This is a previous version of the article published in Desalination. 2024, 576: 117395. https://doi.org/10.1016/j.desal.2024.117395

Metabolic responses to desalination brine discharges in field-transplanted *Posidonia oceanica*: advances for the development of specific early warning biomarkers

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Abstract

Water desalination has become an important process to cope with water scarcity in the Mediterranean basin; however, the endemic seagrass *Posidonia oceanica* may be susceptible to high-salinity brines derived from this industry. To understand how brine affects metabolic processes in *P. oceanica*, transplantation experiments were performed in two sites exposed to a brine dilution plume derived from a desalination plant in Alicante (Spain). *P. oceanica* individuals were transplanted in three locations, i.e., a control site (~37 psu), an intermediate influence site (IB, ~39.5 psu), and a high-influence site (HB, ~42 psu), and were monitored for 6 days. The metabolic endpoints

of reactive oxygen species (i.e., H_2O_2, lipid peroxidation, and ascorbate cycle) and the regulation of genes involved in antioxidant and osmoregulation responses [*please complete sentence*]. The concentration of H_2O_2 and thiobarbituric acid reactive substances (TBARS) increased, while that of ascorbate (ASC) decreased in HB, indicating excessive ROS production, lipid peroxidation, and antioxidant consumption. Genes related to osmoregulation (*SOS1, SOS3, AKT2/3*) and antioxidant response (*GR, APX, FeSOD, MnSOD,* and *STRK1*) were upregulated in brine-exposed plants, especially at the early stages of the experiment. This novel approach has provided a battery of biomarkers that may serve as early warning tools for rapid action mitigation to avoid the negative effects of salinity on *P. oceanica* at the population and community levels. This approach can be also globally applied to relevant macrophytes in environmental monitoring programs to address other stressors and their isolated or combined contribution to marine pollution.

Keywords: Seagrass; Desalination; Oxidative stress; Gene expression; biomonitoring

Introduction

Coastal areas (i.e., ~100 km from the shoreline) are inhabited by 40% of world's population, with a higher human presence in the tropical and temperate regions (Barragán & de Andrés, 2015). As a result, numerous human activities taking place in these areas can harm the marine environment, especially the shallow coastal ecosystems (e.g., coastal development, sewage loads, and plastic pollution) (Crain et al., 2009). Moreover, temperate areas are among the most vulnerable to water scarcity in the context of global warming, as a result of changes in precipitation rates, groundwater salinization, and increased demand of freshwater (Huang et al., 2021; Kummu et al., 2016; van Vliet et al., 2021). The development and implementation of desalination technologies, especially seawater reverse osmosis (SWRO) plants, seems to be a promising solution to address water scarcity in these regions of the world. However, the environmental impact associated with the discharge of brines produced by the desalination process into coastal waters is currently causing the greatest concern and is attracting increasing attention. Brines are the residue of the SWRO process and consist mostly of concentrated seawater, which, without pre-dilution, can double the natural salinity levels of the discharges (Fernández-Torquemada et al., 2009). These brines may also contain trace concentrations of nutrients, metals as well as chemical compounds

used in the desalination process (e.g., antifouling, antifoaming agents, and biocides). However, recent findings have suggested that most biological impacts associated with brines are mainly caused by increased salinity (Blanco-Murillo et al., 2023b). Brine production has increased globally from 11.6 million m^3/day in 2000 to 95 million m^3/day in 2018 (Jones et al., 2019), especially in mid-latitude populated regions, such as the Mediterranean basin (Darre & Toor, 2018; Jones et al., 2019; Palomar & Losada, 2010). Therefore, the development of the desalination industry and its expected global growth in the future require a deeper understanding of the associated impacts on marine ecosystems and the improvement of the current protocols used for environmental monitoring.

Among the vulnerable marine ecosystems affected by brine discharges, seagrass meadows are one of the most studied (Fernández-Torquemada et al., 2019; Sandoval-Gil et al., 2023; Xevgenos et al., 2021). This is because seagrasses play an essential ecological role as habitat-forming species, provide numerous ecosystem services, and are particularly vulnerable to environmental change (de los Santos et al., 2020; Unsworth et al., 2014). In fact, salinity is one of the most relevant factors determining the distribution, ecology, and development of seagrasses (Sandoval-Gil et al., 2023). Most studies investigating the effect of increased salinity on seagrasses have been limited to mesocosm experiments using artificial salts (Blanco-Murillo et al., 2023; Cambridge et al., 2017; Marín-Guirao et al., 2013), and only a few field studies have been conducted in seagrass meadows close to the brine discharge area prior to its regression (Sola et al., 2020; Capó et al., 2020; Gacia et al., 2007; Portillo et al., 2014; Ruiz et al., 2009). Moreover, the field studies have mainly focused on population metrics (e.g., percentage cover, shoot density), shoot morphometry (e.g., growth, foliar surface, necrosis marks), or physiology (e.g., nutrient concentration, carbohydrate content), while information is lacking about the effects of brine-associated hypersalinity at the sub-cellular and metabolic levels (biomarkers) (Roca et al., 2016; Sandoval-Gil et al., 2023; Tsioli et al., 2022). In this regard, the use of experimental transplants may allow the most reliable simulation of natural conditions while modifying the intensity of the studied stressor (Garrote-Moreno, Fernández-Torquemada, et al., 2014; Muñoz et al., 2023; Rodríguez-Rojas et al., 2020). Moreover, it has been demonstrated that the combination of a well-designed field transplantation with the analysis of sub-cellular and metabolic responses is the most effective strategy serving as an early warning tool.

Specifically, these responses can be linked to physiological and ecological effects caused by adverse phenomena at the individual or population levels (Ankley et al., 2010) and can be also used to attribute the consequences of a response to specific stressors when several are present at the same time (Muñoz et al., 2023; Rodríguez-Rojas et al., 2020; Sáez et al., 2015). Most of these experiments have been conducted using macroalgae; therefore, it is necessary to address the potential usefulness of biomarkers in transplanted seagrasses as environmental biotechnology tools, especially in the context of desalination discharges.

Posidonia oceanica is the most abundant and ecologically relevant seagrass species in the Mediterranean Sea (Sandoval-Gil et al., 2023). This seagrass is a stenohaline organism with a narrow optimal salinity range of 37–38.5 practical salinity units (psu) for a normal physiological development (Fernández-Torquemada et al., 2005; Sánchez Lizaso et al 2008; Ruiz et al., 2009), although certain populations were shown to be able to thrive at higher salinity levels (up to 51.5 psu) (Azcárate-García et al., 2023; Mancuso et al., 2023; Marín-Guirao et al., 2017; Nguyen et al., 2023). Increases in salinity have been shown to alter ion composition in *P. oceanica* and cause physiological stress beyond certain threshold levels (e.g., photochemical depletion and growth reduction, among others) (Garrote-Moreno et al., 2015; Sandoval-Gil et al., 2012). However, the sub-cellular mechanisms adopted by this seagrass to cope with osmotic stress are poorly understood. A study by Blanco-Murillo et al. (2023a) on *Zostera chilensis* based on mesocosm experiments revealed that, under hypersaline exposure (+3 and +6 psu), this species displayed a higher production of reactive oxygen species (ROS) and antioxidants as well as a higher expression of genes related to ROS metabolism (ROM) and osmotic regulation. More recently, similar responses have been detected in *P. oceanica* exposed to real desalination brine (+6 psu) (Blanco-Murillo et al., 2023b). The results of the above studies highlight the potential of testing these responses as environmental biotechnology tools for the early detection and mitigation of environmental distress, serving also as biomarkers to evaluate the specific effects of desalination brine on seagrasses.

The aim of this study was to determine the responses of *P. oceanica* to intermediate and high exposure to a real brine discharge and assess its metabolic and cellular tolerance mechanisms and thresholds. To this end, *P. oceanica* was transplanted along a salinity gradient in the area of influence of brine discharges released from a SWRO desalination

plant, and the response of the transplants was studied for up to 6 days. Transplantation experiments allow to recreate a natural environment under specific known conditions; for example, in this case a *P. oceanica* meadow suddenly exposed to a brine discharge. Specifically, we i) evaluated ROS production, oxidative damage, antioxidant content, and osmotic biochemical and molecular responses in plants at different salinity thresholds following exposure to the brine dilution plume, and ii) determined the suitability of these descriptors as biomarkers to monitor the extent of desalination brine discharges and predict their potential impacts.

Materials and Methods

1. Study location and experimental design

The experimental sites were selected at the same depth (3 m) after performing salinity measurements using a conductivity-temperature-depth meter (CTD; RBR Concerto, RBR, Canada). Three different locations were chosen based on historical data (Fernández-Torquemada et al., 2005) and continuous measurements made using a conductivity and temperature meter (CT; ALEC Infinity, Alec Electronics, Japan) during the transplantation experiments. The first was a control location with regular salinity levels (~37 psu), while the other two were sites characterized by intermediate brine exposure (IB; ~39.5 psu) and high brine exposure (HB; ~42 psu) (Figs. 1a and 2b). The experiments were conducted between November 20 and 27, 2021.

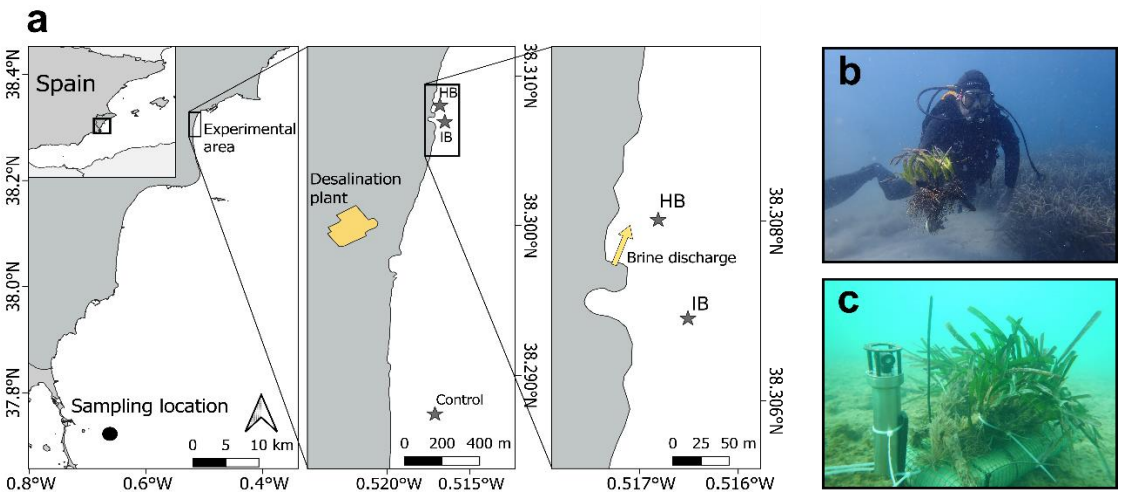
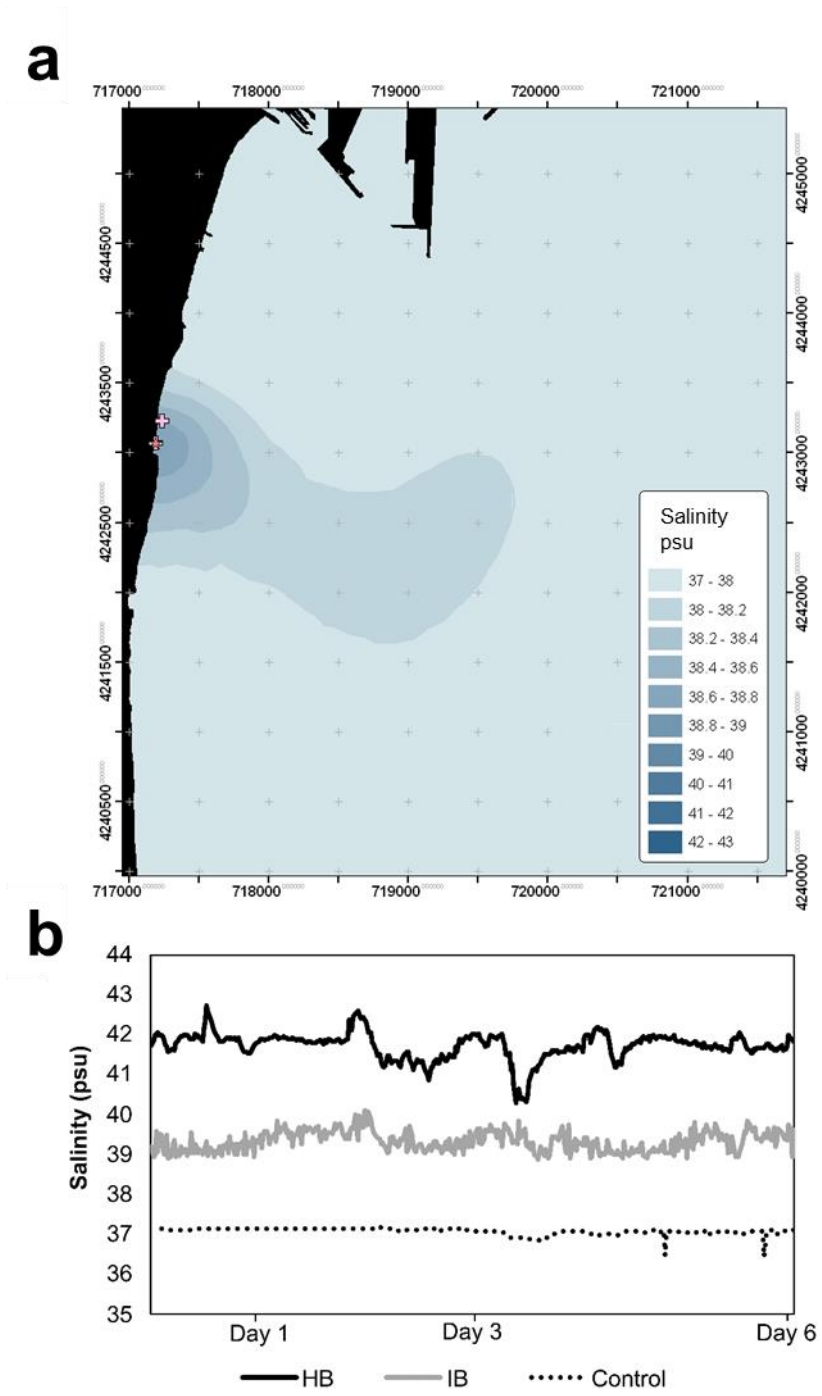


Figure 1: Maps showing the donor meadow (sampling location) and the experimental area (a). The arrow indicates the brine discharge point, and IB and HB represent the intermediate and high brine exposure sites, respectively. On the right-hand side are included images of a SCUBA diver sampling *P. oceanica* ramets (b) and a sample of a transplantation unit in the field (c).



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153 **Figure 2:** Salinity of the brine discharge plume measured using a CTD instrument at a desalination plant
154 in Alicante (a). Salinity data recorded in the experimental sites using a conductivity and temperature (CT)
155 meter during the experimental period (b).

156 Plants were collected from a healthy meadow (Isla Grossa, Murcia), a Specially
157 Protected Area of Mediterranean Importance, and transported in darkness and under
158 constant aeration to the experimental area (Alicante) within 4 h (Fig. 1a). Two *P.*
159 *oceanica* ramets consisting of one plagiotropic and 15–25 orthotropic shoots were
160 attached to a concrete anchor fitted with a transplantation unit (TU). All transplants

were closely placed in the control location for a 7-day acclimatation period and subsequently transported by divers to the HB and IB experimental sites, which were located 71 m and 103 m from the discharge area, respectively (Fig. 2a). Three TUs were installed by SCUBA divers at each site (Fig. 1c). The desalination plant of Alicante releases discharge waters directly to the shore through an open channel. The brine flow oscillates between 800 and 1400 l/s and is partially diluted at a ratio of 1.5–5 parts of seawater to each part of brine. A detailed description of the discharge and plume characteristics is included in Fernández-Torquemada et al. (2009).

Leaf samples were collected 1, 3, and 6 days after the start of the transplantation experiments. The first two mature leaves of a single shoot from both individuals in each TU were sampled each day. The first 5 cm as well as the leaf apex were removed before storing the sample to avoid response variability due to leaf age (Ruocco et al., 2019). The tissue destined for biochemical analyses was rapidly frozen in liquid nitrogen and subsequently transported to the laboratory and stored at –80ºC, while the samples to be subjected to relative gene expression analysis were stored in RNAlater (Invitrogen™), kept at 4ºC for 24 h and then stored at –20ºC, according to the manufacturer's instructions.

2. Hydrogen peroxide (H_2O_2) determination

The H_2O_2 content in *P. oceanica* leaf tissues was determined using a modified version of the protocol described in Sáez et al. (2015). The samples were ground using liquid nitrogen before analysis, and 20 mg was added to 100 µL of 10% trichloroacetic acid (TCA), 150 µL of 10 mM potassium phosphate buffer (pH 7.0), 50 µL of FAPRB lysis buffer from the FavorPrep™ Plant Total RNA Mini Kit (FAVORGEN), and 500 µL of 1 M potassium iodide. The samples were then vortexed for 15 min using glass beads (3 mm) and centrifuged for 15 min at 12,000 ×g and 4ºC. Their absorbance was measured at 392 nm using a spectrophotometer SpectroStar Nano (BMG LABTECH). Standard curves were obtained using commercial H_2O_2 (Sigma Aldrich Merck, St Louis, MO, USA).

3. Determination of thiobarbituric acid reactive species (TBARS)

ROS production can lead to lipid peroxidation in the cellular membrane. Polyunsaturated fatty acids are oxidized by hydroxyl radicals (·OH) leading to the production of malondialdehyde (MDA), which can be determined by reaction with

thiobarbituric acid (TBA). A total of 20 mg of ground biomass using liquid nitrogen was added to 500 µL of 10 % trichloroacetic acid (TCA) and vortexed for 15 min using glass beads (3 mm). The mixture was then centrifuged at 17800 ×g for 15 min at 4°C. Subsequently, 200 µL of supernatant was removed and added to 200 µL of 0.5 % TBA, and the solution was incubated for 30 min at 90°C. Absorbance was measured by taking of 200 µL to a microplate reader (SpectroStar Nano, BMG LABTECH) at 532 nm. Commercial MDA (Sigma Aldrich Merck, St Louis, MO, USA) was used to obtain standard curves.

4. Ascorbate (ASC) and dehydroascorbate (DHA) contents

As an indicator of the antioxidant capacity of *P. oceanica*, the ASC and DHA forms of total ASC (reduced and oxidized, respectively) were determined following the protocol by Benzie and Strain (1999) with some modifications. First, to measure ASC, 10 mg of ground biomass using liquid nitrogen was added with 300 µL of 0.1 M HCl and 300 µL of FAPRB lysis buffer from the FavorPrep™ Plant Total RNA Mini Kit (FAVORGEN). Samples were then vortexed for 10 min and centrifuged at 21000 ×g for 10 min at 4°C. Subsequently, 10 µL of the supernatant was removed and added to 290 µL of FRAP buffer [300 mM sodium acetate buffer at pH 3.6, 20 mM $FeCl_3$, and 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ)]. This solution reacted with ASC forming a colorimetric complex that was measured spectrophotometrically at 593 nm (BMG LABTECH). Second, total ASC was obtained by reducing 250 µL of supernatant with 2.5 µL of 100 mM dithiothreitol (DTT) and incubating the solution for 1 h at room temperature. After incubation, 2.5 µL (w/v) of N-ethylmaleimide was added to stop the DTT-mediated reaction. Then, 10 µL of extract was again added to 290 µL of FRAP buffer and measured at 593 nm. DHA content was determined by calculating the difference between total ASC and ASC. L-ASC (Sigma Aldrich Merck, St Louis, MO, USA) was used to obtain standard curves.

5. RNA extraction and qPCR

A set of nine genes of interest related to antioxidant defense and osmoregulatory mechanisms were examined in terms of their response in *P. oceanica* under different brine exposures (Tab. 1). It should be noted that these selected genes had been already successfully tested in terms of their response to brine-mediated increased salinities in *P. oceanica* in recent mesocosm experiments (Blanco-Murillo et al., 2023b). Leaf samples

were selected as defined by Blanco-Murillo et al. (2023b), taking the first mature leaf of each individual and cutting out a 4-cm fragment after removing the first 5 cm and leaf apex. Samples were stored in RNAlater at 4ºC for 24 h and were then frozen at −20ºC. A total of 50 mg of leaf biomass was powdered using liquid nitrogen, and RNA was extracted using the Aurum™ Total RNA mini kit (BIORAD) following the manufacturer's instructions. RNA purity and integrity were assessed using a spectrophotometer (SpectroStar Nano) at a 260/280 ratio and 1.2% agarose bleach electrophoresis, respectively.

The Quant-iT RiboGreen RNA assay kit (Invitrogen, Waltham, MA, USA) was used to determine RNA concentration by fluorescence in a QFX fluorometer (DeNovix, Wilmington, DE, USA). Samples were then standardized using 350 ng of RNA before synthetizing cDNA using a cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fischer Scientific) with a final sample volume of 20 µL. The PCR (qPCR) solution was prepared by adding 2 µL of cDNA to 0.5 µL of each primer (forward and reverse), 10 µL of Green PCR Mix SYBR (Agilent Technologies, Santa Clara, CA, USA), and 7 µL of nuclease-free water. The reaction was performed on a qPCR Magnetic Induction Cycler (MIC; Bio Molecular Systems, Queensland, Australia) under the following conditions: initial denaturation at 95°C for 5 min, annealing consisting of 40 cycles of 95°C for 10 s, 55°C for 10 s, 72°C for 40 s, and final extension at 72°C for 10 min. The quantification cycle (Cq) values of each gene were subsequently used to determine relative gene expression based on the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001):

$$\text{Fold expression change (FC)} = 2^{(|\,(-\Delta Cq\ \text{treatment}) - (-\Delta Cq\ \text{control})\,|)}$$

To measure FC, 18S rRNA was chosen as a housekeeping gene, as indicated in Serra et al. (2012) and confirmed in Blanco-Murillo et al. (2023b).

Table 1: List of the *P. oceanica* genes of interest analyzed via qPCR.

Gene	**Coding protein**	**Sequence 5'-3'**
STRK1	Salt tolerance receptor-like cytoplasmic kinase 1	CGCCGCGCTCCAACCAAGGA
		CGACGTGGAGCCGCTCGCTT
CAT	Catalase	CTCCGGCCGTCTCGGCCTTG
		GTGCTCCGTGGCGGCACTCT
Mn SOD	Mn Superoxide dismutase	CGGCTCGAGCGCGCCGTAAT

		GAAGCTCCCACGCCCGCACA
Fe SOD	Fe Superoxide dismutase	TGGTATCCCAGAGTTTGGCGGCTCA
		TGGAGTGGCACCCTCGCCTCA
APX	L-ascorbate peroxidase	CGCCTCGCGTGGCATTCAGC
		TCAGGCCCGCCGGTGATCTC
GR	Glutathione reductase	AGGAAGCCCAGAAAGTGTTGCCT
		TCCCAGCCACCAATAGCTCAAGT
SOS1	Salt overlay sensitive 1 Sodium/hydrogen exchanger 7	TGGGTTCTGGCATCCGTCTTTGGG
		GGGCAACGACAGCAACAGGATCGG
SOS3	Salt overlay sensitive 3	TGTTTCTGGTTCTTGATGCTGCTCTGC
		TCTTCCTTGTGAATGAGCCCGTCGT
AKT2/3	Potassium channel AKT2/3	ACCTCGTCAGCGAAGCCCTCGAA
		CCGCGGATGAGGCCCATGACC
18S	Ribosomal RNA 18S	GAGAAGGAAGCTGCTGAAATG
		GAACAGCACAATCAGCCTGAG

6. Statistical analyses

A two-way ANOVA was performed with treatment (three levels: control, HB, and IB) and time (three levels: 1, 3, and 6 days) as fixed factors to determine differences in the responses of all measured parameters. Data normality and homoscedasticity were tested using the Kolmogorov–Smirnov and Bartlett tests, respectively (Underwood, 1997). The post-hoc Tukey-HSD test was conducted to determine the statistical significance of differences between means.

Results

1. ROS production and oxidative damage

H_2O_2 levels in *P. oceanica* leaf tissues revealed a significant interaction between treatment and time factors. The HB group showed significantly higher values compared to the control in all sampling days, while the values for the IB group were significantly higher than those in the control plants only at day 3 (Fig. 3a). Overall, the H_2O_2 values were higher on day 6 than on days 1 and 3, especially in the HB group. TBARS analysis revealed significantly higher lipid peroxidation levels in the HB treatment compared to

the control and IB. As for the effect of time, all treatments showed significantly higher H_2O_2 levels on day 6 compared with day 3, but not with day 1 (Fig. 3b).

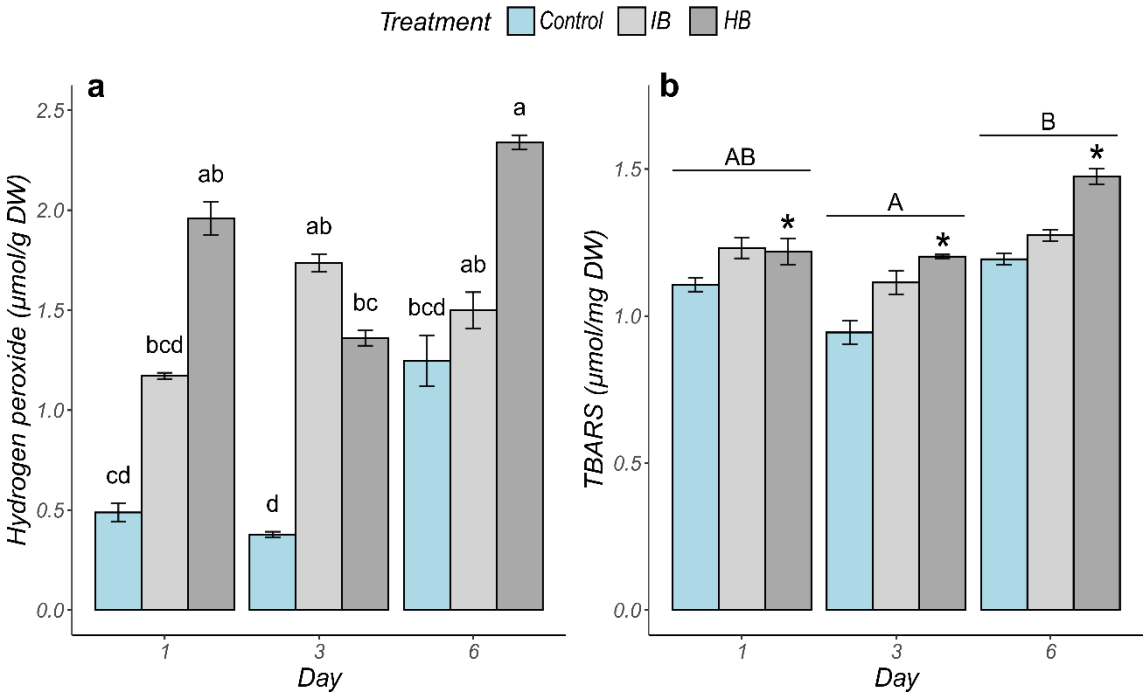

Figure 3: Hydrogen peroxide (H_2O_2) (a) and TBARS (b) contents in *P. oceanica* leaf samples from the three experimental treatments: Control (~37 psu), IB (~39 psu), and HB (~42 psu). Barplots represent the mean of each variable, and error bars show the standard error. Uppercase letters represent significant differences at the 95% confidence interval ($p < .05$) between days 1, 3, and 6. Lowercase letters represent significant differences between groups when the interaction factor was significant. Asterisks (*) indicate significant differences between treatments.

2. Antioxidant levels

Despite the significant differences detected, total ASC (ASC + DHA) was lower in both brine exposure treatments (IB and HB) compared to the control; however, at day 6, the values in HB were not significantly different from those in the control. Moreover, the total ASC values in the control and IB treatments displayed a decreasing trend with time, while the opposite pattern was observed for HB (Fig. 4). ASC levels presented significant differences for the treatment factor, but with a decreasing trend only in plants under HB, and relatively constant values between days (Fig. 4). DHA levels were significantly higher in the control than in brine-exposed plants (HB and IB) on days 1 and 3, while on day 6, they were lower in IB plants than in the control and HB plants. In

general, DHA levels were higher than ASC levels in the control compared to the pattern observed in the brine-exposed plants throughout the experimental period (Fig. 4).

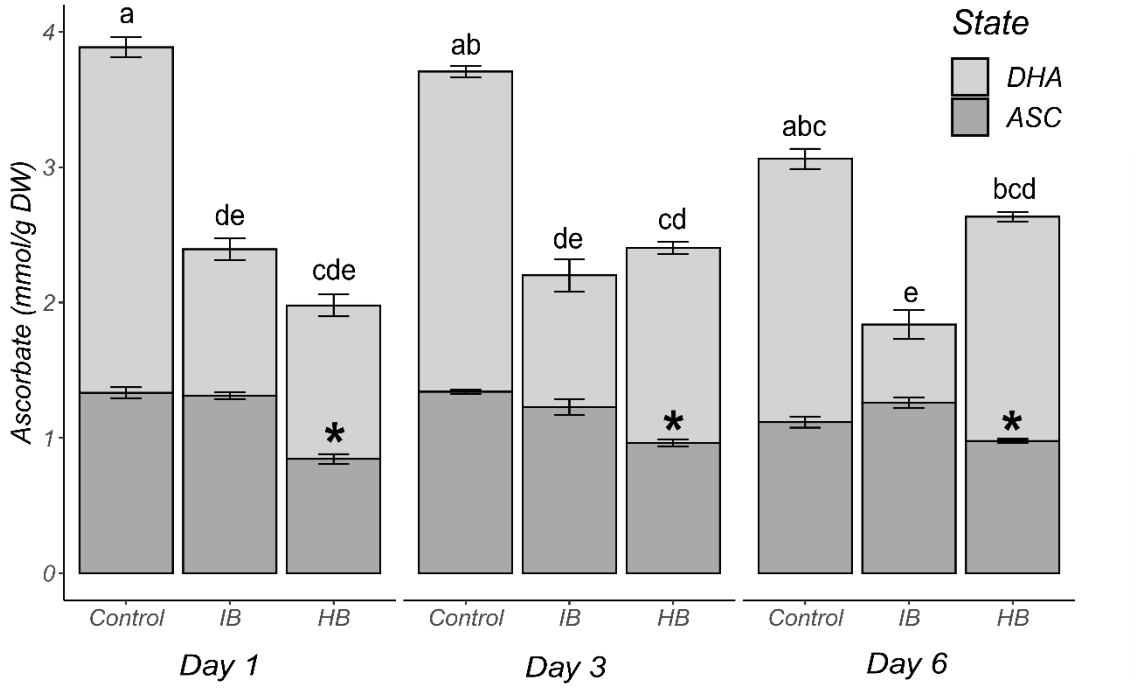

Figure 4: ASC and DHA levels in *P. oceanica* leaves in the three experimental treatments: Control (~37 psu), IB (~39 psu), and HB (~42 psu). Barplots represent the mean of each variable, and error bars show the standard error. Lowercase letters represent significant differences ($p < .05$) between groups when factor interaction was significant. Asterisks (*) indicate significant differences between treatments.

3. Expression of genes related to osmotic and oxidative stress regulation

In regard to genes involved in the oxidative stress response, a general expression structure was observed throughout the experimental period. The brine-exposed plants at IB and HB showed the highest levels of expression at day 1 after transplantation, which decreased significantly at days 3 and 6 (Fig. 5 a-f). Beyond general patterns, downregulation was observable at both brine-influenced sites for *APX* and *CAT* only at day 6 (Fig. 5c and f, respectively) and at IB for *GR* . Indeed, *GR* and *STRK1* were the only oxidative response genes to present a significantly higher expression in HB than IB at all experimental times (Fig. 5d and e).

In regard to the genes involved in osmotic regulation, in both brine-exposed sites, *SOS1* exhibited downregulation throughout the experimental period, with a slight recovery

observed on day 6 in IB. Although no statistical differences were detected, HB plants presented higher relative expression levels on days 1 and 3 (Fig. 5g). *SOS3* was more expressed in HB plants than in IB plants throughout the experimental period, although the gene expression trend declined until day 6 (Fig. 5h). Even though no patterns were observed at days 1 and 3, *AKT* was significantly more upregulated in HB plants than in IB plants at day 6 (Fig. 5i). *STRK1* showed a more significant upregulation in HB plants than in IB plants throughout the experimental period. A slight decrease in the expression of this gene was detected at day 3 and 6 compared to day 1, maintaining the pattern of upregulation in HB (Fig. 5d).

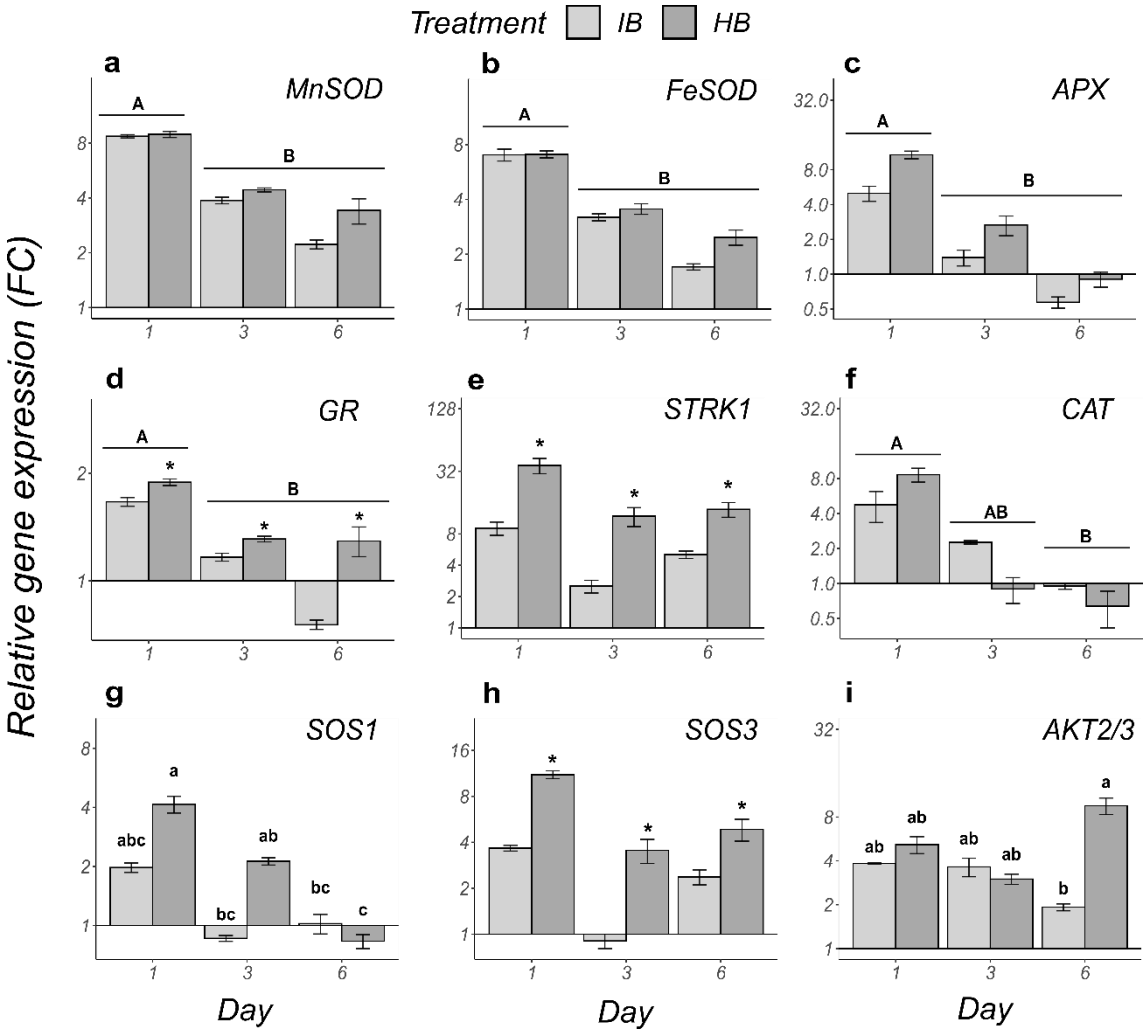

Figure 5: Relative levels of expression of genes related to osmotic regulation and oxidative stress in *P. oceanica* under two salinity exposures in the field, i.e., IB (~39 psu), and HB (~42 psu), compared to the control treatment. The expression of the following genes was measured: *MnSOD* (a); *FeSOD* (b), *APX* (c), *GR* (d), *STRK1* (e), *CAT* (f), *SOS1* (g), *SOS3*(h), and *AKT2/3* (i). Uppercase letters represent significant differences at the 95% confidence interval ($p < .05$) between days (1, 3, and 6). Lowercase

letters represent significant differences between groups when factor interaction was significant. Asterisks (*) show significant differences between treatments.

Discussion

This study examined the cellular and molecular responses of *P. oceanica* growing in two sites exposed to brine effluents derived from a seawater desalination plant. Leaf tissues in the brine-influenced sites accumulated ROS, showed signs of oxidative damage, and exhibited a reduction in some of their resources for cellular antioxidant defense. These effects were proportional to the degree of influence of the brine discharge and were therefore more pronounced in plants closer to the discharge point and exposed to higher salinity levels (~42 psu vs ~39.5 psu). The activation of genes related to osmoregulatory mechanisms was also directly correlated with brine exposure, as these genes were activated in proportion to the salinity level experienced by the plants. The transcription of oxidative stress genes was more evident after 1 day of exposure, reflecting a rapid response to oxidative stress under hypersaline conditions.

In seagrasses, H_2O_2 increments as a consequence of hypersalinity have been reported in *Thalassia tesudinum* exposed to salinities of +15 psu above natural levels for 14 days (Trevathan et al., 2011) and in *Zostera chilensis* exposed to salinities of +3 and +6 psu above baseline salinities (Blanco-Murillo et al., 2023). However, the study by Blanco-Murillo et al. (2023b) under mesocosm conditions showed that *P. oceanica* exposed to salinities of up to +6 psu above the standard 37 psu due to brine discharge from a desalination plant did not exhibit any increase in H_2O_2 levels. In contrast, in this experimental study on field, the H_2O_2 levels in *P. oceanica* were higher than those in the control even in sites with lower salinities (39 and 42 psu) compared to the 43 psu used in Blanco-Murillo et al. (2023b). Hydrodynamics, herbivory (which was observed during sample collection), and water turbidity could be main environmental factors contributing to ROS production in *P. oceanica* transplants. Moreover, in Alicante bay, several anthropogenic activities have historically co-occurred with the operation of the desalination plant, causing the *P. oceanica* meadow to decline in the last decades (Blanco-Murillo et al., 2022) and potentially increasing stress responses due to the influence of other coastal activities (e.g., sewage discharges).

Once in the cytosol, H_2O_2 can be transformed into hydroxyl radicals (·OH) via the Fenton reaction (Bartosz, 1997). These reactive ROS can interact with plasmatic membranes, and this interaction can be measured as TBARS levels, which represent a proxy of lipid peroxidation (i.e., oxidative damage). In this study, lipid peroxidation was higher in HB plants and increased at day 6, which indicated that the intensity and duration of brine exposure were directly related to ROS production and oxidative damage. Previous results from the mesocosm experiment by Blanco-Murillo et al. (2023b) also showed higher lipid peroxidation under a brine exposure of +6 psu, although it was stable throughout the 10-day experimental period. On the other hand, Capó et al. (2020) showed that in a local population of *P. oceanica* under the influence of brine discharges, TBARS levels increased by 54.5% and 108.3% at +1.5 and +2.8 psu above natural levels, respectively. Even though laboratory experiments used brine exposures with salinities as high as +6 psu above natural levels (Blanco et al. 2023b), lipid peroxidation has been observed to be greater in local *P. oceanica* populations (Capó et al. 2020) and transplanted *P. oceanica* (i.e., this investigation) nearby desalination discharges. In terms of observed effects, Capó et al. (2020) reported shorter leaves in the site closest to the brine discharge, which was also observed in *P. oceanica* by Gacía et al. (2007) and attributed to enhanced herbivory by the fish *Sarpa salpa* and the sea urchin *Paracentrotus lividus*. In this study, although measurements were not made, signs of herbivory and shorter leaves were observed in the *P. oceanica* transplants at the HB site (Fig. S1). Moreover, it is important to consider that several studies have reported higher diversity and/or abundances of fish nearby desalination discharges, and the reasons for this phenomenon are still not fully understood (Kelaher et al., 2020; Sola et al., 2020). The mechanic constrains and tissue destruction caused by herbivory may be an added pressure to other anthropogenic impacts that can manifest in greater lipid peroxidation in local or transplanted *P. oceanica* growing near desalination discharge areas.

To manage the oxidative damage caused by the exposure to harmful brine levels, *P. oceanica* cells attempt to scavenge H_2O_2 via enzymatic and non-enzymatic antioxidant mechanisms. In this study, HB plants showed reduced ASC levels, suggesting that this antioxidant enzyme was consumed to cope with ROS production triggered by brine and other factors. Blanco-Murillo et al. (2023b) had also detected a significant ASC consumption in *P. oceanica* under mesocosm conditions, which further confirms the

important role of this antioxidant in the defense of this seagrass against ROS produced under hypersaline and stressful conditions. The decrease of total ASC (ASC+DHA) in the leaves of plants exposed to brine could be due to an impairment of both the metabolic recycling and de novo synthesis of this antioxidant in *P. oceanica* cells. In fact, ASC synthesis is highly dependent on the functioning of the electron transport chain (Millar et al., 2003), which is very sensitive to ionic stress caused by increased salinity. This dependence was observed in the lower electron transport rates in *Z. chilensis* under +3 and +6 psu hypersaline conditions (Blanco-Murillo et al., 2023a). Therefore, considering also the presence of other potential environmental pressures, the ASC values detected were consistent with the effects exerted by brine in the study area.

The superoxide ion (O_2^-) is the first ROS formed in stressed plants (Bose et al., 2014), and its conversion to H_2O_2 is catalyzed by FeSOD in the chloroplasts and MnSOD in the mitochondria (Van Camp et al., 1995). Our results revealed that *MnSOD* and *FeSOD* were markedly upregulated at the early stages of exposure to brine in IB and HB plants, and their expression later decreased. This finding corresponded with the results regarding the regulation of these genes obtained by Blanco-Murillo et al. (2023b) in the mesocosm experiments at 43 psu. Interestingly, Capó et al. (2020) measured the lowest SOD activity in local *P. oceanica* at the site with higher salinity within the brine-influenced area; in contrast, non-enzymatic ROS scavenging mechanisms were the strongest at the site with highest salinity. It is known that long-term exposure to environmental pressures, including excessive salinity, can lead to epigenetic modifications in seagrasses and, consequently, to the variation of gene expression patterns (Shen et al., 2022). Indeed, the observed intra-specific differences between the local *P. oceanica* population sampled by Capó et al. (2020), which had been affected by desalination discharges since 1994, and our transplanted *P. oceanica*, which originated from a non-impacted area, in terms of the response to excessive salinity appear to be related to the development of epigenetic adaptations in the former. The decrease in the expression of metal substrate SODs beyond day 1 was also observed in the mesocosm experiments by Blanco-Murillo et al. (2023b) and can be attributed to a sufficient enzyme stock being transcribed at the earliest stage of strong gene upregulation.

The signaling protein STRK1 is sensitive to salinity increments and promotes CAT synthesis to cope with excessive H_2O_2 concentrations (Yang & Guo, 2018; Zhou et al., 2018). *STRK1* and *CAT* exhibited a higher relative expression in HB plants, which

417 demonstrated that they played a role in the salinity-dependent mechanism to cope with
418 H_2O_2 production as an osmotic pressure response. Therefore, H_2O_2 concentrations and
419 STRK1 phosphorylation activity appeared to trigger *CAT* transcription, especially on
420 day 1. Both genes exhibited a similar trend of upregulation throughout the
421 transplantation period, which was also observed in previous laboratory experiments
422 (Blanco-Murillo et al., 2023b), confirming their co-dependence. On the other hand,
423 H_2O_2 scavenging is also known to be performed by APX, which reduces H_2O_2 by
424 oxidizing ASC to DHA. ASC can then be restored through the oxidation of reduced
425 glutathione (GSH) to glutathione disulfide (GSSG); in turn, GSSG is reduced back to
426 GSH by glutathione reductase (GR) using NADPH as a substrate, which is all part of
427 the ASC-GSH (or Foyer–Halliwell–Asada) cycle (Foyer & Noctor, 2011). In the present
428 study, *APX* was more upregulated in plants under the HB treatment, which indicated a
429 salinity-correlated transcription. These results are also consistent with lower ASC levels
430 in HB plants, indicating an APX-mediated consumption to cope with H_2O_2 excess. *GR*
431 upregulation was shown to be associated with the activation of GSH regeneration under
432 brine exposure, which has been confirmed to occur in local *P. oceanica* growing near
433 desalination discharge areas (Capó et al., 2020). GSH can be then consumed to restore
434 ASC levels through the Halliwell–Asada cycle. It is important to mention that the
435 patterns of upregulation observed during the transplantation period where in line with
436 those observed in the previous mesocosm experiments (Blanco-Murillo et al., 2023b).
437 However, the levels of expression tended to decrease toward the end of the experiments
438 in the latter, supporting the hypothesis that other environmental pressures in addition to
439 the brine discharges induced the responses observed in this study.

440 In addition to ROM, ion balance is essential for the physiological and metabolic
441 functioning of plants cells. In particular, K^+ uptake and Na^+ exclusion are essential
442 mechanisms for seagrasses to cope with brine-derived osmotic pressure, as intracellular
443 Na^+ excess can be highly toxic (Garrote-Moreno et al., 2014). To defend themselves
444 against toxicity, plant cells have developed signaling mechanisms to prevent osmotic
445 stress, among which the Salt Overlay System (SOS) enzymatic complexes have been
446 demonstrated to be essential. For instance, SOS3 is a Ca^{2+} binding protein that enhances
447 the transcription of *SOS1*, which in turn encodes for a Na^+/H^+ antiporter protein. This
448 protein mediates Na^+ extrusion through the plasmatic membrane, implying the
449 extracellular intake of H^+ (Hadi & Karimi, 2012; Yang & Guo, 2018). AKT2/3 is a

specific transport protein that increases K^+ capture, a process that is essential to maintain plant metabolic functioning (Dennison et al., 2001). K+ is used as cofactor by enzymes associated with several biochemical pathways, and its substitution by Na+ can cause severe metabolic impairment (Steven, 1985). In this study, all genes related to osmotic regulation, such as *SOS1*, *SOS3*, and *AKT2/3*, displayed a pattern of marked upregulation at day 1, with greater expression in HB than in IB, followed by a trend of moderate decrease at later sampling times. The exception was *AKT2/3*, which, although being upregulated at day 1, exhibited the highest transcript levels at day 6 in HB plants. Despite the trends in the regulation of these genes were similar to those observed in the previous mesocosms experiments with *P. oceanica* under 43 psu for up to 10 days (Blanco-Murillo et al., 2023b), the decrease in *SOS1*, *SOS3*, and *AKT2/3* regulation in *P. oceanica* transplants after day 1 in our field experiments was less marked. Certainly, overexpression was maintained for most of the time. Therefore, these results obtained are in line with [*please specify*] and are representative of environmental salinity levels, considering that the *P. oceanica* plants transplanted in this study were subjected to average salinities of 39.5 psu in IB and 42 psu in HB.

Overall, the data obtained in this study point to the presence of a battery of biochemical and molecular biomarkers that represent cellular stress, which can be extrapolated to higher levels of biological organization (e.g., physiology, population). The present study comprehensively described a set of reliable brine-monitoring biomarkers in *P. oceanica*, and this strategy can be also applied to other habitat-forming organisms in different temperate and tropical latitudes, such as corals (Marques et al., 2023), macroalgae (Muñoz et al., 2023; Rodríguez-Rojas et al., 2020), and other seagrasses (Blanco-Murillo et al., 2023a; Capó et al., 2020). Moreover, compared with previous mesocosm-derived data, our results showed that using biomarkers related to osmotic and oxidative stress can provide information on brine-specific responses and also identify other potential combined effects when more stressors are present, respectively. While osmoregulatory responses are more specific as a defense against brine discharges (Blanco-Murillo et al. 2023a; Blanco-Murillo et al, 2023b), oxidative stress in seagrasses can be triggered by different isolated and combined environmental stressors other than brines, such as metal pollution, invasive species, or increasing temperatures (Malea et al., 2019; Sureda et al., 2008; Tutar et al., 2017). Therefore, the biomarkers identified in this study can be used to detect changes in osmotic pressure due to brines

and identify its specific contribution when multiple stressors are present. In addition, they can provide early warning signs of stress to take action upon eventual further physiological, population, and community affection. The results of this study can contribute to future developments not only in environmental management at desalination plants, but also in the broader fields of aquatic pollution and ecotoxicology. Indeed, next generation sequencing technologies open new possibilities in terms of the identification of biomarkers associated with other stress factors and the establishment of preventive measures. Finally, the findings presented in this study may be applied in the field of bioengineering, for example to develop biosensors. Such technological advancement will allow us to fully ascertain [*please specify what*] and greatly improve aquatic environmental surveys.

Conclusions

The *P. oceanica* plants transplanted near the desalination discharge areas showed signs of oxidative stress and damage. This finding was confirmed by the consumption of antioxidants and by the patterns of gene regulation of enzymes involved in the reactive oxygen metabolism. However, the regulation of specific osmotic regulation genes (i.e., *SOS1*, *SOS3*, and *AKT2/3*) and the results obtained under controlled conditions in previous investigations demonstrated that the stress experienced by *P. oceanica* was not related only to brine discharges, but was apparently due to a greater extent to other co-existing natural and/or anthropogenic environmental pressures.

These descriptors have been successfully tested as biomarkers to monitor the effects of desalination discharges on *P. oceanica*. They should also be applied in the future as part of environmental monitoring programs and incorporated to ad hoc legal frameworks regarding the operation of desalination plants in the Mediterranean Sea.

Acknowledgement

This investigation was funded by Marie Skłodowska-Curie Action (888415) granted to C.A. Sáez. F. Blanco-Murillo was supported by a grant from Universidad de Alicante (Grant ID: FPUUA98). F. Rodríguez-Rojas was financed by the ANID project FONDECYT #11220425. C.A. Sáez was also financed by project ANID InES I+D 2021

(INID210013). I. Sola was funded by a grant from European Union-Next Generation EU (MARSALAS21-30).

CRediT Authorship Contribution Statement:

F. Blanco-Murillo: Conceptualization, Investigation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing. L. Marín-Guirao: Investigation, Data curation, Formal analysis, Writing - review & editing. F. Rodríguez-Rojas: Investigation, Formal analysis, Data curation, Writing - review & editing. E. Carbonell-Garzón: Investigation, Formal analysis. I. Sola: Investigation, Formal analysis. J. L. Sánchez-Lizaso: Conceptualization, Writing - re- view& editing. C.A. Sáez: Conceptualization, Investigation, Writing - original draft, Writing - review & editing.

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