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Ecophysiological and cellular stress responses in the cosmopolitan brown macroalga *Ectocarpus* as biomonitoring tools for assessing desalination brine impacts

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ABSTRACT

Seawater desalination via reverse osmosis (SWRO) is highlighted as one of the most feasible solutions for obtaining freshwater. However, brine produced by SWRO is generally discharged to the subtidal area potentially causing detrimental effects on benthic organisms. In this study, we evaluated for the first time, ecophysiological and cellular responses of brown macroalgae as diagnosis tools to assess environmental impacts of desalination, through transplantation experiments with the cosmopolitan brown alga *Ectocarpus*. Transplants located at 10 and 30 m from the discharge point of a desalination plant located in Antofagasta, Chile, showed impaired photosynthetic parameters (ETR, *Fv/Fm*, α_{ETR} and ETR_{max}) and oxidative stress responses like accumulation of H_2O_2 and enhanced lipid peroxidation. Also, increased salinity produced high accumulation of ascorbate but a decrease in glutathione content. Also, genes encoding for enzymes related to salinity tolerance, *SOS2*, and oxidative stress, *SOD*, *APX*, *PRX* and *GR*, were highly up-regulated in transplanted *Ectocarpus*, especially at 10 m from the brine discharge. Altogether, our results demonstrate that *Ectocarpus* is a sensitive species to brine impacts, and that the transplantation method combined with its physiological and molecular responses are reliable tools to incorporate in environmental monitoring plans to address for desalination brine impacts on coastal ecosystems.

1. Introduction

In the last decades, terrestrial water fluxes have been severely affected by direct anthropogenic intervention (e.g. water withdrawals from domestic, agricultural, and industrial activities) [1]. On the other hand, Climate Change driven by massive emissions of greenhouse gases, among others, have been changing the distribution of the weather patterns all over the globe and influencing water cycle dynamics, causing an important impact in water availability and demand worldwide [2,3]. To address the latter, seawater desalination via reverse osmosis (SWRO), has become a feasible solution, which comprises the removal of salt and minerals from saline water. Nowadays, approximately 7% of the world's population is dependent on desalinated water for daily purposes, but it is expected to grow up to 14% by 2025, and 25% by 2050 (United Nations, 2015, [4]). However, and despite the important use of desalination, this procedure may cause impacts on

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coastal ecosystems, since it can generate large quantities of high salinity brines that are usually discharged into the subtidal zone [5,6]. As brine is denser and twice saline than seawater, it sinks to the bottom and can cause detrimental effects on benthic organisms, mainly through osmotic stress [7,8]. It has been observed that brine-associated impacts are highly species- and ecosystem-specific; thus, the consequences of brine discharges recorded by research so far cannot be directly extrapolated to the impacts caused by desalination projects worldwide [7]. Moreover, different investigations have evidenced that biological stress mediated by brines is principally related with osmotic stress derived from the excess of salt [9–11].

Despite the long history of desalination plants worldwide, most environmental impact data available is related with ecological effects (e.g. [6,12–14]). Available studies demonstrate that the ecological impacts of the hypersaline stream are influenced by several factors including salinity levels, discharge method, dilution rate and tolerance of surrounding marine organism [7,15]. For example, sensitive benthic fauna has been identified, as certain polychaeta [16] and equinoderms [17]. Also, different brine tolerance ranges were observed in reef-building corals species, as well as in associated bacteria; for example, triggering partial coral bleaching [13]. Other study demonstrated that benthic bacteria can be affected by brine effluents in a site-specific manner, where discharge method and local stressors influenced the abundance and diversity of these communities [18]. In photoautotrophs, laboratory approaches have shown that saline excess above 39.1 psu versus control value of 37.5 psu (average Western Mediterranean Sea salinity) results in a reduction of the seagrass *Posidonia oceanica* vitality, in terms of leaf grow, necrotic spots, and leaves premature senescence; moreover, when exposed to 45 psu, approximately 50% of the seagrasses died in two weeks [19]. Similar results were obtained with the Mediterranean seagrass *Cymodocea nodosa*, which showed detrimental effects when exposed to increased salinities in laboratory-controlled conditions [20], and during in situ transplantation nearby brine discharges [21]. Also, field experiments nearby a pilot desalination plant in Spain demonstrated the same tendency as the laboratory experiments, disclosing significant damages on *P. oceanica* viability (at 38.4 psu) and meadow structure (at 39.1 psu) [22].

Until now, most research on the effects of brines on coastal macrophytes has been restricted to seagrasses and their communities; however, no studies have described yet brine impacts on other ecologically-relevant macrophytes, such as macroalgae. Macroalgae (seaweeds) are main primary producers and bioengineers in coastal environments from inter-tropical to polar latitudes [23]. Considering the important biological role of brown macroalgae as habitat forming organisms (e.g. kelp forests) in temperate coastal rocky shores worldwide, and the lack of knowledge about the potential detrimental impact of the desalination industry on them, it is highlighted as a relevant research subject to understand their response mechanisms to address hypersalinity, as well as to extrapolate those to potential ecological and economic consequences.

Peters et al. [24] proposed *Ectocarpus* sp. (formerly *Ectocarpus siliculosus*, and henceforth referred as *Ectocarpus*) as a general model organism for the study of brown macroalgae. Features include its small size, high fertility, rapid growth and the fact that the entire life cycle can be completed under controlled laboratory conditions; these aspects not only makes *Ectocarpus* a reliable model organism for macroalgae, but also a good prospect to biomonitor anthropogenic impacts. *Ectocarpus* has been previously used as a biomonitoring organism in order to address the consequences of metal pollution through combined laboratory and field transplantation experiments [25–27], thus, is highlighted as suitable to address for other impacts; e.g., desalination brine discharges.

In this study, we analysed the ecophysiological (photosynthetic activity) and metabolic (oxidative stress damage and tolerance) responses of *Ectocarpus*, through in situ transplantation experiment to evaluate the space-temporal exposure against brine discharges from a desalination plant, in order to understand tolerances strategies and potential damage; and evaluate these as potential tools for monitoring brine impacts.

2. Material and methods

2.1. Ectocarpus culture

Ectocarpus strain Es524 (Culture Collection of Algae and Protozoa CCAP 1310/333), isolated from Caleta Palito, Chañaral (26°16′29.2″S; 70°39′38.4″W), Chile, was grown in polycarbonate flasks (10 L, Nalgene), using clean filtered and autoclaved seawater enriched with Provasoli nutrients [28], and with a filtered stream of air continuously circulating (0.2 µm pore size filter). Growth conditions comprised a photoperiod of 12 h/12 h light/dark, 120 µmol photons m^2 sec^{-1}, and 16 °C constant temperature in a culture chamber. Culture media was renewed every two weeks to maintain media nutrient supply. Biomass was collected after 5 months for transplantation devices implementation.

2.2. Transplantation device and field experiments

Transplant devices were based in the prototype designed by [25], with certain modifications. Briefly, plastic containers with rubber sealing lid (12 cm long, 8 cm wide and 5 cm high). A hundred perforations (~1 mm) for water free flux and exchange between inside and outside the box. Inside the plastic containers, three 76 mm flat width semipermeable dialysis tubing cellulose membranes (Sigma-Aldrich, molecular weight cut-off = 14 KDa, D9402) were set containing ~100 g fresh weight of *Ectocarpus* thalli and sealed with elastic bands at each end (see Fig. 1A, B).

Three transplant devices each with three dialysis bags containing *Ectocarpus* samples were placed in each of the two sites selected (autumn 2019) nearby the brine discharge pipe from the desalination plant "Aguas Antofagasta", Antofagasta, Chile (23°32′36.7"S; 70° 24′14.3″W) which produces 600 L s^{-1} of freshwater for human consumption (Fig. 1C). Sites were located 10 m and 30 m away from the discharge pipe, both at 15 m depth, respectively. A pristine control site was located at the same depth in the Bolsico Bay, within Santa María Island sector (23°33′14″S; 70°24′24″W) (Fig. 1C). All transplants were installed by scuba divers using ropes attached to concrete anchors (Fig. 1B). Salinities and temperatures were recorded by a conductivity and temperature meter (CT; ALEC infinity) for 24 h in each transplantation site. *Ectocarpus* transplants were collected at 3 days, and the rest at 7 days; the latter being the end of the experiments. All samples were immediately subjected to photosynthetic activity analyses, and a portion frozen in liquid nitrogen and later stored at −80 °C for further biochemical and molecular analyses. It is important to mention that the transplants at 30 m were lost for day 7, likely due to the strong current and weather conditions of the area; thus, data for this site were taken only at day 3.

2.3. Physiological analyses

In vivo chlorophyll *a* fluorescence associated with Photosystem II was determined using portable pulse amplitude modulated (PAM) fluorometer (Junior-PAM, Walz GmbH, Germany). Thalli of *Ectocarpus* were collected from transplanted and control transplants (initial time) and monitored on days 3 and 7.

In order to obtain rapid light curves (RLC) for each treatment, parts of the macroalgae were put into 5 mL incubation chambers [29]. The F_o (basal fluorescence) and F_m (maximal fluorescence) were measured after 15 min in darkness to obtain the maximal PSII quantum yield (F_v/F_m) as a photoinhibition indicator [30], being $F_v = F_m$-F_o, F_o the basal fluorescence of 15 min dark-adapted thalli and F_m maximal fluorescence after a saturation light pulse of > 4000 µmol m^{-2} s^{-1} [31].

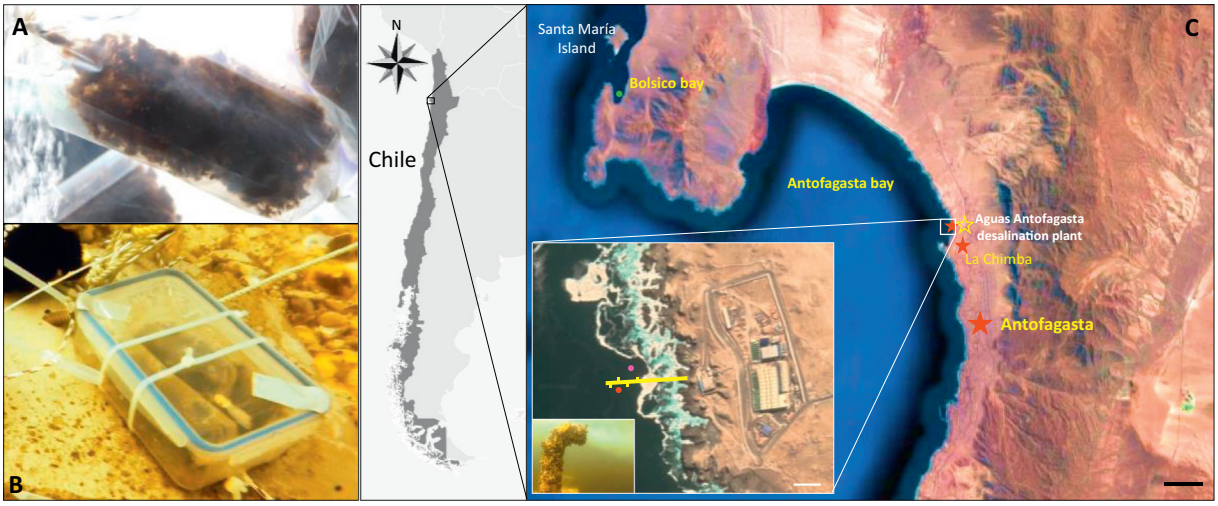

Fig. 1. Transplantation device and assessed sites nearby a desalination plant in Antofagasta, Chile. A, *Ectocarpus* biomass inside the dialysis tubing membrane. B, transplantation device consisting in a plastic box attached to a concrete anchor with three dialysis tubing membranes containing *Ectocarpus* biomass. C, Map showing transplantation sites nearby the brine discharge of "Aguas Antofagasta" desalination plant at La Chimba, Antofagasta, Chile, and in a control site in the Bolsico Bay, Santa María Island area. Zoomed map shows the brine discharge pipe in yellow with four brine diffusers (inset photograph), and the position of the transplantation devices installed: site at 10 m from the discharge (red dot), site at 30 m from the discharge (pink dot), and the control site (green dot). Black and white bars at the low right corner of every map represent scales of 5 km and 100 m, respectively. Maps images were taken from Google Earth. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The Electron transport rate (ETR) was calculated according to Schreiber et al. [31] as follows: ETR ($\mu mol\ electrons\ m^{-2}s^{-1}$) = $\Delta F/Fm' \times E \times A \times F_{II}$.

Where E is the incident PAR irradiance, A, is the thallus absorbance [30] and F_{II} is the fraction of chlorophyll *a* associated to PSII (400–700 nm), being 0.8 in brown algae [29,32].

The ETR parameters such as maximum electron transport rate (ETR_{max}), an estimator of photosynthetic production, and the initial slope (α_{ETR}) of ETR versus irradiance function, an estimator of photosynthetic efficiency, were obtained from the tangential function reported by Eilers and Peeters [33]. The saturation irradiance for ETR (Ek_{ETR}) was calculated from the intercept between ETR_{max} and α_{ETR}.

2.4. Quantification of hydroxide peroxide and TBARS

Quantification of hydroxide peroxide (H_2O_2) was carried out spectrophotometrically according to Sáez et al. [25]. Briefly, 100 mg of frozen biomass was homogenized to powder in a mortar with liquid nitrogen and mixed with 1 mL of 10% TCA in the presence of glass beads (3 mm), vortexed for 10 min and centrifuged for 10 min at 16,000*g* at 4 °C. Then, 100 µL of supernatant was incubated with 100 µL of 50 mM K_2HPO_4 buffer and 100 µL of 1 M KI. Absorbance was measured at 390 nm in a 96-well microplate in a microplate reader SPECTROStar Nano (BMG LABTECH). Calibration curve was constructed using commercial H_2O_2 (Sigma-Aldrich).

Thiobarbituric acid reactive substances (TBARS) were determined as a proxy for lipid peroxidation according to Sáez et al. [25]. Supernatant was obtained as for H_2O_2 measurements. Then, 200 µL supernatant was mixed with 200 µL 0.5% TBA diluted in 10% TCA and heated at 95 °C for 45 min. Absorbance at 532 nm was measured using 200 µL of the mixture in the microplate reader. Calibration curve was constructed using commercial malondialdehyde (Sigma-Aldrich) as standard.

2.5. Quantification of ascorbate and glutathione

The content of the antioxidant molecules, the reduced/oxidized pair: ascorbate (ASC) and dehydroascorbate (DHA) was measured according to Sáez et al. [25]. Briefly, 150 mg of frozen biomass was homogenized to powder in a mortar with liquid nitrogen and mixed with 1.2 mL of 0.1 M HCl in the presence of glass beads (3 mm), vortexed for 10 min and centrifuged for 10 min at 21,000*g* at 4 °C. For reduced ASC quantification, 10 µL of lysate was mixed with 290 µL of FRAP solution (300 mM sodium acetate, 20 mM $FeCl_3$ and 10 mM 2,4,6-Tri(2-pyridyl)-s-triazine) and absorbance measured at 593 nm immediately. As well, total ascorbate (reduced + oxidized) was determined by reducing all DHA within the extracts by incubating 500 µL of the lysate with 100 mM DTT for 1 h. Then, the reaction was stopped with 10% of N-ethylmaleimide. Total ascorbate was measured as for ASC and concentrations of DHA was obtained by subtracting ASC levels from total ascorbate. L-ASC (Sigma-Aldrich) was used as standard.

On the other hand, reduced glutathione (GSH) and oxidized (GSSG) were quantified according to Sáez et al. [25]. For total glutathione, 200 mg of frozen biomass was homogenized to powder in a mortar with liquid nitrogen, mixed with 300 µL of 0.1 M HCl in the presence of glass beads (3 mm), vortexed for 10 min, and centrifuged for 15 min at 7400*g* at 4 °C. Then, extracts were neutralized with 250 µL of 50 mM NaH_2PO_4 and, 50 µL of neutralized extract was mixed with 250 µL of the reaction buffer containing: 0.1 M NaH_2PO_4 (pH 7.5), 6 mM EDTA, 0.34 mM NADPH, 0.4 mM DTNB, and 1 U of glutathione reductase (Sigma-Aldrich). Changes in the absorbance were measured at 412 nm for every 20 s for 5 min in the microplate reader. To quantify GSSG, a volume of 50 µL of neutralized supernatant was incubated with 4-vinylpyridine for 45 min and subsequently measured as for total GSH. Commercial GSH was used for standard curves (Sigma-Aldrich).

2.6. RNA purification, cDNA synthesis and qPCR

RNA extraction was performed using 100 mg of ground tissue with the RNeasy Mini Kit (Quiagen) according to the manufacturer instructions. RNA purity was determined spectrophometrically by 260/280 ratio using the LVIS nanoplate in the SPECTROStar Nano (BMG LABTECH). RNA quantification was carried out by fluorescence using Quant-iT RiboGreen RNA Assay Kit (Invitrogen) in a QFX Fluorometer (DeNovix) and integrity was evaluated by 1% agarose gels using the "bleach gel" method [34]. Only samples with a 260/280 ratio > 1.9, concentrations above 100 ng/µL and good integrity were used for qPCR.

Synthesis of cDNA was carried out with 500 ng of total RNA using

the ProtoScript First Stand cDNA Synthesis Kit (New England BioLabs), according to the manufacturer instructions.

Then, for qPCR reaction, 25 ng of cDNA (1 μL) were used together with 0.25 μM of each primer and the Brilliant II SYBR Green QPCR Master Mix (Agilent Technologies) 1 × (Final volume of 20 μL). qPCR program consisted in: initial denaturation at 95 °C for 5 min; 40 cycles of: 95 °C for 30 s, 55–60 °C for 30 s and 72 °C for 40 s; and final extension at 72 °C for 10 min. All reactions were performed in a MIC qPCR Magnetic Induction Cycler (Bio Molecular Systems). Genes analysed encoded for: salt overlay sensitive 2 (*SOS2*), superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), peroxiredoxin (*PRX*), and glutathione reductase (*GR*). Primer sequences were for *SOS2*: forward 5′-CGCGACCGCTGTTCCGCC-3′ and reverse 5′-ACGCCGCACGCCCAC ACG-3′; *SOD*: forward 5′-GGGTAAAAGCCTGGACGAGA-3′ and reverse 5′-TCTTGAACGCCTCGAACGAC-3′; *APX*: forward 5′-TCAACCACGAAG CCAACCTT-3′ and reverse 5′-CTTTCGCCCGTACCTCATGT-3′; *PRX*: forward 5′- TTTTCGCTCCTGGGTTTGGT-3′ and reverse 5′- TCATCCTC ACACGCAAGTCC-3′; *GR*: forward 5′-GCATCAACAGCGATGGGTTC-3′ and reverse 5′-GGTCGAGTATGTCGCTGAGG-3′; and 18S rRNA: forward 5'-AATTTGACTCAACACGGG-3′ and reverse 5′-TACAAAGGGCAGGG ACG-3′. Relative expression analyses were based in the $2^{-\Delta\Delta Ct}$ method using 18S rRNA as housekeeping gene [35].

2.7. Statistical analyses

The effects of the in situ treatments on the ecophysiological and cellular metabolic responses of *Ectocarpus* were assessed using analysis of variance, where three biological replicates where considered (one biological replicate corresponded to mean values of the three technical replicates in each transplant device) [36]. One fixed factor was considered for day 3, with three levels: control, and 10 m and 30 m from the discharge pipe. For day 7 with two levels: control and at 10 m from the discharge pipe. In addition, two fixed factors were considerate to compare the responses between 3 and 7 days, only for control and site 1. Student Newman Keuls tests (SNK) were performed on significant ANOVA. Homogeneity of variance was tested using Cochran tests and by visual inspection of the residuals. All data conformed to homogeneity of variance. Analyses were performed by using SPSS v.21 (IBM, USA).

3. Results

3.1. Salinities and photosynthetic activity as in vivo chlorophyll a fluorescence

Salinities recorded to site 1, site 2 and control site, shown in Table 1. Salinities in site at 10 m and at 30 m from the discharge were 2.38 and 1.5 psu higher, respectively, compared to that in the control site.

The electron transport rate (ETR) was analysed as a productivity indicator in *Ectocarpus* samples exposed to the brine effluent (Fig. 2). ETR at day 3 decreased considerably in samples located at 10 m and 30 m from the brine discharge pipe compared to control samples (Fig. 2A). After 7 days, the results showed an increase to values of nearly 20 μmol e^{-} m^{-2} s^{-1} in the control site, whereas in site at 10 m from the brine outfall these were < 3 μmol e^{-} m^{-2} s^{-1}(Fig. 2B); the latter, still higher than the same measurement at day 3 (Fig. 2A).

The maximal quantum yield (*Fv/Fm*) decreased significantly (compared to controls) in *Ectocarpus* transplants exposed to brines (10 and 30 m), although with no differences with each other (Fig. 3A). At 7 days, transplants at 10 m presented significantly lower levels of *Fv/Fm* relative to controls at the same time and at 3 days of experiments (Fig. 3A). Patterns in α_{ETR} were similar to those observed for *Fv/Fm*; however, no differences were detected between controls and transplants at 30 m at 3 days of experiments (Fig. 3B). Finally, trends in ETR_{max} were comparable to those in α_{ETR}, although controls at day 7 were significantly higher than at the same site at day 3 (Fig. 3C).

3.2. Quantification of oxidative stress and damage parameters

To understand how brine discharge impacted in *Ectocarpus* oxidative stress metabolism, we analysed the accumulation of H_2O_2 as an approach to extrapolate reactive oxygen species (ROS) generation, and TBARS as a proxy for lipid oxidative damage in the samples installed nearby a desalination discharge (Fig. 4). Concentrations of H_2O_2 at 10 m after 7 days of experiments were significantly higher than in controls at both 3 and 7 days; also, the latter did not present significant differences with each other (Fig. 4A). When analysing the TBARS content at 3 days, these significantly increased only in transplants located at 30 m from the discharge, compared to controls and at 10 m from the brine outfall (Fig. 4B). At 7 days, TBARS increased significantly in controls compared to the same site at 3 days and at 10 m from the discharge at day 7; these last two sites did not show significant differences (Fig. 4B).

3.3. Quantification of antioxidant molecules

In order to elucidate the *Ectocarpus* potential strategies to face osmotic stress triggered by augmented salinities due to a brine effluent, we measured the content of two relevant antioxidant molecules present in photoautotrophs: glutathione and ascorbate, in both reduced and oxidized forms (Fig. 5). After 3 days of transplantation experiments, total glutathione decreased significantly compared to controls at 10 m, and increased significantly in samples at 30 m (Fig. 5A). No differences were detected in total glutathione between controls and 10 m samples at days 3 and 7 (Fig. 5A). Levels of GSSG were significantly higher in relation to GSH in all experimental sites and times, with over 70% of total glutathione as GSSG; the exception was at 30 m in day 3, although concentrations of GSSG were still significantly higher than GSH (Fig. 5A).

For total ascorbate levels at 3 days, there was a significant increase in *Ectocarpus* transplanted at 10 m of the brine discharge compared to controls and 30 m samples, both of which did not display differences in this molecule content (Fig. 5B). At 7 days, total ascorbate decreased in controls compared to the same site at day 3; at 10 m, total ascorbate increased showing no significant differences with controls at day 3 (Fig. 5B). Regarding ASC/DHA relations, there was a marked trend denoting higher values for DHA over ASC at all sites and times (Fig. 5B). However, while DHA content was ~70% of total ascorbate in control treatments at both days, in 10 m transplants DHA content was always above 90%.

Table 1
Transplant sites location, salinities and temperatures.

Sites	Location	Distance from brine discharge pipe	Salinity (psu)	Temperature (°C)
Site 1	La Chimba, Antofagasta	10 m	36.79 ± 0.06	16.01 ± 1.16
Site 2	La Chimba, Antofagasta	30 m	35.91 ± 0.07	15.99 ± 0.42
Control site	Bolsico Bay, ISM sector	Not applies	34.41 ± 0.05	15.81 ± 0.83

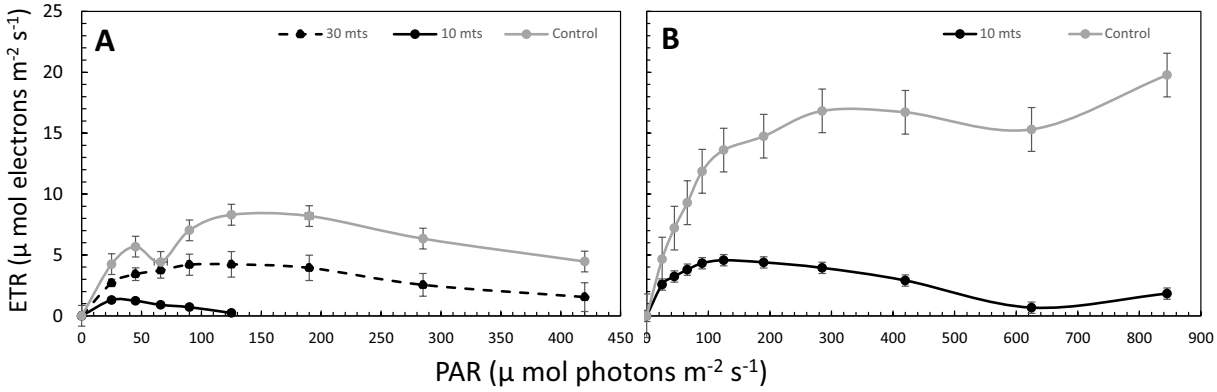

Fig. 2. Electron transport rate in *Ectocarpus* samples transplanted nearby brine discharges from a desalination plant. Electron transport rate obtained after 3 (A) and 7 (B) days of brine exposure at differences distances from the discharge pipe: 10 m (black line), 30 m (dashed line), and control site (grey line).

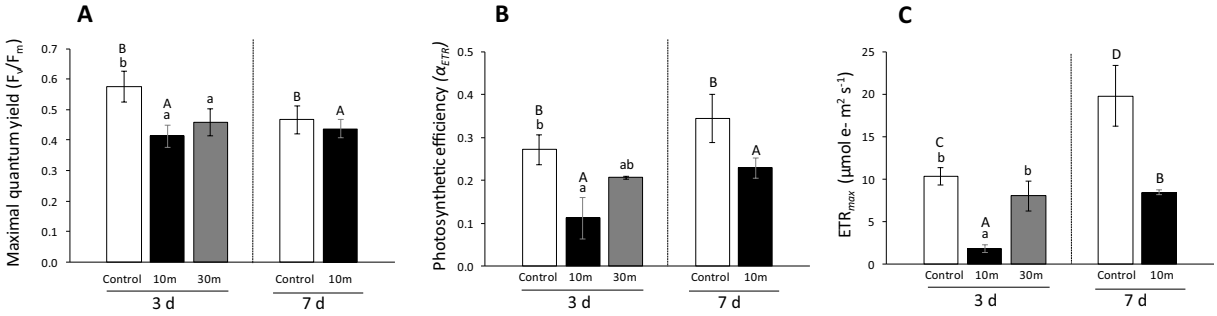

Fig. 3. Physiological responses as Maximal quantum yield (F_v/F_m), Photosynthetic efficiency (α_{ETR}) and Maximal electron transport rate (ETR_{max}) in *Ectocarpus* samples transplanted nearby brine discharges from a desalination plant. F_v/F_m (A), α_{ETR} (B) and ETR_{max} (C) were measured in *Ectocarpus* samples at two distances (10 m and 30 m) from the brine discharge pipe of a desalination plant located in Antofagasta, Chile, at 3 and 7 days. Plots are shown as means ± SE, $n = 3$. Lowercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and different distances from the brine discharge after 3 days (left panel). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and site at 10 m from the brine discharge at 7 days of experiments.

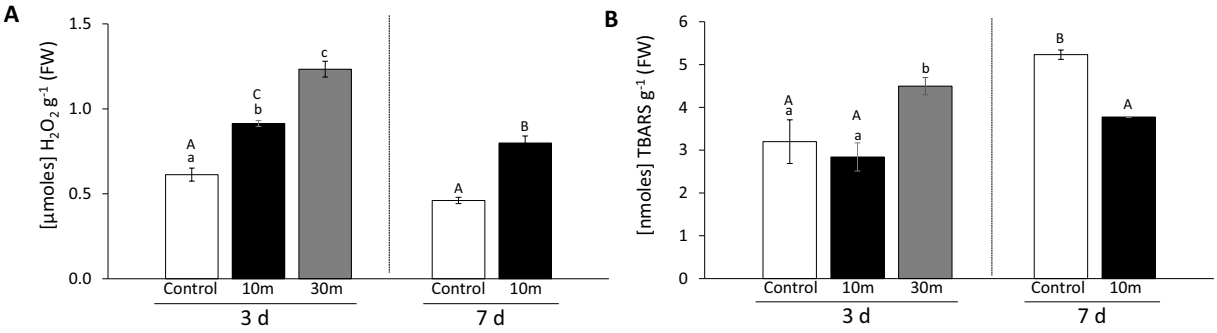

Fig. 4. Quantification of H_2O_2 and TBARS in *Ectocarpus* samples transplanted nearby brine discharges from a desalination plant. Accumulation of H_2O_2 (A) and TBARS (B) were measured in *Ectocarpus* samples at two distances (10 m and 30 m) from the brine discharge of a desalination plant located in Antofagasta, Chile, at 3 and 7 days. Plots are shown as means ± SE, $n = 3$. Lowercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and different distances from the brine discharge after 3 days (left panel). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and site at 10 m from the brine discharge at 7 days of experiments.

3.4. Tolerance-response genes expression profiles

Expression of candidate genes related to hypersalinity and oxidative stress were analysed in order to understand the molecular response to brine exposure in transplants of *Ectocarpus* (Fig. 6). The salt overlay sensitive 2 encoding gene (*SOS2*), related to hypersalinity stress response in photoautotrophs, showed an up-regulation at both sites exposed to the brine effluent after 3 days, compared to controls (Fig. 6A). At 7 days, relative expression values at 10 m did no present significant differences with controls at either 3 or 7 days (Fig. 6A). Regarding the expression of genes associated with oxidative stress response *SOD*, *APX*, *PRX* and *GR*, there was clear pattern displaying a significant up-regulation of all these genes after 3 days at 10 m from the brine discharge (Fig. 6B,C,D,E). Conversely, at day 3 samples located at 30 m from discharge pipe did not exhibit significant changes in the expression compared to the controls (Fig. 6B,C,D,E). At 7 days, most antioxidant enzyme-encoding genes showed higher expression compared controls at 3 and 7 days, although less up-regulated than at 10 m in day 3; the exception was *GR*, with no significant differences with controls at both experimental times (Fig. 6B,C,D,E).

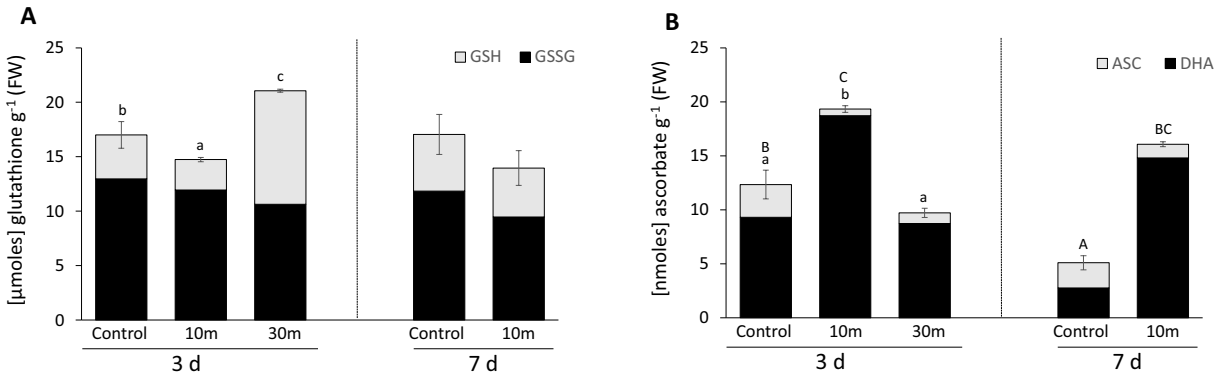

Fig. 5. Quantification of total glutathione and total ascorbate in *Ectocarpus* samples transplanted nearby brine discharges from a desalination plant. Accumulation of total glutathione (A) and ascorbate (B) in their reduced (GSH and ASC) and oxidized (GSSG and DHA) forms were measured in *Ectocarpus* samples at two distances (10 m and 30 m) from the brine discharge of a desalination plant located in Antofagasta, Chile, at 3 and 7 days. Plots are shown as means ± SE, $n = 3$. Lowercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and different distances from the brine discharge after 3 days (left panel). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and site at 10 m from the brine discharge at 7 days of experiments.

4. Discussion

Studies, protocols and strategies to monitor the biological consequences of desalination brine discharges are still not fully optimized to be reliably incorporated into environmental monitoring programmes. Nevertheless, most of those are limited to survival rates, growth, and effects on community structure. To the extent of our knowledge, this is the first study encompassing physiological, metabolic and molecular aspects in response to direct exposition of brine effluents from a desalination plant, in this case on a macroalga species.

After brine exposition of transplanted *Ectocarpus,* we found that the electron transport rate (ETR), which reflects gross PSII photosynthesis, was severely impaired, especially in transplants at 10 m from the brine discharge at both 3 and 7 days. This suggest a possible disruption in the chloroplast electron transport chain, inducing ROS over-production (as evidenced in our H_2O_2 measurements) [37]. For instance, it has been registered for the unicellular freshwater green microalga *Scenedesmus obliquus* that different salt concentration (from 0 to 25 psu of NaCl, control salinity 0 psu) generated a decrease in the ETR after 4 days [38]. Similarly, in the green microalga *Chlamydomonas reinhardtii* a significant reduction on the ETR was observed after 100 mM and 300 mM NaCl treatment (~6 and ~18 psu, respectively, control salinity 0.02 psu) after 24 h [39]. In our study, we also found that *Fv/Fm*, α_{ETR}, and ETR_{max} were decreased in *Ectocarpus* samples at both sites tested nearby the brine discharge, at 3 and 7 days, suggesting a brine-derived damage to the antenna complex. This process has been shown to induce impaired photosynthetic efficiency and capacity in macroalgae [40,41]. In contrast to our results, in the seagrass *P. oceanica, Fv/Fm* and α_{ETR} were not altered at 39, 41 and 43 psu (control salinity 37 psu) during one week of mesocosms experiments [42], and not even after three months exposed to 43 psu [12]. On the other hand, hypersalinity treatments using brine directly (54 psu) for two weeks in *P. australis*, showed a significant decrease in *Fv/Fm*, α_{ETR}, and ETR_{max} when compared to control salinity of 37 psu, although this responses were not observed when using diluted brine at 42 and 46 psu [43]. In accordance with our results, Zheng et al. [44] showed that photosynthetic oxygen

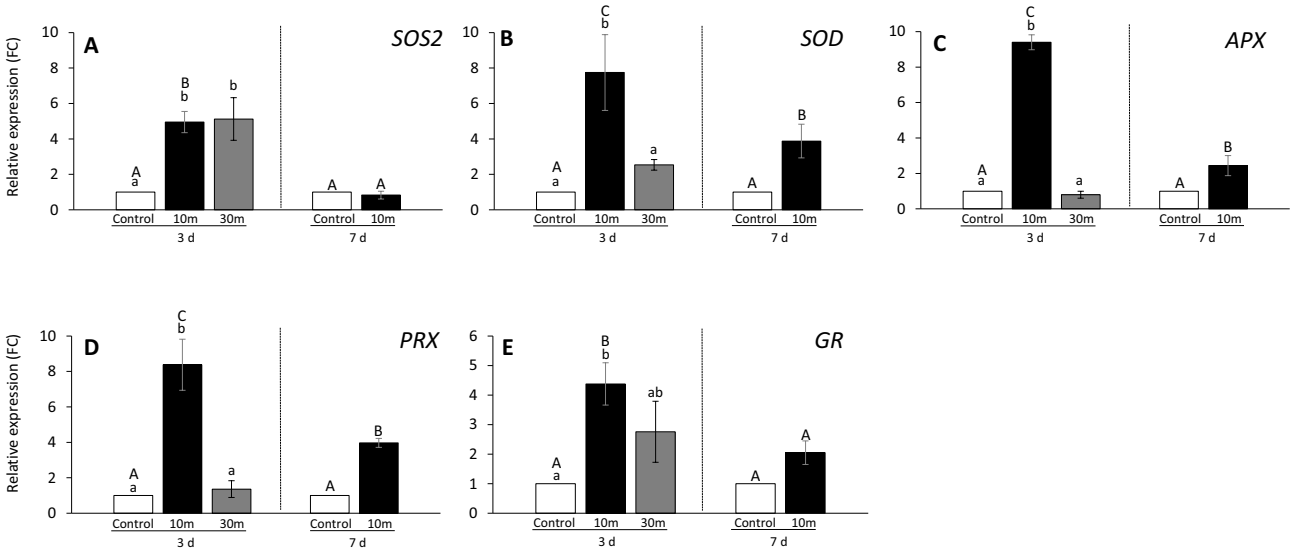

Fig. 6. Relative expression of hypersalinity and oxidative stress-related genes in *Ectocarpus* samples transplanted nearby brine discharges from a desalination plant. Relative expression of *SOS2* (A), *SOD* (B), *APX* (C), *PRX* (D) and *GR* (E) were measured in *Ectocarpus* samples at two distances (10 m and 30 m) from the brine discharge of a desalination plant located in Antofagasta, Chile, at 3 and 7 days. Plots are shown as means ± SE, $n = 3$. Lowercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and different distances from the brine discharge after 3 days (left panel). Uppercase letters represent significant differences at 95% confidence interval ($p < .05$) between controls and site at 10 m from the brine discharge at 7 days of experiments.

evolution rate, another indicator of antenna complex state, decreased under 35 psu (control salinity 25 psu) in green macroalga *Ulva prolifera* after 6 days.

It is well established and characterized that salt excess can lead to oxidative stress and damage to several macromolecules in plants. Principal reasons include cell dehydration and uncontrolled uptake of Na^+, which in turn, disrupt several metabolic pathways that require H_2O molecules, and changes in ionic state of most enzymes, respectively (e.g. [45,46]). We demonstrated that H_2O_2 was over-accumulated at 3 and 7 days in *Ectocarpus* transplanted at both 10 and 30 m from the brine discharge, suggesting the induction of an oxidative stress state. Interestingly, H_2O_2 content was higher in samples of site at 30 m than at 10 m from the brine discharge, suggesting that different saline concentration thresholds are necessary to activate ROS detoxification systems. These systems are regulated by specific ROS amounts, which in turn activate tolerance mechanisms [47]. Indeed, 10 m samples did not showed an increase in TBARS content, suggesting that efficient tolerance mechanisms were activated, such as high ascorbate content and up-regulation of genes related to oxidative stress as evidenced in our results. H_2O_2 excess can directly catalyse the Fenton reaction, producing the highly oxidising hydroxyl radicals, which rapidly target lipids. Our results on lipid peroxidation demonstrated considerable levels of oxidative damage in *Ectocarpus* transplanted at 30 m from the brine outfall at 3 days of experiments, likely due to insufficient activation of ROS detoxification systems. Similarly, to our results, TBARS content within the thallus in *U. fasciata* was higher when treated with ~60 psu for 4 days [48]. For instance, exposure to ~60 psu for 6 days (control salinity ~30 psu) drastically increases H_2O_2 levels in *U. prolifera*, which was also correlated with high levels of lipid peroxidation [49]. A recent investigation in the microalga *Dunaliella salina* evidenced that hypersaline stress beyond 2 M of NaCl (~117 psu, control salinity ~15 psu) until exponential growth phase, was accompanied by an increase in intracellular total ROS content [50].

To counteract oxidative damage, photoautotrophs have evolved developing a large battery of antioxidant molecules, such as glutathione and ascorbate, which interact closely in the Foyer-Halliwell-Asada cycle [51]. Dissimilar patterns were observed between total glutathione and ascorbate at 3 days of transplantation experiments, trend that was maintained after 7 days in 10 m samples. While the highest total glutathione was recorded in 30 m samples, in the case of total ascorbate this was observed at 10 m from the brine outfall. The latter could be explained by a distinct salinity-dependent activation of de novo synthesis and recycling of these metabolites [46]. Although different magnitudes of salt concentration were used by Lu et al. [48], similar responses were registered in *U. fasciata* after 24 h of hypersalinity treatments of ~60 and ~90 psu (control salinity ~30 psu), with increased and decreased content of total glutathione and ascorbate, respectively. Furthermore, similarly to *Ectocarpus* transplants at 30 m from the discharge, high salinity conditions (~60 psu for 6 days) triggers a rapid accumulation of total glutathione but not of ascorbate pools in *U. prolifera* [49]. These responses are also accompanied by an increase in the activities of enzymes that occupy these molecules as substrates to neutralize ROS, such as GR and APX, indirectly suggesting changing rates in GSH/GSSG and ASC/DHA ratios [48,49]. Indeed, the predominance of the oxidized forms of glutathione and ascorbate, was a marked trend in all the samples at all treatments, supporting an oxidative stress scenario. Although, there are no studies accounting for GSH/GSSG and ASC/DHA under hypersaline treatments in macroalgae, other abiotic stresses, such as copper excess, have revealed severe changes in these antioxidant molecules ratios in *Ectocarpus*, through laboratory and field assessments [25,26], where accumulation of oxidized forms of glutathione and ascorbate were the major tendency.

In terms of ecotoxicological and environmental diagnosis applications, differential expression of certain stress-related genes have considered to be useful biomarkers for the screening the state impacts of chemical and pollutants during ecological risk assessments [25,52]. In our study, we observed that *SOS2*, involved in signalling under salt stress, was up-regulated in *Ectocarpus* transplants at 10 and 30 m from the brine discharge, but only at 3 days of experiments, suggesting the time-frame specificity of *SOS2* in an early induction for the activation of the hypersalinity-tolerance response in *Ectocarpus*. There are no reports evaluating the expression of the *SOS* genes in aquatic photoautotroph. However, studies in *Arabidopsis thaliana* demonstrated that *SOS1* was overexpressed under 100 mM NaCl (~ 8 psu, control salinity ~1.5 psu) for three weeks [53]. Also, *SOS1*, *SOS2* and *SOS3* showed up-regulation in different tissues of *A. thaliana* roots when exposed for 5 days to 140 mM NaCl (~ 8 psu, control salinity ~1.5 psu) [54]. Regarding the expression of oxidative stress enzymes, there was a notorious up-regulation of all transcripts in *Ectocarpus* transplanted at 10 m from the brine discharge, pattern observed at 3 and 7 days of experiments, which is also in agreement with all oxidative stress, damage and antioxidant responses recorded as part of this investigation. Although, transcripts levels in samples located at 10 m from the brine discharge were maintained high in comparison with control samples, at 7 days they decreased significantly in almost all cases, suggesting the beginning of an acclimation process, where translated enzymes content were enough to counteract oxidative stress or that other set of genes are necessary for the late tolerance response. Interestingly, transcript levels at 30 m *Ectocarpus* did not demonstrate changes in expression profile patterns, supporting the hypothesis of the need to reach certain ROS stress thresholds to induce the expression of these genes. In *U. fasciata*, an increase in the transcripts *SOD*, *APX* and *GR* was evidenced upon hypersalinity stress (~ 90 psu, control salinity 30 psu) after 3 h [55]. Likewise, *SOD* and *APX* were overexpressed in *P. yezoensis* after high salt stress (~6 psu, control salinity ~0.6 psu) for 12 h [56]. Other study demonstrated that transcription of *PRX1* and *PRX2*, from the halotolerant alga *Dunaliella viridis*, can be differentially up-regulated after hypersalinity shock of 3 M of NaCl (~175 psu, control salinity ~58 psu) after 48 h and 6 h, respectively [57]. Encompassing all our results, it is evident that an average salinity of ~37 psu at 10 m from the brine discharge pipe is enough to activate transcriptional machinery of antioxidant enzymes in *Ectocarpus*.

5. Conclusions

In this study, we demonstrated for the first time that short-term brine exposure from desalination industry can provoke detrimental effects at the ecophysiological and cellular level, principally in terms of hypersalinity and oxidative stress, using transplants of a macroalga species. In this sense, we suggest that the best physiological and molecular biomarkers for *Ectocarpus* transplants include: photosynthetic parameters such as *Fv/Fm*, α_{ETR}, and ETR_{max}; oxidative stress parameters as H_2O_2 and glutathione and ascorbate content (and redox status); and gene expression profiles of genes related to oxidative and salt stress. In this regard, *Ectocarpus* transplants nearby a brine discharge can display physiological metabolic responses that highlight the biological effects of salinity excess. This approach not only contribute to understand mechanisms of tolerance to hypersalinity and brine discharges in macroalgae, but also highlight the *Ectocarpus* transplantation protocol and measurements as valuable tools for environmental biotechnology to address diagnosis, prevention and monitoring of the state impacts of desalination.

Credit Author Statement

Fernanda Rodríguez-Rojas: Conceptualization, Investigation, methodology, Formal analysis, Writing - original draft, Visualization, Funding acquisition. Américo López: Investigation, Methodology, Formal analysis. Paula Celis-Plá: Investigation, Methodology, Formal analysis. Pamela Muñoz: Investigation, Methodology. Enzo García-Bartolomei: Investigation, Writing - review & editing. Fernando Valenzuela: Investigation. Rodrigo Orrego: Investigation, Writing - review & editing. Adoración Carratalá: Investigation, Writing - review & editing. José Luis Sánchez-Lizaso: Conceptualization, Formal analysis,

Methodology, Investigation, Resources, Writing - review & editing. Claudio Sáez: Conceptualization, Investigation, Methodology, Visualization, Writing - review & editing, Supervision, Funding acquisition.

CRediT authorship contribution statement

Fernanda Rodríguez-Rojas:Conceptualization, Investigation, Methodology, Formal analysis, Writing - original draft.**Américo López-Marras**:Investigation, Methodology, Formal analysis.**Paula S.M. Celis-Plá**:Investigation, Methodology, Formal analysis.**Pamela Muñoz**:Investigation, Methodology.**Enzo García-Bartolomei**:Investigation, Writing - review & editing.**Fernando Valenzuela**:Investigation.**Rodrigo Orrego**:Investigation, Writing - review & editing.**Adoración Carratalá**:Investigation, Writing - review & editing.**José Luis Sánchez-Lizaso**:Conceptualization, Formal analysis, Methodology, Investigation, Resources, Writing - review & editing.**Claudio A. Sáez**:Conceptualization, Investigation, Methodology, Visualization, Writing - review & editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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