Chemosphere 341 (2023) 140061

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Desalination brine effects beyond excess salinity: Unravelling specific stress signaling and tolerance responses in the seagrass *Posidonia oceanica*.

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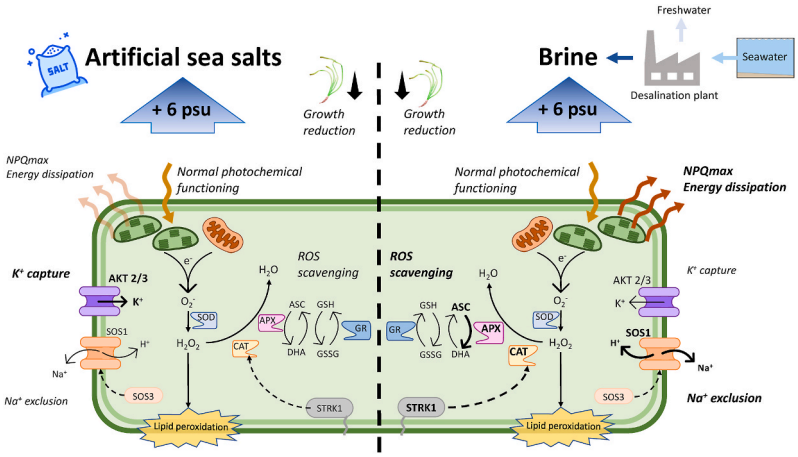
HIGHLIGHTS

- *Posidonia oceanica* the most ecologically relevant seagrass in the Mediterranean basin.
- Salinity increment (artificial salts vs desalination brine) responses were analyzed.
- Hypersalinity resulted in growth reduction, lipid peroxidation, and *SOS1* expression.
- Brine increased NPQ_{max}, ascorbate consumption (ASC) and *STRK1* and *CAT* transcription.
- Brine triggered specific responses have the potential to be used as specific biomarkers.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Giulia GUERRIERO

ABSTRACT

Desalination has been proposed as a global strategy for tackling freshwater shortage in the climate change era. However, there is a concern regarding the environmental effects of high salinity brines discharged from desalination plants on benthic communities. In this context, seagrasses such as the Mediterranean endemic and ecologically important *Posidonia oceanica* have shown high vulnerability to elevated salinities. Most ecotoxicological studies regarding desalination effects are based on salinity increments using artificial sea salts, although it has been postulated that certain additives within the industrial process of desalination may exacerbate a negative impact beyond just the increased salinities of the brine. To assess the potential effect of whole effluent brines on *P. oceanica*, mesocosm experiments were conducted within 10 days, simulating salinity increment with either

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https://doi.org/10.1016/j.chemosphere.2023.140061
Received 11 June 2023; Received in revised form 1 September 2023; Accepted 2 September 2023
Available online 7 September 2023

artificial sea salts or brines from a desalination plant (at 43 psμ, 6 psμ over the natural 37 psμ). Morphometrical (growth and necrosis), photochemical (PSII chlorophyll *a* fluorometry), metabolic, such as hydrogen peroxide (H_2O_2), thiobarbituric reactive substances (TBARS) and ascorbate/dehydroascorbate (ASC/DHA), and molecular (expression of key tolerance genes) responses were analyzed in each different treatment. Although with a still positive leaf growth, associated parameters decreased similarly for both artificial sea salt and brine treatments. Photochemical parameters did not show general patterns, although only *P. oceanica* under brines demonstrated greater energy release through heat (NPQ). Lipid peroxidation and upregulation of genes related to oxidative stress (*GR*, *MnSOD*, and *FeSOD*) or ion exclusion (*SOS3* and *AKT2/3*) were similarly incremented on both hypersalinity treatments. Conversely, the ASC/DHA ratio was significantly lower, and the expression of *SOS1*, *CAT, and STRK1* was increased under brine influence. This study revealed that although metabolic and photochemical differences occurred under both hypersalinity treatments, growth (the last sign of physiological detriment) was similarly compromised, suggesting that the potential effects of desalination are mainly caused by brine-associated salinities and are not particularly related to other industrial additives.

1. Introduction

Seagrass meadows are one of the most ecologically important and valuable coastal ecosystems worldwide. Their environmental and socioeconomic importance is based on their associated ecosystem services, from habitat engineers, carbon sequestration, sediment stabilization, and fisheries support, among others (e.g. Cullen-Unsworth et al., 2014; de los Santos et al., 2020; Hemminga and Duarte, 2000; Unsworth et al., 2019). Despite known seagrass importance to both natural and human systems, these ecosystems have experienced a significant decline in their extent during the last decades as a consequence of human activities (Adams et al., 2020; Blanco-Murillo et al., 2022; Marbà et al., 2014).

Seagrasses are marine angiosperms specially abundant in temperate and tropical regions, where they regularly occur in areas surrounded by high human populations and significantly disturbed environments (Halpern et al., 2008; Short et al., 2007). Moreover, these areas are specially suffering from the effects of the ongoing climate change (Schewe et al., 2014). An obvious example of this is the Mediterranean Sea, which has been recognized as a hotspot for the cumulative impacts of human activities and global change (Coll et al., 2010; Crain et al., 2008; Gissi et al., 2021). One of the major consequences of overpopulated warm-temperate coasts under a climate change context is water shortage (Schewe et al., 2014). To address this problem, desalination technologies, combined with water reuse, have become the main nonconventional freshwater sources worldwide (Darre and Toor, 2018). Reverse osmosis (RO) is the most frequently used desalination technology and seawater (SW) is the main intake for freshwater production in desalination processes around the world (Darre and Toor, 2018). SWRO desalination has the potential to affect coastal ecosystems because of the discharge of large volumes of brine with high salinity into coastal waters (Fernández-Torquemada et al., 2019; Ihsanullah et al., 2021; Petersen et al., 2018; Roberts et al., 2010). The brine resulting from the desalination process is composed mainly of concentrated seawater, reaching salinities that, in most cases, double the levels of the source (Panagopoulos and Haralambous, 2020). The potential of brines to affect coastal biological communities depends mainly on the dilution capacity of the recipient water body, the technology and logistics of discharge and the tolerance thresholds of resident organisms (Ihsanullah et al., 2021; Roberts et al., 2010). Tolerance thresholds are the limits of a certain environmental stressor to which an organism can adapt and keep normal physiological and metabolic performance. The main harmful effects of these discharges have been attributed to the osmotic pressure and oxidative stress produced by the excess salinity on living organisms. However, brines may also contain other chemicals used in the industrial desalination process (i.e., antiscalants, detergents, antifoaming agents, or dechlorination products), as well as higher concentrations of metals and/or nutrients depending on the characteristics of the seawater source (e.g., a desalination plant installed in a polluted area) (Fernández-Torquemada et al., 2019; Panagopoulos and Haralambous, 2020). However, the effect of these components on coastal marine organisms has rarely been studied as most studies to date have focused on the hypersaline and hyperosmotic component of brines or the whole effluent or on full exposure to all potential stressors (Clark et al., 2018).

Several studies on seagrass tolerance to desalination discharges have been conducted using artificial sea salts to increase salinity under controlled laboratory conditions, focusing exclusively on the hypersalinity effects of brines (e.g., Cambridge et al., 2017; Garrote-Moreno et al., 2014, 2015; Marín-Guirao et al., 2013a,b; Oscar et al., 2018; Sandoval-Gil et al., 2012a, 2012b; Trevathan et al., 2011). These approaches have provided essential information on species-specific tolerance thresholds and on how salinity affects seagrass physiology. Salinity stress has been found to generate osmotic unbalance and to trigger reactive oxygen species (ROS) overproduction and oxidative damage in plants, thus activating a set of responses related to ion exchange and reactive oxygen metabolism (ROM) (Munns and Tester, 2008). When the energy cost associated with the activation of this secondary metabolism exceeds energy production, cellular and tissue degradation processes (*e.g.* foliar necrosis) may initiate, which may eventually lead to plant death in the mid/long term (Fernández-Torquemada et al., 2005). However, few studies have focused on seagrass molecular signaling and metabolic responses against hypersalinity to complement morphological and physiological descriptors (Sandoval-Gil et al., 2022). These responses at lower levels of biological organization do not only contribute to our knowledge of seagrass biology, but they can also can be used as biomarkers in environmental monitoring programs (EMP), which allow stress detection before severe affection leads to plant death, and subsequent meadow regression.

Some seagrass species have been found to be relatively tolerant to high salinity ranges, such as *Zostera marina* (Biebl and McRoy, 1971; Salo et al., 2014), *Posidonia australis* (Cambridge et al., 2017), *Cymodocea nodosa* (Sandoval-Gil et al., 2012a), and *Thalassia testudinum* (Tomasko et al., 1999; Tomasko and Hall, 1999); whereas others behave as stenohaline organisms, such as the Mediterranean endemic *Posidonia oceanica* (Sandoval-Gil et al., 2023). The high sensitivity of the latter species to salinity changes together with its great ecological and economic relevance, has led to its use as a reference organism to assess the impact of brine discharges in the Mediterranean Sea (Sánchez-Lizaso et al., 2008).

Despite the scientific advances made in the last decades on the effects and mechanisms of tolerance to increased salinity in seagrasses (Sandoval-Gil et al., 2023), there is still a large gap of knowledge on how SWRO-brines, rather than simply salts, affects seagrasses. So far, only one study has addressed this issue (Cambridge et al., 2019). This study found that desalination brine induced more detrimental symptoms of physiological stress in *P. australis* compared to similar increased salinities but was reached using artificial sea salts. Indeed, *P. australis* under direct brine influence (54 psμ) for 15 days showed photochemical depletion and growth reduction compared to artificial salts. These findings arose questions on the metabolic responses triggered specifically by brine and on how a less tolerant species such as *P. oceanica* would respond to this source of impact. In spite of the latter, Cambridge et al. (2019) exposed *P. australis* to extremely high salinity increments

(+9 and + 17 psμ), raising the question as to whether these treatments, beyond the origin of the increased salinities, are realistic in terms of the viability in such high concentrations around in desalination discharges.

Considering the need to address realistic brine responses and the possibility of desalination discharge consequences beyond increased salinities, this study aimed to assess the biological responses of the stenohaline seagrass *P. oceanica* to hypersalinity reached with seawater mixed with artificial sea salts compared to real brine from an SWRO plant through mesocosm experiments. Our main objectives were the following: (1) to assess the ecophysiological and metabolic responses subjected to hypersalinity reached with either artificial salts or desalination brines, (2) to further ascertain whether brine exposure triggers stress responses higher than those of hypersalinity reached with artificial sea salts, and (3) to identify brine-specific functional indicators and biomarkers as possible biotechnology tools for desalination environmental monitoring programs (EMPs).

2. Materials and methods

2.1. Sample collection and mesocosm set-up

Posidonia oceanica ramets were collected in October 2021 in a healthy meadow with low human-influence near Isla Grosa (SE Spain). Plant ramets composed of a plagiotropic (horizontal) apical shoot and 18–28 orthotropic (vertical) shoots were transported in coolers (darkness) with local seawater to the mesocosm facilities of the Oceanographic Center of Murcia (Spanish Institute of Oceanography, IEO-CSIC) within 3 h. The experimental system consisted of independent 200 L aquaria as described by Marín-Guirao et al. (2011) and Sandoval-Gil et al. (2012a). Upon arrival at the laboratory, 18 plant fragments of similar size were individually planted in plastic net pots (22 × 40 × 10 cm) covered with emulated sediments. Two pots were randomly allocated inside each of the nine selected aquaria filled with prefiltered seawater.

Plants were kept to acclimatize for 10 days under constant conditions: 2 °C ± 0.1 °C water temperature, 37 ± 0.1 salinity, and a 12:12 h light:dark photoperiod at saturating irradiance (200 ± 20 μmol quanta $m^{-2}s^{-1}$), using a tungsten-halogen light source (Aqualight-400). Water quality was controlled during the experiment by continuous chemical and physical filtration and partial seawater renewal (30%) on days 3 and 6 after the beginning of the experiment.

2.2. Experimental design

After the acclimatation period, three aquaria (n = 3) were assigned to each of the three selected salinity treatments: 37 psμ (control), 43 psμ produced with Instant Ocean artificial salts (artificial) and 43 psμ produced by diluting SWRO brine from the San Pedro Desalination plant (brine). Salinity increments were set at 43 psμ, considering the commonly detected level of nearby brine discharges in the Mediterranean Sea (e.g. Fernández-Torquemada et al., 2005; Gacia et al., 2007). Indeed, similar levels have been previously used in ecotoxicology studies with marine angiosperms (e.g., Marín-Guirao et al., 2011, 2013; Sandoval-Gil et al., 2012b). During the experiment salinity was controlled using a conductometer (PROSolo, YSI Inc., USA). Results of a basic chemical analysis of seawater and brine are attached in Supplementary Material 1.

Plant sampling for biochemical and transcriptomic parameters, as well as ecophysiological measurements was conducted on days 1, 3, 6, and 10 after the start of the experiment. Plant growth and morphology were characterized only at the end of the experiment (day 10). Plant tissue for biochemical and gene expression analyses was collected from the first and second mature leaves (*i.e.*, the youngest photosynthetically active leaves) of one shoot per pot. Leaf sampled area and shoot position were standardized as shown in Fig. 1 to avoid distinct responses due to tissue age and physiological differentiation (Ruocco et al., 2019, 2021). The first 5 cm and the apical end of each leaf were discarded, and the epiphytes were gently removed using a razor blade. Leaves were then immediately frozen in liquid nitrogen and stored at −80 °C for further biochemical analyses (see Fig. 1). For gene expression analysis, epiphyte-free 4 cm long leaf segments were entirely submerged in RNAlater (Invitrogen™), kept at 4 °C for 24 h and then stored at −20 °C according to the manufacturer's instructions.

2.3. Photosynthetic performance

Photosynthetic performance was estimated by fluorescence using a Diving-PAM portable fluorometer (Walz, Germany). Measurements were made on three shoots per pot at a fixed position on the first mature leaf at each sampling time (Fig. 1).

In each sampling period, the maximum (F_m) and minimum fluorescence (F_o) values of dark-adapted leaves were measured in order to calculate the photosystem II (PSII) maximum quantum yield [$F_v/F_m = (F_m - F_o)/F_m$)]. Rapid light curves (RLC) were measured at noon i.e., after 4 h in the presence of light. Nine incremental irradiances (0, 13, 56, 134, 181, 280, 416, 655, and 830 mmol photons $m^{-2}s^{-1}$) were measured during a 20 s exposure and data were processed using PAM WinControl program (Walz, Germany). Gross photosynthesis was calculated as the maximal electron transport rate (ETR_{max}), and α_{ETR} as photosynthetic efficiency, was calculated following the tangential model function by

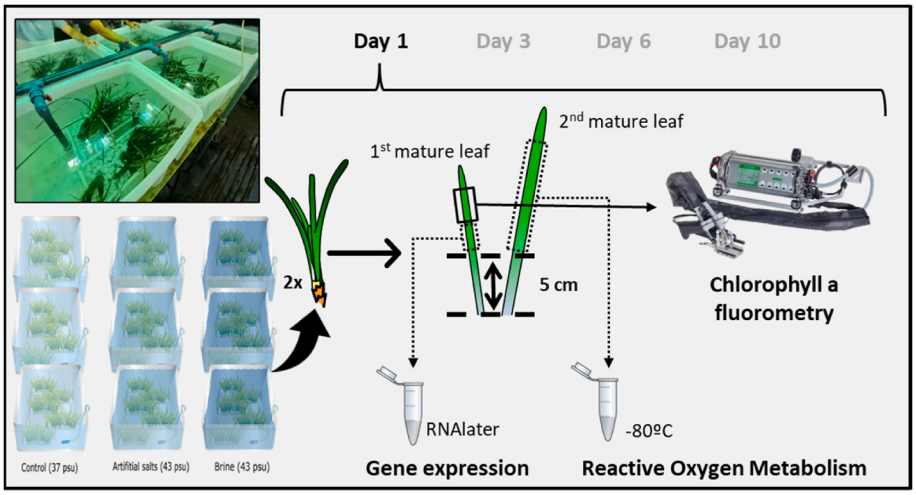

Fig. 1. Scheme showing the techniques for each aquarium and sampling time.

Eilers and Peeters (1988), which has already been used for the measurement of photochemical performance in seagrasses (Pazzaglia et al., 2020). ETR_{max} and α_{ETR} intersection allowed the determination of the saturating irradiance (Ek_{ETR}). Nonphotochemical quenching (NPQ_{max}) is an indicator of energy excess dissipation, and it was calculated as $NPQ_{max} = (F_m–F_m)/F'_m$, with F'_m being the maximum fluorescence of light-adapted leaves (Schreiber et al., 1995).

2.4. Hydrogen peroxide (H_2O_2) determination

Among ROS, the more representative species under salt stress is H_2O_2 (Schmidt et al., 2013). H_2O_2 content was measured according to Sáez et al. (2015), with some modifications for *P. oceanica*. Twenty mg of grounded biomass were added to 100 μL of 10% trichloroacetic acid (TCA), 150 μL of 10 mM potassium phosphate buffer (pH 7.0), 50 μL of lysis buffer, and 500 μL of 1 M potassium iodide. The mixture was vortexed using glass beads (3 mm) for 15 min and then centrifuged for 15 min at 12,000×*g* at 4 °C. The supernatant (300 μL) was placed in a microplate for absorbance measurements at 392 nm (SpectroStar Nano, BMG LABTECH). Commercial H_2O_2 (Sigma Aldrich Merck, St Louis, MO, USA) was used for standard curves.

2.5. Quantification of thiobarbituric acid reactive substances (TBARS)

ROS overproduction can cause the oxidation of cellular components such as lipids, proteins or nucleic acids (Bartosz, 1997). TBARS are commonly used as a proxy of lipid peroxidation (e.g., Muñoz et al., 2020; Sáez et al., 2015). Grounded biomass (20 mg) was added to 500 μL of 10% TCA, vortexed for 15 min using glass beads (3 mm), and then centrifuged at 17800×*g* for 15 min at 4 °C. Two hundred μL of supernatant were mixed with 200 μL of 0.5% thiobarbituric acid (TBA) and incubated for 30 min at 90 °C. Two hundred μL were sampled and measured at 532 nm in a microplate reader (SpectroStar Nano, BMG LABTECH). Commercial malondialdehyde (MDA, Sigma Aldrich Merck) was used for constructing standard curves.

2.6. Ascorbate (ASC) and dehydroascorbate (DHA) determination

Living cells have antioxidants that help in preventing oxidative damage by scavenging ROS excess, such as the ASC/DHA couple (Foyer and Noctor, 2011). ASC and DHA were measured following the protocol developed by Benzie et al. (1999), with some modifications for *P. oceanica*. Leaves were grounded using a mortar and liquid nitrogen. Ten mg of fresh weight biomass were added to 300 μL of 0.1 M HCl and 300 μL of lysis buffer and vortexed for 10 min. Samples were then centrifuged at 21,000×*g* for 10 min at 4 °C. Ten μL of the supernatant were sampled and mixed with 290 μL of tripyridyl triazine (Fe III TPTZ), and sample absorbance was rapidly measured at 593 nm in a microplate spectrophotometer (SpectroStar Nano, BMG LABTECH). This absorbance level was correlated with ASC concentration. For total ascorbate (ASC + DHA) determination, 250 mL of the initial supernatant were incubated for 1 h at room temperature (~21 °C) after adding 2.5 μL of 100 mM dithiothreitol (DTT), turning all DHA to ASC. Then, 2.5 μL (w/v) of N-ethylmaleimide were added to stop the reaction and 10 μL were added again to 290 μL of Fe III TPTZ and taken to the spectrophotometer. DHA was calculated from the difference between the total ascorbate and ASC. L-ASC (Sigma Aldrich Merck, St Louis, MO, USA) was used for standard curves.

2.7. Gene expression analyses

Plant cells have different strategies to respond to salinity increments, such as developing ion exclusion or uptake mechanisms, or synthetizing enzymes that help in scavenging excessive ROS contents (Munns and Tester, 2008). To detect some of these responses, nine genes were selected for primer design because of their role in osmotic regulation and oxidative response (Table 1). Fifty mg of liquid nitrogen-grounded biomass were used for RNA extraction using a BIORAD Aurum™ Total RNA mini kit, following the manufacturer's instructions. To test RNA purity and integrity, extracted material was analyzed using the 260/280 ratio and 1.2% agarose bleach gel (Aranda et al., 2012), respectively. A Quant-iT RiboGreen RNA assay kit (Invitrogen, Waltham, MA, USA) was used before taking samples to a QFX Fluorometer (DeNovix, Wilmington, DE, USA) for precise quantification of RNA. Knowing the concentration of each sample, 350 ng of RNA were used to synthetize cDNA with a cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fischer Scientific), and adding RNase-free water to each sample adjusting the volume to 20 μL. Real-time quantitative PCR (RT-qPCR) reactions were performed using 60 ng (2 μL) of cDNA, 0.25 μM of each primer (forward and reverse), and 17.5 μL of Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara,CA, USA), adjusting the final volume to 20 μL. For the RT-qPCR a MIC qPCR Magnetic Induction Cycler (Bio Molecular Systems, Queensland, Australia) was used. The program configuration was set as follows: initial denaturation at 95 °C for 5 min and then 40 cycles of 95 °C for 10 s, 55 °C for 10 s, 72 °C for 40 s, and ending with a final extension at 72 °C for 10 min. All primers were designed using a reference transcriptome of *P. oceanica* available in the HUB-AMBIENTAL Research Centre (Dr. Rodríguez-Rojas personal communication), using the bioinformatic tool of GenScript for real-time PCR primer design (https://www.genscript.com/tools/real-time-pcr-taqman-primer-design-tool). Relative gene expression analyses were expressed as fold change (FC) based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Gene 18S rRNA was used as housekeeping as proposed by Serra et al. (2012) and confirmed its reliability in this investigation.

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

2.8. Statistical analyses

Photochemical parameters (Fv/Fm, ETR_{max}, α_{ETR}, Ek_{ETR}, and NPQ) and reactive oxygen metabolism (ROM) (H_2O_2, TBARS, ASC and DHA) were analyzed using a two-way ANOVA with treatment (three levels: control, artificial salts, and brine) and time (four levels: day 1, day 3, day 6, and day 10) as orthogonal fixed factors. Gene FC data were analyzed using the same statistical methodology except for the Treatment factor which consisted of only two levels (artificial salts and brine). Growth length, growth biomass, and necrosis surface were tested using a one-way ANOVA with treatment as the tested factor. In all cases, data

Table 1
List of genes of interest and primer sequences designed in this study that were analyzed in *Posidonia oceanica* using qPCR.

Gene	Coding protein	Sequence 5′–3′
SOS1/ NHX7	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
STRK1	Salt tolerance receptor-like cytoplasmic kinase 1	CGCCGCGCTCCAACCAAGGA CGACGTGGAGCCGCTCGCTT
CAT	Catalase	CTCCGGCCGTCTCGGCCTTG GTGCTCCGTGGCGGCACTCT
Mn SOD	Mn/Fe Superoxide dismutase	CGGCTCGAGCGCGCCGTAAT GAAGCTCCCACGCCCGCACA
Fe SOD	Cu/Zn Superoxide dismutase	TGGTATCCCAGAGTTTGGCGGCTCA TGGAGTGGCACCCTCGCCTCA
APX	l-ascorbate peroxidase	CGCCTCGCGTGGCATTCAGC TCAGGCCCGCCGGTGATCTC
GR	Glutathione reductase	AGGAAGCCCAGAAAGTGTTGCCT TCCCAGCCACCAATAGCTCAAGT
18S	Ribosomal RNA 18S	GAGAAGGAAGCTGCTGAAATG GAACAGCACAATCAGCCTGAG

normality and variance homoscedasticity were tested using a KS test and a Bartlett test, respectively, and data transformations were made when needed (Underwood, 1997). ANOVA results are included in Supplementary Material 2. When significant results were found in the ANOVA, a Tukey HSD test was used to compare both the levels and interaction factors. To identify how the measured parameters were related to the studied treatments and times, a principal component analysis (PCA) based on Euclidean distance was performed. One PCA was performed for biochemical and photosynthetic parameters and another for the gene expression data. PCAs were conducted separately because gene relative expression data did not have control samples. All statistical analyses were performed using R software (v 3.6.0) and package "sciplot" for PCA ordination.

3. Results

Leaf growth (elongation) was significantly lower in the brine treatment compared to control, whereas artificial salts showed intermediate values (Fig. 2a). Growth as leaf biomass production was significantly lower in both hypersalinity treatments compared to the control, but without significant differences between them (Fig. 2b). The necrotic leaf surface area did not show significant differences among treatments, although plants from the control and artificial treatments showed the lowest and highest values respectively (Fig. 2c).

Fv/Fm showed no significant differences between treatments and values kept stable through the experimental period. The RLCs revealed no significant differences in ETR_{max}, α_{ETR}, and Ek_{ETR} among treatments. Fv/Fm significantly increased on days 6 and 10, whereas ETR_{max} and Ek_{ETR} presented no significant differences between salinity treatments, although higher values were detected on the day 3 compared to day 10. α_{ETR} also presented an increment on day 3, showing significant differences with that on day 1, but no differences were found in the treatment factor. Given the patterns in the latter parameters, these were included as Supplementary Material 3. Conversely, NPQ_{max} presented significantly higher values particularly in brine treatments, with a tendency of increased levels on day 1 and stabilizing at later sampling times, although always greater than controls and salinities reached with artificial salts (Fig. 3).

H_2O_2 levels decreased in both increased salinity treatments relative to the controls, with no significant differences between them (Fig. 4a). Quantification of thiobarbituric acid reactive substances (TBARS) revealed higher lipid peroxidation in plants from both hypersalinity treatments compared to control plants, although without significant differences between them and with constant values throughout the experimental period (Fig. 4b).

Plants from both hypersalinity treatments showed lower total ascorbate values than those of the controls. Although the concentration of ascorbate in its oxidized state (DHA) showed no differences between treatments, its concentration in the reduced state (ASC) was

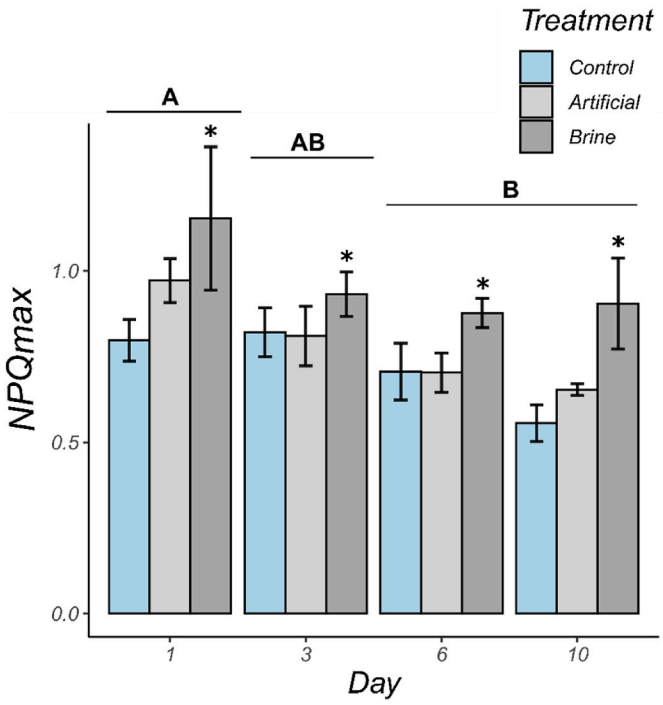

Fig. 3. Nonphotochemical quenching (NPQ) in *P. oceanica* leaves under experimental salinity treatments: control (37 psμ), artificial Salts (43 psμ), and brine (43 psμ) at four sampling times (days 1, 3, 6 and 10). Bars represent the mean of each variable with their respective standard errors (SE). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between days. Asterisks (*) show significant differences between treatments.

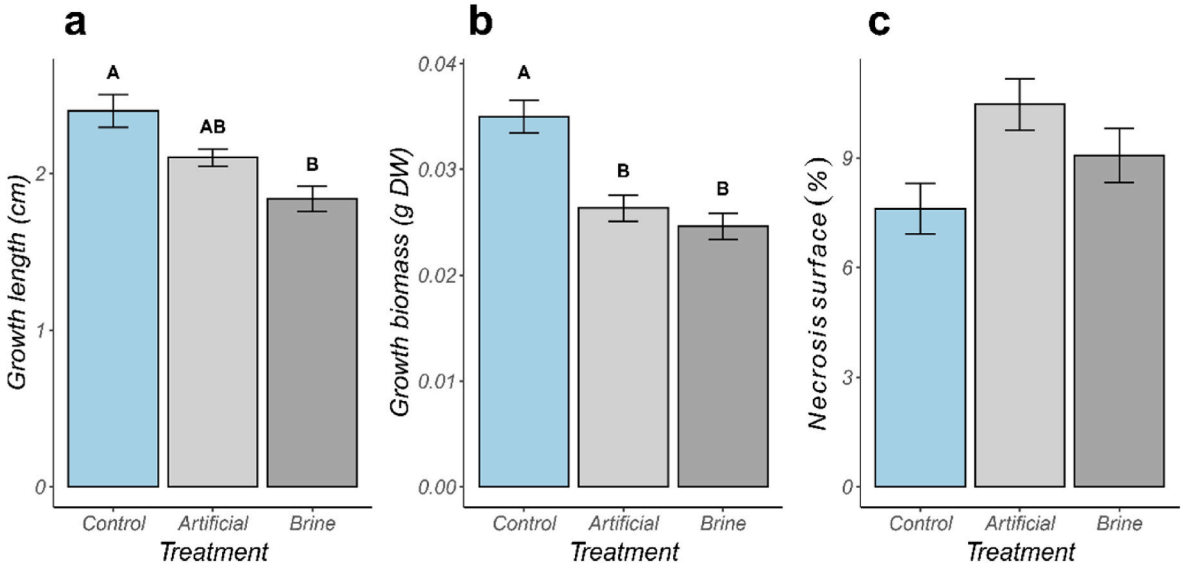

Fig. 2. Growth length (a), biomass (b), and necrosis (c) of *P. oceanica* samples under experimental salinity treatments: control (37 psμ), artificial Salts (43 psμ), and brine (43 psμ). Barplots represent the mean of each variable and error bars show the standard error. Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between treatments.

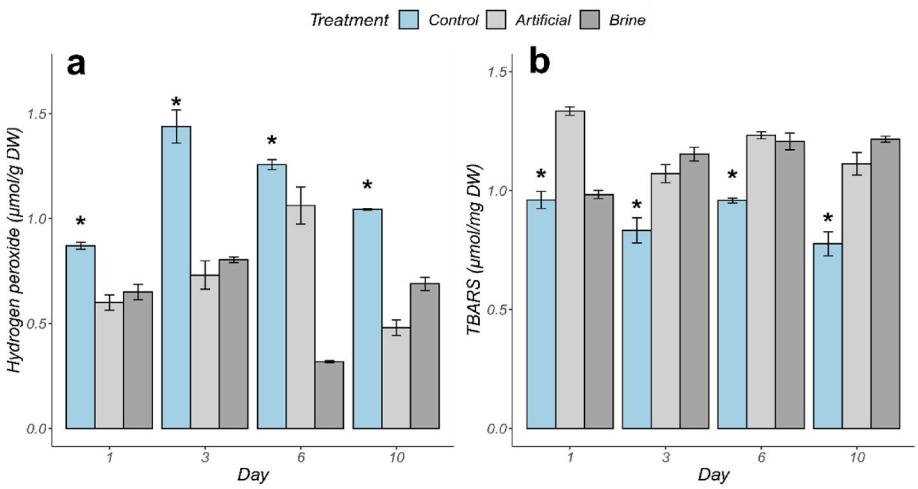

Fig. 4. (a) Hydrogen peroxide (H_2O_2) and (b) thiobarbituric acid reactive substances (TBARS) contents in *P. oceanica* leaves under experimental salinity treatments: control (37 psµ), artificial Salts (43 psµ), and brine (43 psµ) at four sampling times (days 1, 3, 6 and 10). Bars represent the mean of each variable with their respective standard errors (SE). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between days. Asterisks (*) show significant differences between treatments.

significantly lower in plants under brine influence, with reduced percentages between 41% and 75.1% compared to control (Fig. 5). A general decline along the experimental period was detected in plants in control and artificial salt treatment.

In relation with genes associated with osmotic regulation, the gene *SOS1* presented the highest upregulation on the day 3 in plants under brine exposure, with no significant difference among salinity-originated treatments (Fig. 6a). In relation to *P. oceanica* under artificial salts, a marked downregulation of *SOS1* was observed on day 6 but later turning into levels close to gene silencing as well as occurred under brine exposure (Fig. 6a). Even if numeric variability mediated no significant differences for *SOS3*, a trend of overexpression, similar between increased salinity treatments, could be observed on day 1, followed by a general downregulation at later experimental times (Fig. 5b). The expression of *AKT2/3* presented its higher values for artificial salts on days 1 and 10, being significantly higher than those under brine treatments (Fig. 5c). Both hypersalinity treatments followed a similar trend, with higher relative expression on day 1, a decline until day 6 reaching their minimum values and a subsequent recovery to regulation levels similar to the initial ones on day 10 (Fig. 6c). *STRK1* was the only gene that presented clear opposite trends between treatments, with higher relative expression values in plants under brine exposure, except on day 10, and a general downregulation in the artificial salt treatment (Fig. 6d).

Concerning the expression of genes related to ROM, *CAT* relative expression reached its maximum values on days 1 and 3 in plants under brine exposure, although it showed signs of downregulation beyond 6 days (Fig. 7a). The main trend on *CAT* under artificial salts was a near gene silencing effect, in spite of a marked downregulation observed on day 6 (Fig. 7a). Both *FeSOD* and *MnSOD* presented similar patterns between treatments brine and artificial salt treatments, with a higher expression on day 1 and lower (but sill upregulation) on day 10; at intermediate times, a clear overexpression on day 3 was observed, but only under brine treatments (Fig. 7b and c). The expressions of *APX* and *GR* also followed similar trends between brine and artificial salt treatments: starting with the highest and nonsignificant overexpression on day 1, then a pattern of silencing on day 3, and similar upregulation on day 6 that continued on day 10, although in the latter only for brine treatments (Fig. 7d and e).

A PCA biplot represents the parameters that characterized each treatment and time (Fig. 8). Brine samples were mainly correlated with TBARS, NPQ, and low ASC/DHA ratio, whereas artificial salts presented low ETR_{max}, low Ek_{ETR}, and high α_{ETR} values, except for day 1, which presented more similarities with brine treatments. Regarding gene regulation, noticeable differences were found between treatments and a general decline in relative expression during the experimental time. Generally, genes *STRK1, SOS, CAT* and *APX* were more expressed in brine samples, whereas *AKT2/3* seemed to be more upregulated in plants under hypersalinity produced by artificial salts.

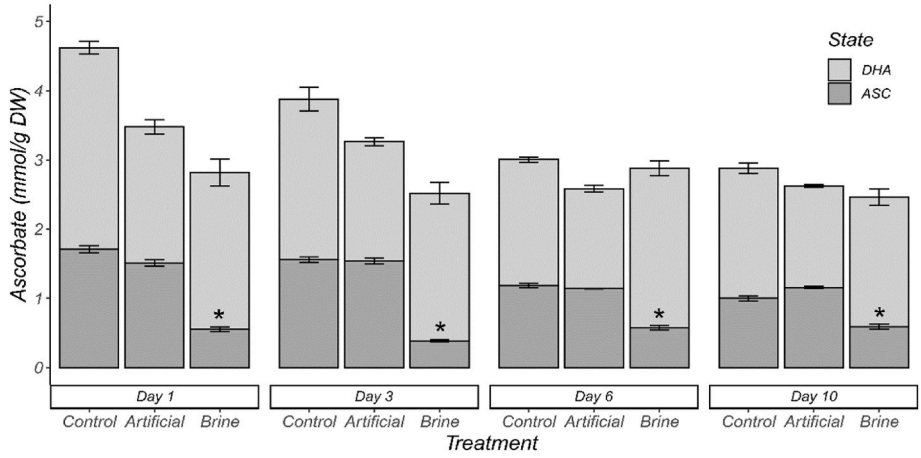

Fig. 5. Leaf ascorbate (ASC) and dehydroascorbate (DHA) content in *P. oceanica* leaves under experimental salinity treatments: control (37 psµ), artificial Salts (43 psµ), and brine (43 psµ) at four sampling times (days 1, 3, 6 and 10). Bars represent the mean of each variable with their respective standard errors (SE). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between days. Asterisks (*) show significant differences between treatments.

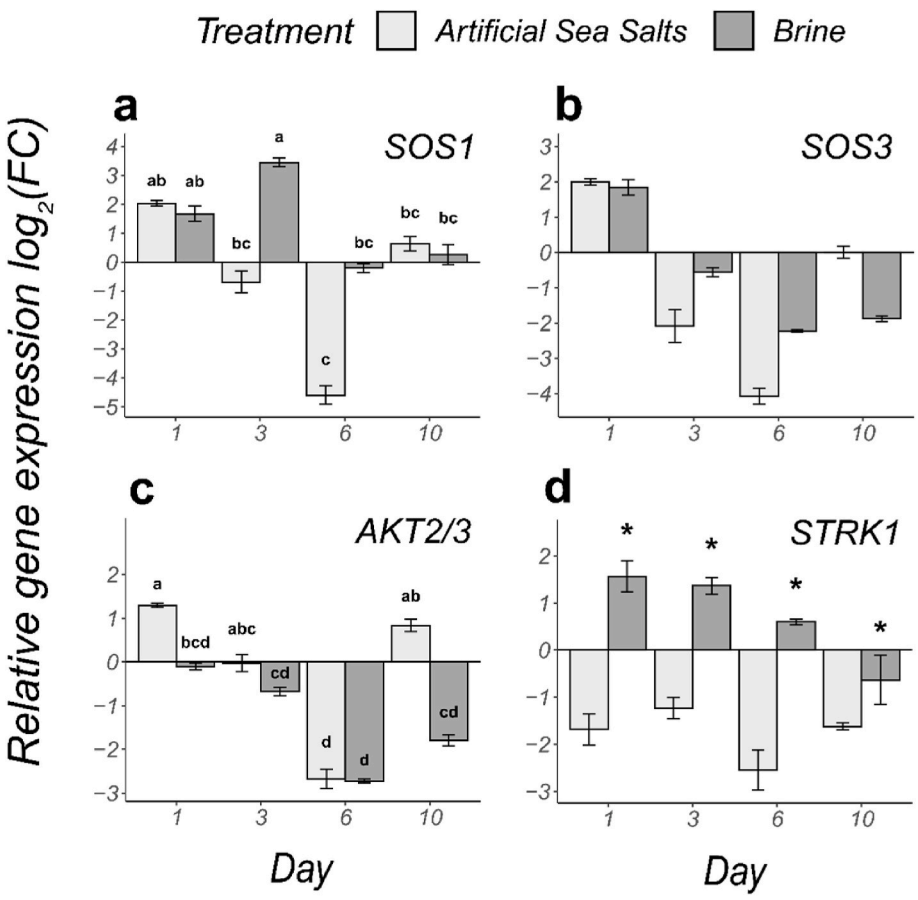

Fig. 6. Relative expression of osmotic regulation genes in *P. oceanica* under two different excess salinity treatments, mediated by either artificial salts or desalination brines. The genes measured were *SOS1* (a); *SOS3* (b), *AKT2/3* (c), and *STRK1* (d). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between days (1, 3, 6, and 10). Lowercase letters represent significant differences between groups when factor interaction was significant. Asterisks (*) show significant differences between treatments.

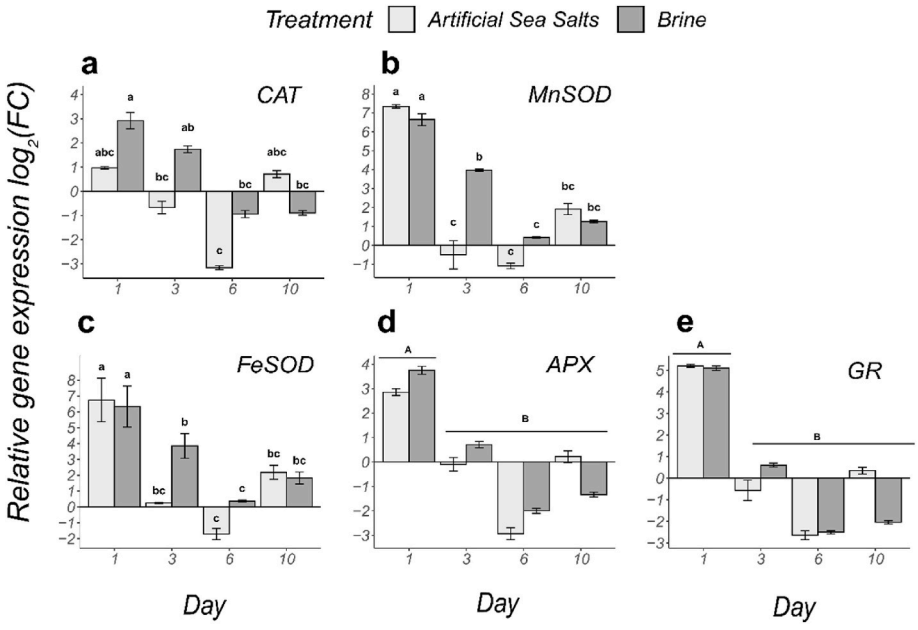

Fig. 7. Relative expression of oxidative stress-related genes in *P. oceanica* under two different excess salinity treatments, mediated by either artificial salts or desalination brines. Genes measured were *CAT* (e), *MnSOD* (f), *FeSOD* (g), *APX* (h) and *GR* (i). Upper case letters represent significant differences at 95% confidence interval ($p < .05$) between days (1, 3, 6 and 10). Lower case letters represent significant differences between groups when factor interaction was significant. Asterisk (*) show significant differences between treatments.

4. Discussion

Desalination brine discharges are a complex and relatively new source of marine pollution and their effects on marine communities are still incompletely understood (Roberts et al., 2010). In this study we have deepened our knowledge of the potential effects caused by desalination brine discharges on *Posidonia oceanica* meadows, demonstrating for the first time that certain biomarkers respond more actively to exposure to real brine than to exposure to artificial salts. However, the overall metabolic and physiological response of the species followed a common trend regardless of the source of hypersalinity. This indicates that, although the main effects of brine discharges on seagrasses are

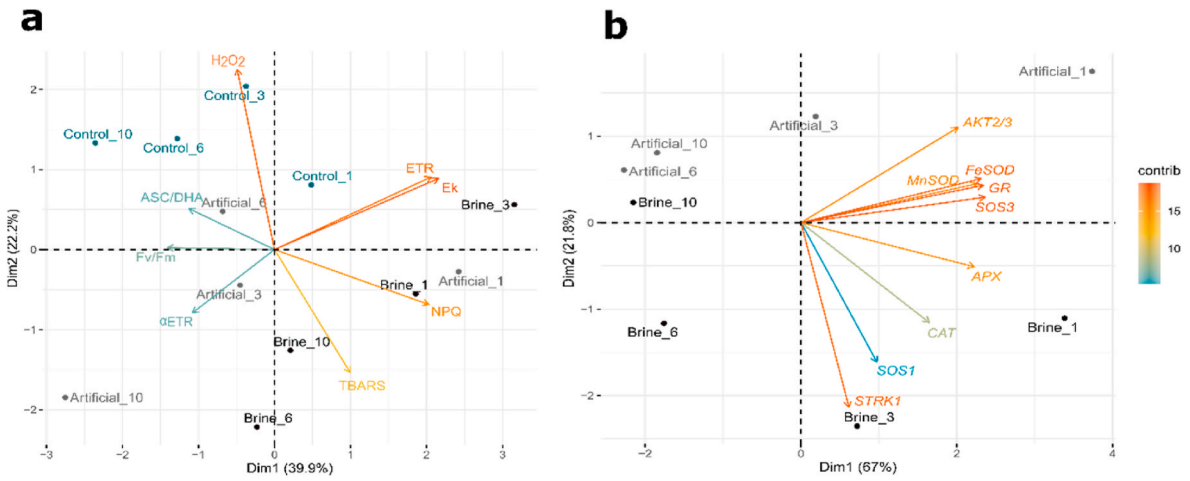

Fig. 8. PCA plot representing the measured parameters and samples by treatment (control, artificial, brine) and time (days 1, 3, 6, and 10). Photochemical (Fv/Fm, ETRmax, αETR, EkETR, and NPQ) and metabolic (ASC/DHA, H2O2, and TBARS) parameters (a), and relative gene expression (b).

caused by the high concentration of salts (ionic and osmotic stress), the industrial additives that brine may contain caused a differential response of certain biomarkers.

We already know that hypersalinity triggers a series of responses in seagrasses, such as synthesis and accumulation of organic osmolytes (i.e., amino acids and soluble sugars), ion exclusion, and activation of the antioxidant response and other defense mechanisms (Cambridge et al., 2017; Sandoval-Gil et al., 2012b). These are high-energy-cost responses that can ultimately have a direct impact on normal plant development and growth (Fernández-Torquemada and Sánchez-Lizaso, 2013; Sandoval-Gil et al., 2012a). In our case, *P. oceanica* plants exposed to both hypersalinity treatments (artificial salt or brine based) of 43 psμ showed reduced rates of leaf production, suggesting the diversion of growth resources to activate mechanisms of protection and repair of damage induced osmotic stress (Sandoval-Gil et al., 2022). Because similar physiological effects could be observed in both hypersalinity treatments in the short term, further studies are needed to ascertain if these differences at the metabolic level imply that brine-induced excess salinity can have different effects compared with that caused by only sea salts at physiological and, therefore, ecological levels.

In this study, plants from both hypersalinity treatments showed a trend of increase in necrotic leaf, although not significant, possibly due to the short duration of the experiments (10 days). In a previous experiment *P. oceanica* plants exposed to salinities above 42.5 psμ for 15 days showed a marked increase in leaf necrosis (Fernández-Torquemada et al., 2005). Therefore, it could be inferred that even if *P. oceanica* plants can withstand high levels of salinity associated with brine discharge for a relatively short period of time, longer exposures could cause irreversible damage to leaf tissues and eventually death of individuals and meadow regression.

In general, the photosynthetic performance (photochemistry) of *P. oceanica* in this study was not altered by exposure to high salinity levels, regardless of whether the salinity was produced by brine or artificial salts. These results agree with those of Cambridge et al. (2019), who also found no photosynthetic changes in *P. australis* under hypersalinity, except after 2 weeks under undiluted brine (56 psμ), which represents an unrealistic condition in the marine environment. Despite the stable photosynthetic performance, nonphotochemical quenching (NPQ) did increase in *P. oceanica* under brine influence compared with that under artificial sea salts and the controls. NPQ is a xanthophyll cycle-mediated photoprotective mechanism, the functionality of which has been demonstrated in *P. oceanica* under hypersaline stress conditions (Marín-Guirao et al., 2013a,b). NPQ induction allows the dissipation of excess energy in the thylakoid membranes, through heat, thus preventing excessive ROS formation (Demmig-Adams and Adams, 1996). This protective mechanism has been shown to be relevant in seagrasses to cope with stress due to increased salt concentration, as reported for *Thalassia testudinum* under 45 psμ for 7 days (Trevathan et al., 2011), *Cymodocea nodosa* under 60 psμ for 7 days (Garrote-Moreno et al., 2015a,b) and in *P. oceanica* under 46 psμ for 7 days (Garrote-Moreno et al., 2015a,b). In this study, this photoprotective response has been shown to be even more sensitive when the increase in salinity was caused by brine from a desalination plant than when hypersalinity was only associated with artificial sea salts, which suggests that at these level responses, other brine components may increase excess energy production in electron transport chains.

Despite the activation of this protective mechanism, plants under hypersaline conditions showed oxidative damage to membranes, as indicated by the levels of TBARS, similarly in both treatments (artificial salt and brine). Similar levels of oxidative damage have also been reported by Capó et al. (2020) in *P. oceanica* plants collected from a population chronically influenced by a brine discharge (40.8 psμ). Interestingly, plants that displayed hypersalinity-induced oxidative damage exhibited lower H_2O_2 content than control plants. This could be explained by the highly efficient H_2O_2 scavenging due to enzimatic (e.g., CAT and APX) or nonenzymatic (e.g., ascorbate) mechanisms under hypersaline conditions, as supported by Vaidyanathan et al. (2003). These authors found an effective H_2O_2 scavenging by CAT at increasing salt levels on rice (*Oryza sativa*) for 48 h while lipid peroxidation was uprising. In our case, only brine-exposed *P. oceanica* showed upregulation of osmotic-related gene *STRK1*, which encodes a receptor protein that is activated by increased salinity and phosphorylates and triggers *CAT* expression; thus, linking osmotic imbalance with antioxidant defense. In fact, *CAT* displayed a similar pattern to *STRK1*, with a strong expression level at the beginning of exposure that progressively decreased over time. Therefore, this strong activation of *CAT* expression could have contributed to the reduced levels of H_2O_2 in brine-treated plants.

In addition, the reduced levels of ASC in brine-affected plants and the consequent reduction in the ASC/DHA ratio indicated a high consumption of the antioxidant (Sofo et al., 2015), confirming its eventual implication in H_2O_2 and ROS in general scavenging. These findings could also be explained by a lower ASC recycling capacity of *P. oceanica* plants under brine exposure compared with those treated with artificial salts. ASC consumption as a response to hypersaline stress has also been detected in the filamentous macroalgal species *Ectocarpus* transplanted in the field for up to 7 days, a brine-influenced site of 2.3 psμ above natural salinities (Rodríguez-Rojas et al., 2020). These results may indicate an enhanced response to oxidative stress in *P. oceanica* plants triggered by brine treatment, which, together with NPQ induction seems

to be effective enough to maintain oxidative damage (TBARS) controlled as in plants exposed to excess salinity mediated by sea salts, as also did at physiological levels of growth and necrosis.

We already know that excessive ion concentrations related to hypersalinity cause electron transport chain malfunction, causing electrons overtransfer to nearby oxygen (Munns and Tester, 2008). Superoxide anion (O_2^-) is usually the first ROS species to be formed, and it is dismutated to H_2O_2 by MnSOD and FeSOD in the mitochondria and chloroplast, respectively (Fridovich, 1997). This accumulation of O_2^- appeared to trigger the overexpression of *MnSOD* and *FeSOD* in *P. oceanica* in both hypersalinity treatments, which may increase H_2O_2 levels to be scavenged by CAT and APX. Indeed, we observed higher regulation of *APX,* together with *CAT,* in the brine treatment, which may also explain the low detected levels of ASC. ASC is oxidized serving as substrate by APX (forming DHA) to reduce H_2O_2. Overexpression of *GR* allows the recovery of glutathione (GSH), which also restores ASC from DHA in the Foyer-Halliwell-Asada (glutathione-ascorbate) cycle (Foyer and Noctor, 2011). Therefore, finding increased *GR* and *APX* is a strong indicator of an effective functioning of the Foyer-Halliwell-Asada cycle in *P. oceanica*. Similar gene regulation responses to hypersaline stress (*SOD*, *CAT*, *APX*, and *GR*) have been described in other macrophytes. For instance, these genes were also upregulated in *Ectocarpus* transplanted to 36 and 38 psμ brine influence (above a natural salinity of 34 psμ) during 3 and 7 days (Rodríguez-Rojas et al., 2020), and in *Z. chilensis* exposed to 37 and 40 psμ (over 34 psμ as control) under controlled conditions for 10 days (Blanco-Murillo et al., 2023); this demonstrates a pattern of antioxidant responses in marine macrophytes upon hypersaline excess beyond phylogenetic lineages.

Regarding genes related to osmotic regulation, the initial overexpression of SOS1 and SOS3 indicates an active Na^+ exclusion response to hypersalinity in the short term. *SOS3* activation stimulates *SOS1* expression which codifies for a plasmatic membrane transport protein that excludes Na^+ by up taking H^+ (Ji et al., 2013). This mechanism protects the cell from the toxicity caused by Na^+ excess and it increased in the presence of brine on day 3. This response was also observed in *Z. chilensis* which showed an increment in *SOS1* relative expression after 3 days of hypersalinity exposure (Blanco-Murillo et al., 2023). To maintain ion homeostasis, Na^+ exclusion is complemented with specific K^+ uptake, mediated by AKT 2/3. In fact, *P. oceanica* has shown to be capable of reducing intracellular Na^+ and increase K^+ levels being exposed to 46 psμ for 7 d, indicating an effective ion transport under osmotic stress (Garrote-Moreno et al., 2015a,b). An increment in cytoplasmic K^+ was also measured in *C. nodosa* under hypersaline conditions (Tsioli et al., 2022), highlighting its role in seagrass osmoregulation. However, *AKT 2/3* was only upregulated in artificial salts on days 1 and 10, which means that K^+ capture might not be the most urgent response. Possibly, basal AKT 2/3 transport proteins in *P. oceanica* membranes are efficient enough to maintain minimum K^+ levels, even under hypersaline stress, thus directing the osmoregulatory main metabolic cost to Na^+ exclusion. The general trend followed by *SOS1*, *SOS3* and *AKT 2/3* expression was a higher transcription on days 1 and 3, a marked down-regulation on day 6 and a certain recovery at the end of the experiment, showing a common metabolic response to hypersalinity. Different ion proportions in brine compared artificial salts may be causing this higher *SOS1* on day 3 and *STRK1* transcription, while downregulating *AKT 2/3*, indicating a certain sensitivity of these osmoregulatory responses to different hypersalinity origins.

Cambridge et al. (2017) analyzed the effects of hypersalinity on *P. australis* to subsequently compare them to real desalination brine (Cambridge et al., 2019), studying photosynthetic performance and leaf water and osmotic potentials. Their findings showed a deleterious and more rapid effect of undiluted desalination brine on *P. australis* compared to hypersalinity reached with artificial salts (56 psμ). By contrast, our work on *P. oceanica*, a more stenohaline species than *P. australis* (Sandoval-Gil et al., 2022), under more realistic brine exposures, demonstrates certain differences in the physiological and metabolic responses between brine and artificial salts. However, plants under both hypersalinity treatments kept photosynthetic performance and oxidative damage at the same level and a similar pattern was observed with physiological endpoints. To this end, it is clear that certain different differences are caused by brine-induced hypersalinity compared to just marine salts, at least on *P. oceanica* at metabolic level, but the chemical and biochemical basis remain unclear. In spite of the latter, SWRO desalination plants pump seawater through semipermeable membranes, which have a low permeability to ions, but still not the same to all of them. There is certain ion selectivity, concentrating more divalent ions and monovalent cations compared to monovalent anions (Biesheuvel et al., 2020; Mukherjee and Sengupta, 2003). Moreover, depending on the needs of the desalination plant, the brine may contain other industrial-associated components, such as antiscalants and antifouling compounds, which may contain trace metals as Fe and Cu; these, when in excess can cause oxidative stress through Fenton reaction (Bartosz, 1997; Foyer and Noctor, 2011). Despite this information, the metabolic and especially the physiological data demonstrate that in the case of *P. oceanica*, deleterious responses at high levels of biological organization may be principally related to brine-associated hypersalinity around desalination discharges.

Our investigation demonstrated that *P. oceanica*'s growth, lipid peroxidation and relative expression of *SOS1*, *FeSOD*, and *MnSOD* actively responded to hypersaline stress despite its origin, and that NPQ, ASC/DHA and the transcription of *CAT* and *STRK1* presented brine-specific responses, as presented in the graphical scheme of Fig. 9. Therefore, these parameters arise as suitable descriptors and biomarkers to identify brine impacts on *P. oceanica* and as potential early-warning indicators of physiological and population stress to be incorporated to EMP. Moreover, most of them seem proper to even differentiate brine impacts where other environmental pressures are present, a necessary aspect to address in future field investigations.

5. Conclusions

Hypersalinity exposure (43 psμ), despite the sources explored in this investigation, of *P. oceanica* for up to 10 days resulted in a growth biomass decline, lipid peroxidation and a short-term upregulation of genes related to ion exclusion and oxidative stress (especially *SOS1*, *FeSOD*, and *MnSOD)*, but no relevant effects were found in photosynthetic performance (Fv/Fm, ETR_{max}, α_{ETR}, and Ek_{ETR}).

Brine exposed plants differentially demonstrated an increase in NPQ, a lower ASC/DHA ratio and higher *CAT* and *STRK1* upregulation compared to plants exposed to the same salinity reached with artificial salts. This investigation demonstrated that although brines can induce biological stress in *P. oceanica*, physiological performance and predictively effects at higher levels of biological organization are principally related to excess salinities contained in desalination discharges.

Credit authorship contribution statement

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

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests. Claudio Sáez and Iván Sola report financial support was provided by

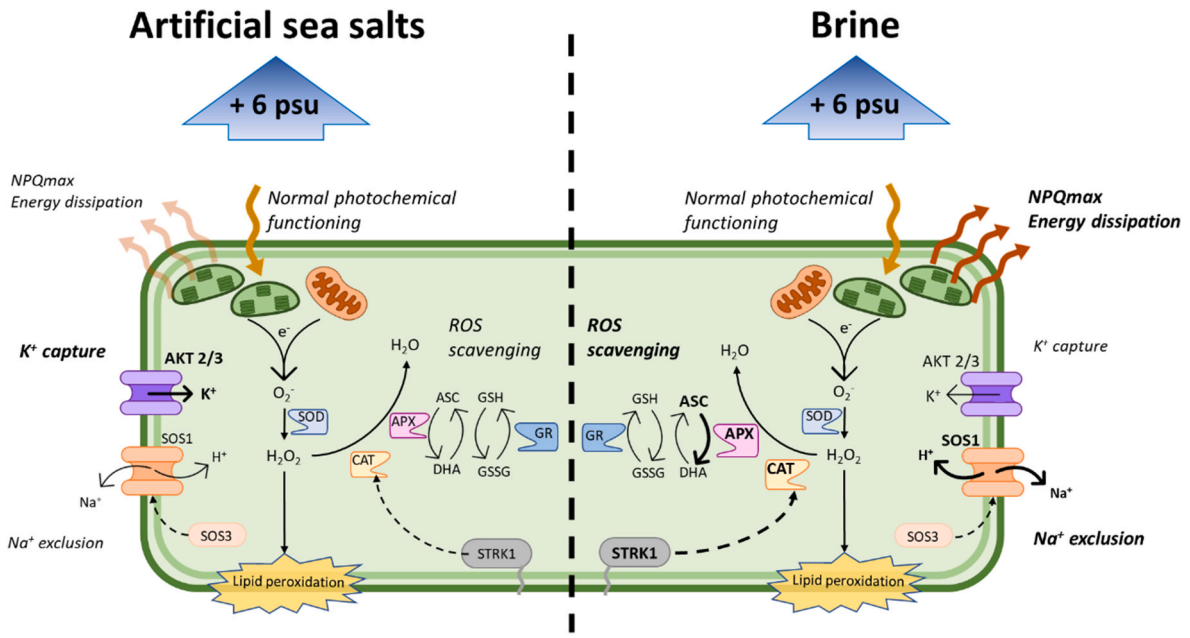

Fig. 9. Scheme showing the physiological and metabolic responses of P. oceanica under hypersalinity conditions reached with artificial salts (left) and desalination brines (right). Intensified processes appear highlighted. NPQ, nonphotochemical quenching; ROS, reactive oxygen species; SOS, salt overly system; SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; STRK1, salt tolerance receptor like kinase; GR, glutathione reductase.

European Commission. Fabio Blanco Murillo reports a relationship with University of Alicante that includes: funding grants.

Data availability

Data will be made available on request.

Acknowledgements

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).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2023.140061.

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