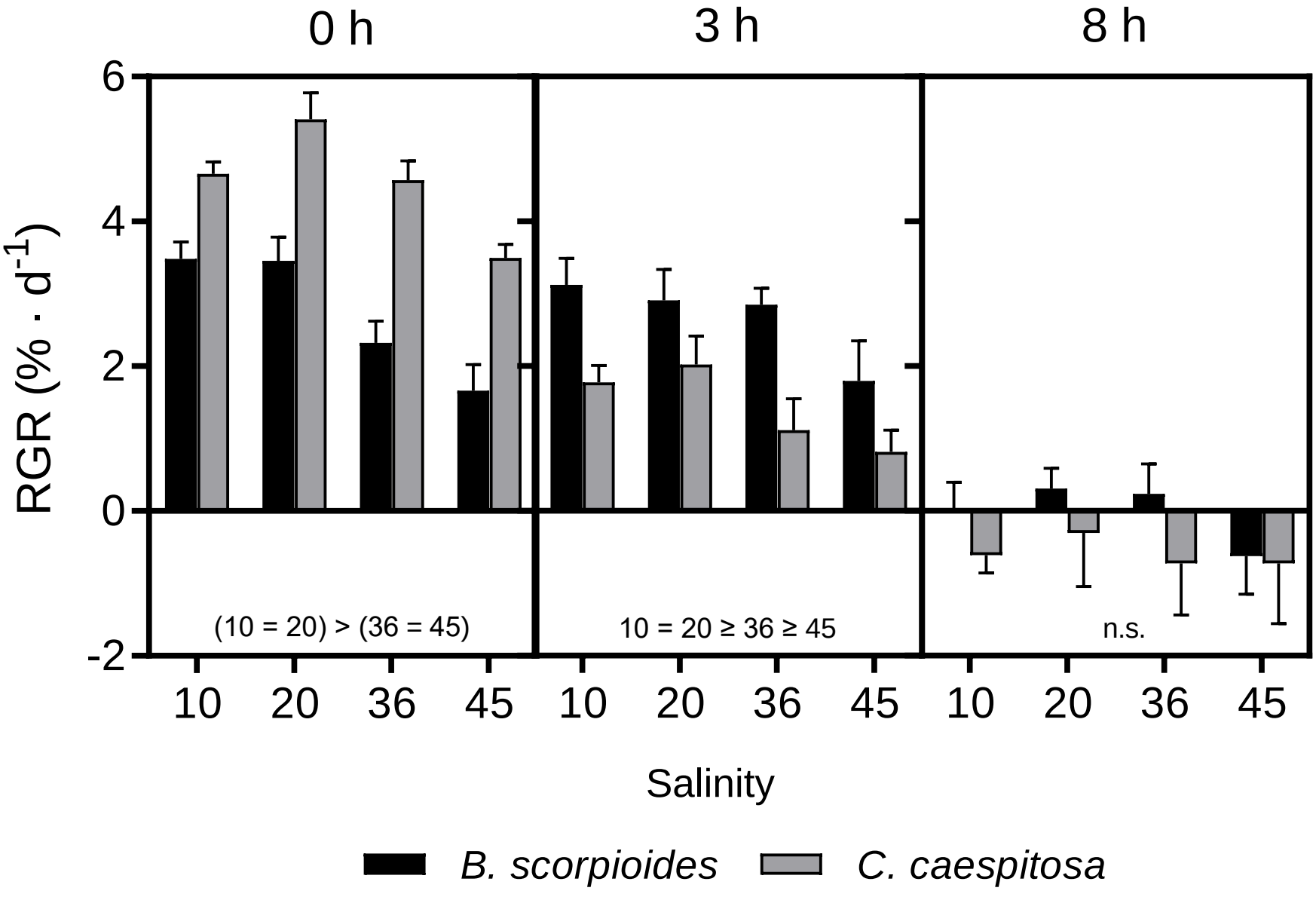
Figure 5. Recovery of F_v/F_m, ETR_m and P_{sat} of *B. scorpioides* and *C. caespitosa* after continuous emersion for 1, 3 and 6 days, from experiment 3.2 (mid-term recovery). Dotted lines indicate initial control values under submersion for each physiological variable. Same letters indicate homogeneous groups among recovery times from 1-way RM ANOVA for each

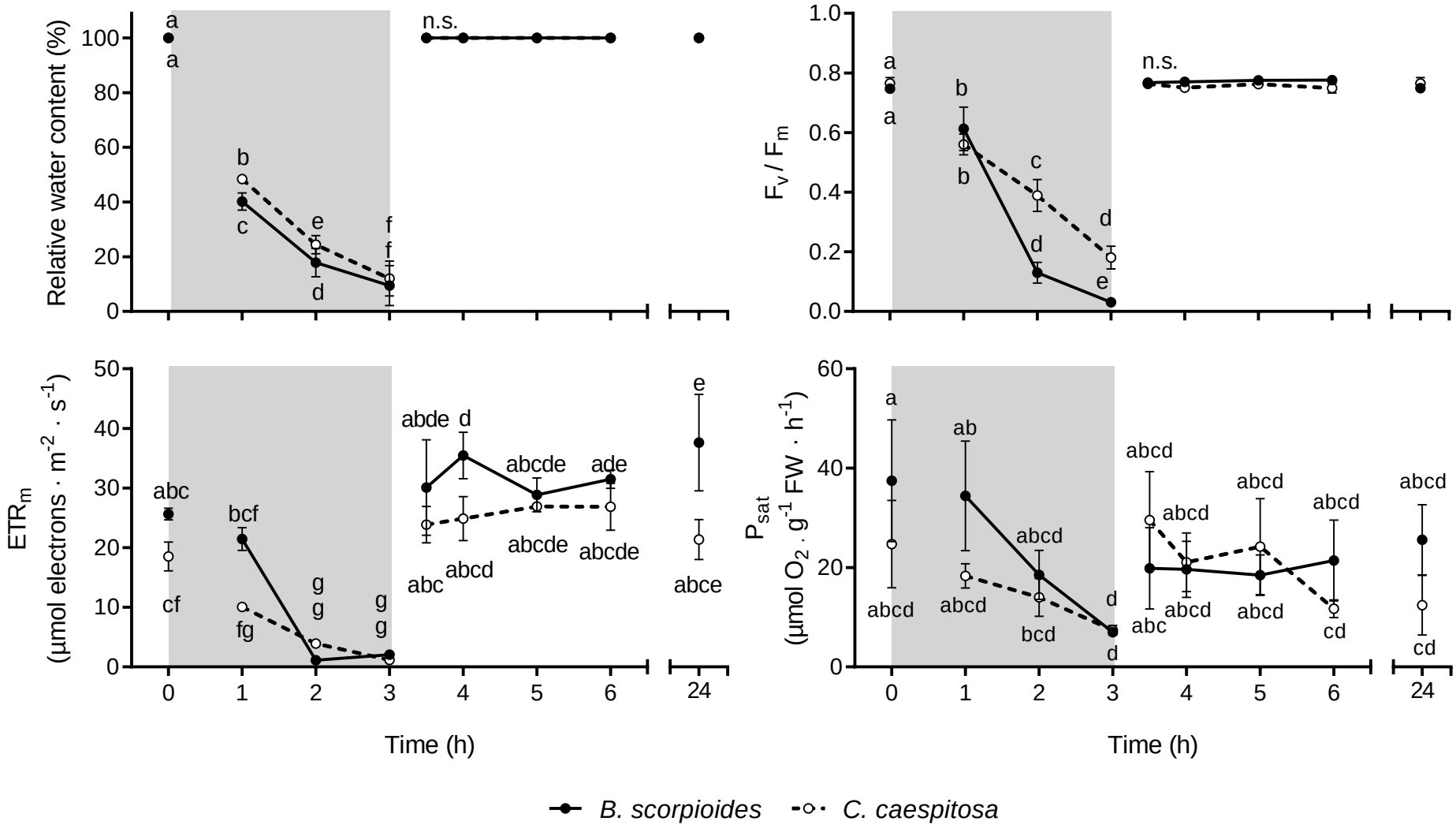
58 species ($P < 0.05$) (lower-case: *B. scorpioides*; upper-case: *C. caespitosa*). Values are mean ±

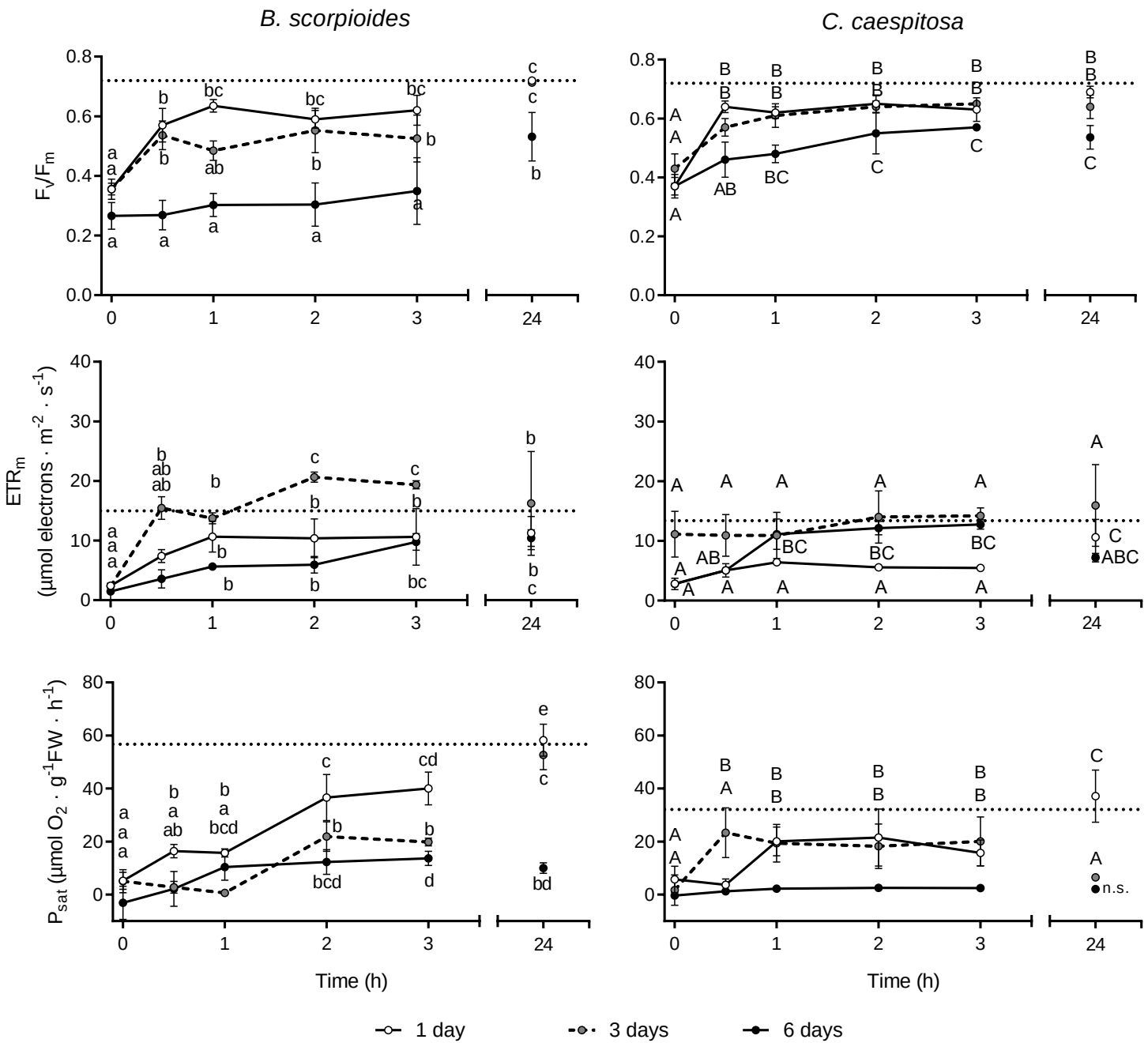
59 SD (n = 4). n. s. non-significant changes.


Blotted treatment
Hydrated treatment
B-15ºC
B-25ºC
C-15ºC
C-25ºC
Relative water content (%)
250
200
150
100
50
0
0
1
2
3
Emersion time (h)
0
1
2
3
Emersion time (h)


15ºC
25ºC
SP × T × E, P < 0.001
RGR (% · d-1)
6
4
2
0
-2
-4
-6
ab
ab
ab
def
fg
g
abd
bdef
abde
a
fg
h
efg
i
abd
efg
0 h
3 h
8 h
H+E
0 h
3 h
8 h
H+E
Emersion treatment
B. scorpioides
C. caespitosa


0 h
3 h
8 h
RGR (% · d-1)
6
4
2
0
-2
(10 = 20) > (36 = 45)
10 = 20 ≥ 36 ≥ 45
n.s.
10
20
36
45
Salinity
B. scorpioides
C. caespitosa


Relative water content (%)
F_v / F_m
ETR_m (µmol electrons · m^{-2} · s^{-1})
P_{sat} (µmol O_2 . g^{-1} FW · h^{-1})
n.s.
Time (h)
B. scorpioides
C. caespitosa


B. scorpioides
C. caespitosa
F_v/F_m
ETR_m (µmol electrons · m^{-2} · s^{-1})
P_{sat} (µmol O_2 · g^{-1}FW · h^{-1})
Time (h)
1 day
3 days
6 days
n.s.