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A risk assessment on *Zostera chilensis*, the last relict of marine angiosperms in the South-East Pacific Ocean, due to the development of the desalination industry in Chile

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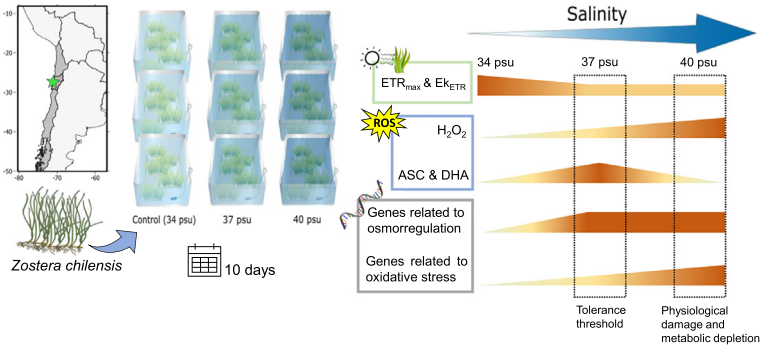
HIGHLIGHTS

- *Zostera chilensis* is a relict seagrass species in the South American Pacific
- Hypersalinity triggers Reactive Oxygen Species production and antioxidant consumption
- Photosystem II electron transport rate and saturation irradiance decreased at higher salinities
- Hypersaline water activates the expression of genes related to osmotic adjustment and mainly of enzymes linked to antioxidant response
- This endemic species might be negatively affected under the influence of a desalination brine discharge

GRAPHICAL ABSTRACT



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ABSTRACT

Seagrasses, which are considered among the most ecologically valuable and endangered coastal ecosystems, have a narrowly limited distribution in the south-east Pacific, where *Zostera chilensis* is the only remaining relict. Due to water scarcity, desalination industry has grown in the last decades in the central-north coasts of Chile, which may be relevant to address in terms of potential impacts on benthic communities due to their associated high-salinity brine discharges to subtidal ecosystems. In this work, we assessed ecophysiological and cellular responses to desalination-extrapolable hypersalinity conditions on *Z. chilensis*. Mesocosms experiments were performed for 10 days, where plants were exposed to 3 different salinity treatments: 34 psu (control), 37 psu and 40 psu. Photosynthetic performance, H_2O_2 accumulation, and ascorbate content (reduced and oxidized) were measured, as well as relative gene expression of enzymes related to osmotic regulation and oxidative stress; these, at 1, 3, 6 and 10 days. *Z. chilensis* showed a decrease in photosynthetic parameters such as electron transport rate (ETR_{max}) and saturation irradiance (Ek_{ETR}) under hypersalinity treatments, while non-photochemical quenching (NPQ_{max}) presented an initial increment and a subsequent decline at 40 psu. H_2O_2 levels increased with hypersalinity, while ascorbate and dehydroascorbate only increased under 37 psu, although decreased along the experimental period. Increased salinities also triggered the expression of genes related to ion transport and osmolyte syntheses, but salinity-dependent up-

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regulated genes were mostly those related to the reactive oxygen species metabolism. The relict seagrass *Z. chilensis* has shown to withstand increased salinities that may be extrapolable to desalination effects in the short-term. As the latter is not fully clear in the long-term, and considering the restricted distribution and ecological importance, direct brine discharges to *Z. chilensis* meadows may not be recommended.

1. Introduction

Marine angiosperms are rare along the South American Pacific, being Chile the only country with reported seagrass populations. That is the case of *Zostera chilensis* (J. Kuo) S.W.L. Jacobs & D.H. Les, an endemic seagrass from Chile, and the only marine angiosperm species of the South-East Pacific described to date (Kuo and den Hartog, 2007; Short et al., 2007). This species have been suggested to be closely related to other Australian seagrasses from the Zosteraceae family (Smith et al., 2018), and grouped within the Temperate Southern Oceans bioregion (Short et al., 2007). However, there is still no complete agreement, mostly due to the scarce and shallowness of the available studies. In this context, *Z. chilensis* was firstly thought to belong to be a cryptic species of *Heterozostera tasmanica* (González and Edding, 1990; Phillips et al., 1983), although a more recent genetic study has linked the Chilean seagrass closer to *Zostera nigricaulis* (Coyer et al., 2013). In spite of the available records, there is still no certainty of the origin of this species. Therefore, and as recognized in the majority of studies in the species (Guiry and Guiry, 2019; Jacobs and Les, 2009), now forward in this article the species will be referred as the endemic *Z. chilensis*. The reason of *Z. chilensis* present distribution is currently unknown. It could be associated with seagrass fragments originated in the western Pacific (Oceania and the Pacific Islands) that may drifted to the American coasts (Smith et al., 2018; Stafford-Bell et al., 2015), or even as remains of seagrass meadows that may have extended along the eastern Pacific (Vélez-Juarbe, 2014). In any case, its current known distribution is restricted to 3 enclosed, protected and shallow bays between the 27°S and 30.3°S latitudes (Sandoval and Edding, 2015), probably due to the absence of more suitable habitats and their competition with the locally dominant kelps and other macroalgae (La Nafie et al., 2012). Despite of its limited extension, *Z. chilensis* plays a relevant ecological role as bioengineering species; as well as a food source for the most southern Pacific population of the green sea turtle *Chelonia mydas* (Álvarez-Varas et al., 2017). However, the restricted distribution of *Z. chilensis* and, the current and future potential human activities nearby their populations, demand to expand our knowledge upon its responses to environmental stress. Indeed, the species has been included in the UICN Red list as an endangered species (Short and Waycott, 2010). Beyond these conservational aspects, the unique biological features highlight *Z. chilensis* as a key species to understand evolutive basis that brought plants to the sea.

Water scarcity is currently affecting more than the 50 % of world's population (Huang et al., 2021; Kummu et al., 2016), and desalination has become one of the most feasible options to cope with the increasing freshwater demand (Eke et al., 2020; Jones et al., 2019). In this context, seawater desalination is one of the main growing industries worldwide, which is also predicted steadily expand as a consequence of global Climate Change, especially in semi-arid and Mediterranean latitudes (Jones et al., 2019; Stanhill et al., 2015). In Chile, water from desalination has increased from 1 to 24 SWRO plants from 1997 to 2018, i.e. from 0.05 to 1.57 hm^3 of discharged brine per day (Sola et al., 2019), and is even believed to duplicate in the next decade (Herrera-León et al., 2021). The main environmental issue desalination operations is associated with its residue, which in direct seawater intake plants, consists in a high salinity brine that is usually directly discharged to the subtidal area; this brine can even duplicate the salinity of the origin desalinated seawater (Panagopoulos and Haralambous, 2020).

The effects of desalination brines on the marine environment has been assessed for many different ecosystems, and its potential detrimental effects have been mostly associated with the osmotic stress induced by the excess salinity (Ihsanullah et al., 2021; Petersen et al., 2018). In this regard, certain seagrasses have proven to be negatively affected by direct brine discharges; indeed, they have been proposed as bioindicators to follow the extent of brine impact in environmental monitoring programs (EMPs) (Cambridge et al., 2017; Sánchez-Lizaso et al., 2008). Most effects of brine-mediated increased salinities on seagrass species have been well studied at the morphological and physiological level, revealing growth reduction, osmolyte accumulation, changes in osmotic potential, or alterations in photosynthetic performance (Fernández-Torquemada and Sánchez-Lizaso, 2006; Marín-Guirao et al., 2013a), but few studies have been carried out focusing on the metabolic or molecular response of seagrasses to increased salinities (Sandoval-Gil et al., 2022). However, previous studies have already provided lights of increments in cellular reactive oxygen species (ROS) and changes the expression of genes related to ion transport in seagrasses under hypersalinity (Capó et al., 2020; Piro et al., 2015).

Beyond the baseline restricted information on *Z. chilensis*, the available records also highlight the little knowledge on molecular and biochemical mechanisms of seagrasses overall to cope with excess salinities, especially those that could be associated with brine discharges. Furthermore, a desalination plant project pending authorisation in Bahía Chascos, where the northernmost described *Z. chilensis* meadow is located (SEIA, 2022).

Thus, given the ecological relevance and potential vulnerability of *Z. chilensis*, the negative effects of brine discharges observed in certain seagrasses, and the predicted increment of this water source in the future (even nearby their ecosystems), it is necessary to further understand its responses and potential prevalence under excess salinities.

To this end, the aim of this investigation was to address for the first time physiological and metabolic responses to hypersalinity were studied in *Z. chilensis*, with a special emphasis of potential consequences of the desalination industry growth nearby the species ecosystems. The purposes of this novel research were: (1) determining physiological, biochemical and molecular mechanisms of this seagrass to eventually cope with extrapolable-desalination brine salinities; (2) identify a hypersalinity tolerance thresholds and, moreover, (3) discern certain responses with potential to be used as environmental biotechnology tools to evaluate the impacts of brine discharges through *Z. chilensis* in behalf of the conservation of this endemic species.

2. Materials and methods

2.1. Sampling and experimental design

Zostera chilensis individuals were collected by SCUBA divers at 1.5 meters depth from Bahía Chascos in the Atacama Region (Fig. 1), and immediately transported (within 12 h) in darkness with aeration until reaching the laboratory. For acclimatation to experimental conditions, individuals (3–5) were distributed in 9 aquaria with prefiltered seawater (Whatman GF/F glass fibre filter, 0.7 μm) for 3 days at 15 °C, 34 psu and 12:12 h photoperiod at 100 ± 10 μmol quanta m^{-2} s^{-1} (Fig. 2).

After the acclimatation period, salinity was increased with artificial salts (Instant Ocean ®) stablishing 3 treatments with 3 replicates each for up to 10 days: control (34 Practical Salinity Units, psu), intermediate salinity (37 psu), and high salinity (40 psu). Experimental salinities were set upon known increments that desalination brine discharges can cause in the nearest water body; these, do not usually exceed 6 psu upon natural salinities of the receiving seawater body (e.g. Gacia et al., 2007; Muñoz et al., 2020; Muñoz et al., 2023a; Muñoz et al., 2023b; Portillo et al., 2014).

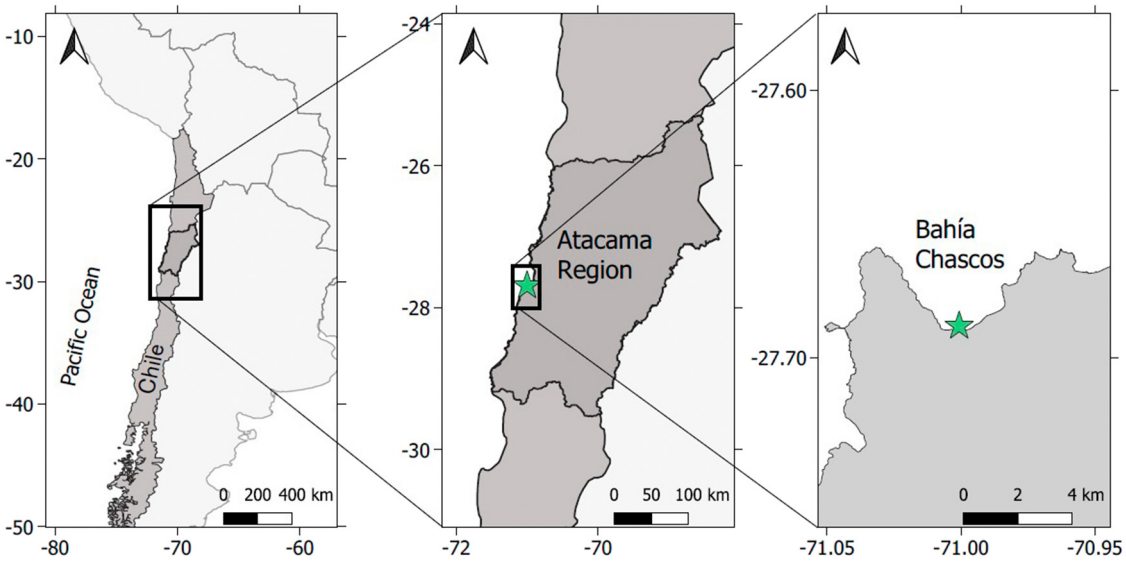

Fig. 1. Map representing *Z. chilensis* sampling location in Bahía Chascos (Atacama Region, Chile). Coordinates are shown in decimal degrees (RCS: WGS84).

Also, to determine the effect at different timescales 4 sampling times were established: 1, 3, 6 and 10 days after the start of the experiments. Water renovation took place after each sampling time, and aquariums were installed with constant aeration and filtration. In each time, photosynthetic performance was assessed, and leaf biomass from every replicate was sampled and rapidly frozen in liquid nitrogen and stored at −80 °C for further biochemical and molecular analyses.

2.2. Photosynthetic performance measures

A Junior PAM fluorometer (blue light) and WinControl-3 software (Walz GmbH, Effeltrich, Germany) were used to assess the photosystem II (PSII) chlorophyll *a* fluorescence. Three *Z. chilensis* shoots were sampled from each treatment and time, in order to measure direct photosynthetic responses. Leaves were stored in dark chambers for 15 min to obtain rapid light curves (RLC) from mid-way along each one of them. and its photosynthetic performance was measured following the protocol developed by Celis-Plá et al. (2014).

Direct fluorescence allowed the calculation of the effective quantum yield (*Df/Fm′*), as the photosystem II chlorophyll *a* fluorescence in the presence of light, where *Fm′* is the maximum fluorescence in the presence of light, *Ft* is the instantaneous fluorescence before application of a saturation pulse and $Df = Fm'\text{-}Ft$ (Figueroa et al., 2014). RLC measured the fluorescence response of the plant when exposed to 12 incremental irradiances of white light (E1 = 25, E2 = 45, E3 = 66, E4 = 90, E5 = 125, E6 = 190, E7 = 285, E8 = 420, E9 = 625, E10 = 845, E11 = 1150, and E12 = 1500 μmol $m^{-2}{\cdot}s^{-1}$), covering the range of harvestable irradiances of seagrasses (Ralph and Gademann, 2005). These curves allow the determination of PSII electron transport saturation and performance after being completely oxidized. Knowing the maximal (F_m) and minimal (F_o) fluorescence, and its difference ($F_v = F_m - F_o$), maximum quantum yield (F_v/F_m) and was calculated for each replicate (Schreiber et al., 1995). Maximal electron transport rate (ETR_{max}), an indicator of maximal photosynthetic capacity, and the photosynthetic efficiency (αETR), were obtained from the Eilers and Peeters' tangential model function (Eilers and Peeters, 1988), which has been already used for the measure of photochemical performance in seagrasses (Pazzaglia et al., 2020). The saturating irradiance (Ek_{ETR}) i.e., the irradiance at which ETR_{max} was saturated was calculated from the intercept between ETR_{max} and α_{ETR}. The Non-photochemical quenching (NPQ_{max}) is a photoprotection indicator related with energy dissipation. This parameter was calculated according to Schreiber et al. (1995).

2.3. Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) was measured following the protocol developed by Sáez et al. (2015). Twenty mg of fresh weight (FW) biomass were grounded using liquid nitrogen and mixed with 100 μL of 10 %

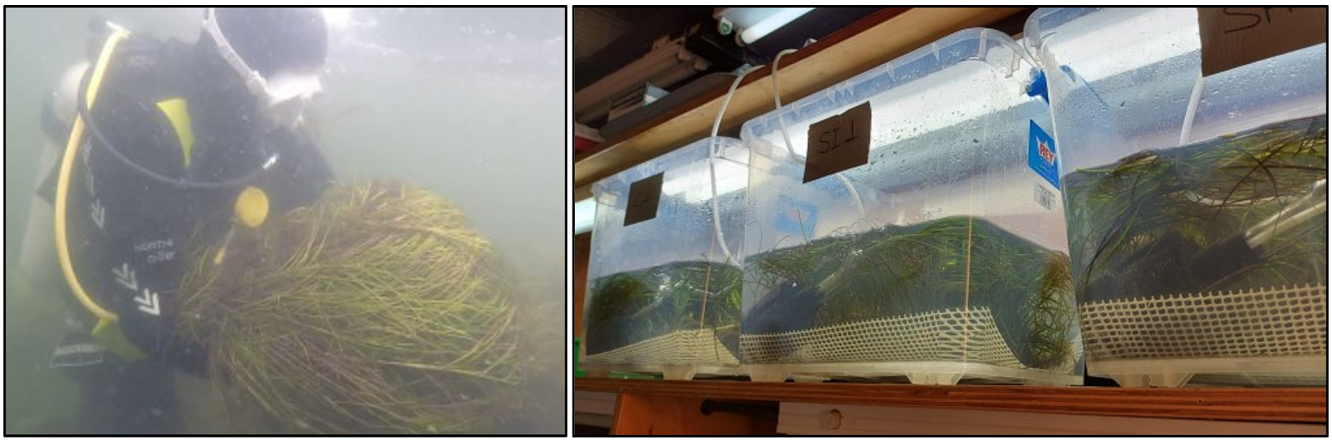

Fig. 2. *Z. chilensis* collection (left) and mesocosm installation (right).

trichloroacetic acid (TCA),100 μL of 10 mM potassium phosphate buffer (pH 7.0), 200 μL of HCl 0.5 M, and 500 μL of 1 M potassium iodide. After vortexing the samples using glass beads (3 mm) in the darkness for 10 min, samples were centrifuged for 15 min at 12,000 ×*g* at 4 °C. Three hundred μL of supernatant were placed in a microplate reader (SpectroStar Nano, BMG LABTECH), and absorbance was measured at 390 nm. Commercial H_2O_2 (Sigma Aldrich Merck, St Louis, MO, USA) was used for standard curves.

2.4. Ascorbate determination

Ascorbate levels were measured in its forms reduced (ASC) and oxidized, also called dehydroascorbate (DHA), based on the methodology proposed by Benzie and Strain (1999). Twenty mg of FW biomass were grounded in a mortar with liquid nitrogen, and 1.2 mL 0.1 M HCl was added before vortexing the sample for 10 min. Mixed samples were centrifuged at 21,000 ×*g* for 10 min at 4 °C. Ten μL of the supernatant were added to 290 μL of tripyridyl triazine (Fe III TPTZ). The absorbance at 593 nm was measured immediately after adding Fe III TPTZ (SpectroStar Nano, BMG LABTECH). For total ascorbate measurements, 300 μL of extract were incubated in the presence of 5 μL of 100 mM dithiothreitol for 1 h. After this time, 5 μL (w/v) of N-ethylmaleimide were added to stop the reaction. The subtraction between total ascorbate and its oxidized form provided details on ASC and DHA levels. L-ASC (Sigma Aldrich Merck, St Louis, MO, USA) was used for standard curves.

2.5. Relative gene expression analyses

Total RNA was extracted from 50 mg of grounded leaf biomass. Extractions were made using a FavorPrep™ Plant Total RNA Mini Kit (Favorgen Biotech Corp.). RNA purity and integrity were determined by 260/280 ratio and electrophoresis in 1.2 % agarose bleach gel (Aranda et al., 2012). For quantification, the Quant-iT RiboGreen RNA assay kit (Invitrogen, Waltham, MA, USA) was used; and measured with a QFX Fluorometer (DeNovix, Wilmington, DE, USA) for final determination of RNA concentrations. cDNA synthesis was carried out using the High-capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fischer Scientific), and adjusting the volume to 20 μL. For the quantitative PCR (qPCR) reactions, 50 ng of cDNA were used, 0.25 μM of each primer and the 1× of the Brilliant II SYBR Green qPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) also adjusted to a final volume of 20 μL. Designed primers sequences and coding proteins are shown in Table 1.

Table 1
List of genes of interest analysed in *Zostera chilensis* by using RT-qPCR.

Gene	Coding protein	Accession number (GeneBank)	Sequence 5′-3′
SOS1/NHX7	Salt overlay sensitive 1 Sodium/hydrogen exchanger 7	KMZ66737.1	CGAGACAGCTTCTTCGAGGGACACG TGTTCCAAGGCAAACGGTTGATGT
P5CR	Pyrroline-5-carboxylate reductase	KMZ57016.1	AGTGGAAGCGGCCCAGCATA GCAGCGGCACACACTGCATT
NHX1	Sodium/hydrogen exchanger 1	KMZ65914.1	GCTGGGTTCCAAGTCAAGAAG GCTGCAAAGATGGCTCCAATGGC
CAT	Catalase		TGCCGACACTCAACGGCATCGT TCGGCATGTCGAGCAGGATCA
Mn/Fe SOD	Mn/Fe Superoxide dismutase	KMZ72128.1	TCGGATCTGGATGGGTTTGGCT CGTGCAGTCGCTGAGTGCCATGA
Cu/Zn SOD	Cu/Zn Superoxide dismutase	KMZ60238.1	GCTGCATGTCCACTGGGCCACA AGCTCAACTCATGCCCACCCT
OC	Ornithine cyclodeaminase	KMZ65553.1	GCACCGCCGCGATATCTGCTCT GCTTCCGCCAGTTCACGGGCTTT
APX	L-ascorbate peroxidase	KR733075.1	CGCCTCGCGTGGCATTCAGC TCAGGCCCGCCGGTGATCTC
GR	Glutathione reductase	KMZ57694.1	GTGCATCACGATTCGCAGCA TGGCCATGAGAACCTCCCAAT
18S	Ribosomal RNA 18S	AY491942.1	GAGAAGGAAGCTGCTGAAATG GAACAGCACAATCAGCCTGAG

qPCRs were made using a MIC qPCR Magnetic Induction Cycler (Bio Molecular Systems, Queensland, Australia). The program configuration was: 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, ending with a final extension at 72 °C for 10 min.

Nine genes of interest were selected (Table 1) to study the molecular response of *Z. chilensis* to hypersalinity. These genes were chosen as candidates for hypersalinity stress indicators as they are involved in some of the main known responses of plants to hypersalinity: osmolyte synthesis, ion transport, and reactive oxygen metabolism (ROM) (Yang and Guo, 2018). Relative expression, or fold change (FC) analyses were based in the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). 18S rRNA was used as housekeeping gene based on the analysis developed by Serra et al. (2012), which showed 18S as the best reference gene under salinity changes according to BestKeeper applet.

2.6. Statistical analyses

Photosynthetic performance metrics were analysed by using a three-way analysis of variance (ANOVA). Both Treatment (34 psu, 37 psu and 40 psu) and Time (day 1, day 3, day 6, day 10) were analysed as fixed and orthogonal factors, and Aquarium (A1, A2, A3) as random factor, nested in Treatment.

$$X_{ijn} = \mu + Tr_i + Ti_j + Tr \times T_{ij} + A(Tr)_{k(i)} + A(Tr)_{k(i)} \times T_{ij} + e_{n(ijk)}$$

H_2O_2, ASC and DHA levels were analysed using a two-way ANOVA with Treatment and Time as fixed factors, and for gene FC the same analysis was performed but the Treatment factor had two levels: 37 psu and 40 psu.

Data normality and homoscedasticity were tested using a Kolmogorov-Smirnov and a Bartlett test, respectively (Underwood, 1997). When data homoscedasticity was not achieves, a significance level of 0.01 was used (see Supplementary data). To address the differences between levels of each factor when significant results were found in the ANOVA, a Tukey HSD test was used (Quinn and Keough, 2002). All analyses were conducted using R software (v3.7).

3. Results

3.1. Photosynthetic performance measures

Df/Fm′ showed no significant differences between days or treatments, and *Fv/Fm* displayed a significant decrease only at 10 days in all treatments (data not shown). Under salinity treatments, ETR_{max} presented significantly lower values in both 37 and 40 psu treatments (Fig. 3a) and a significant increase of this parameter was found on days 6 and 10. Regarding α_{ETR}, control plants presented non-significant lower values compared with both hypersalinity treatments except for day 3 (Fig. 3b). This trend was followed by the irradiance of saturation of ETR (Ek_{ETR}) where control plants presented significantly higher values compared to both hypersalinity treatments (Fig. 3c). This parameter also showed a decline on the 3rd day and a significant increment on days 6 and 10.

As energy dissipation indicator, NPQ_{max} showed that 40 psu treatment on the 3rd day presented significantly higher values, while the same treatment was significantly lower on day 10th (Fig. 3d).

3.2. Biochemical parameters

Hydrogen peroxide values were significantly higher in the 40 psu treatment compared to the control, with a mean increment of 91.1 %. 37 psu treatment showed intermediate values although no significant differences were found with control plants (Fig. 4). Regarding the effect of time, 3rd day showed higher values compared to days 1 and 6, while no differences were found when compared to the 10th day.

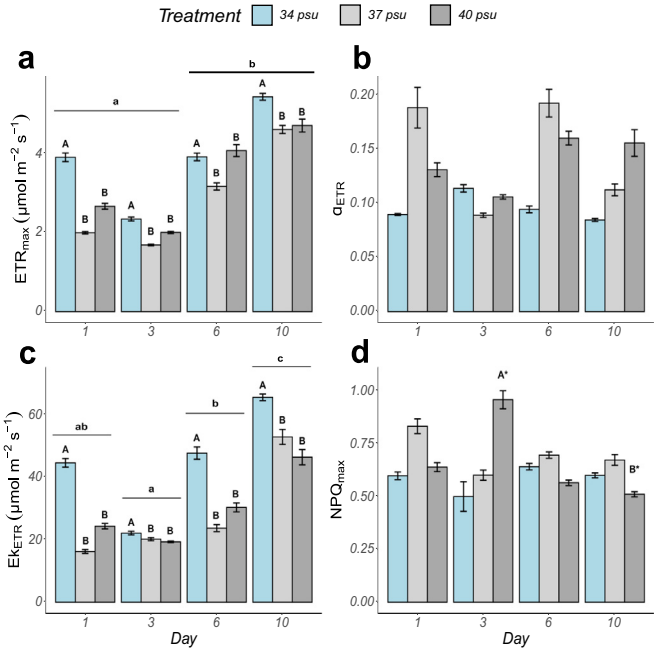

Fig. 3. Maximal electron transport rate (ETR_{max}), photosynthetic efficiency (α_{ETR}), saturation irradiance (Ek_{ETR}) and non-photochemical quenching (NPQ_{max}) in *Z. chilensis* samples under experiments at 34 (blue), 37 (light grey) and 40 (dark grey) psu for 10 days. Barplots represent the mean of each variable and error bars show the standard error. Upper case letters represent significant differences at 95 % confidence interval ($p < .05$) between treatments (34 psu, 37 psu, 40 psu). Lower case letter represents significant differences between days (1, 3, 6, 10). Asterisk (*) show significant differences of a group in the *post-hoc* test when factor interaction was significant.

Both ASC and DHA values were significantly higher in the 37 psu treatment compared to control plants. ASC presented significantly lower results on 10th day compared to the rest of days and DHA showed the same difference but also day 6 was significantly lower compared to day 1 (Fig. 5).

3.3. Relative gene expression

The expression of candidate genes related to oxidative stress and osmotic regulation were analysed to assess the response and tolerance capacity of *Z. chilensis* to hypersalinity.

The Salt Overly Sensitive 1, (*SOS1*) codifies for a transport protein which mediates sodium extrusion via proton intake, thus dealing with Na excess in the cytosol (Ji et al., 2013). Also, the Sodium/hydrogen exchanger 1 encoding gene (*NHX1*) mediates the same antiporter process in the vacuole. Pyrroline-5-carboxylate reductase (P5CR) is an enzyme which catalyses the production of proline, an amino acid which may be used as an organic osmolyte which helps to compensate osmotic unbalance. Ornithine cyclodeaminase (OC) is an essential enzyme in proline synthesis, which is an organic osmolyte used to maintain osmotic balance.

Regarding genes relative to oxidative stress response, superoxide dismutases (Mn/Fe SOD, Cu/Zn SOD) are enzymes in charge of the conversion of superoxide anion into H_2O_2 in the cytosol, and catalase (CAT) degrades hydrogen peroxide, forming water and molecular oxygen. Ascorbate peroxidase (APX) also consumes H_2O_2 by oxidizing ASC to DHA and glutathione reductase (GR) restores reduced glutathione levels which are essential for DHA reduction into ASC in the Halliwell-Asada cycle.

There is a general pattern of up-regulation in both hypersalinity treatments with the only exception of *NHX1* after 24 h of experiment and *OC* on days 1 (40 psu) and 6 (37 psu). While the 40 psu treatment showed higher relative expression in most genes for days 3 and 6, it was lower on day 10 in the case of *SOS1, P5CR, CAT* and *NHX1*.

Only *GR* and *Cu/Zn SOD* showed differences between treatments with a higher up-regulation at 40 psu and also a significant increase in expression on the 10th day. *NHX1* presented significantly higher values on day 3 compared to day 1 while *P5CR* and *SOS1* showed higher expression on the 3rd day compared to the rest of the days. For *CAT*, the interaction between factors was significant because 40 psu treatment on day 3 differed significantly from the rest of values, showing a higher expression. *OC* also presented significant interaction, in this case because on the 10th day the 40 psu treatment presented significantly higher values compared to the rest of measures (Fig. 6).

4. Discussion

Our results reveal that salinities over 3 and 6 psu above the natural levels generate oxidative stress and trigger physiological and biochemical tolerance responses from *Z. chilensis*; thus, showing a degree of vulnerability of this species to salinity increments. In this regard, it has been observed that salinity excess can lead to osmotic stress and cause a variety of tolerance responses in plant cells (Yang and Guo, 2018), and seagrasses are not an exception (Touchette, 2007). The alteration of environmental salinity might condition seagrass survival and development, becoming a potential cause of meadow regressions observed in different ecosystems around the world; certainly, salinity excess derived from desalination brine discharges has been involved in these different processes (Marín-Guirao et al., 2013b; Sandoval-Gil et al., 2012b).

In regard to photosynthetic responses in *Z. chilensis*, *Df/Fm'* and *Fv/Fm* displayed a small or none influence of excess salinity, in spite that it has been observed that quantum yields can be reduced as a consequence of salinity increments in different macroalgae and seagrasses; however, strong responses in this parameters have been mostly associated with extreme salinity conditions (i.e. more than 50 psu) (e.g. Garrote-Moreno et al., 2015; Tsioli et al., 2022). Moreover, Marín-Guirao et al. (2011) found no significant effects on *Fv/Fm* when exposing *Posidonia oceanica* to 43 psu, and a decline in ETR_{max} which also coincides with our results. *Z. chilensis* experienced a reduction in ETR_{max} similarly in both hypersalinity treatment, possibly as a consequence of electron transport chain malfunction due to changes in membrane potential (Sudhir and Murthy, 2004). This reduction of gross photosynthesis has been reported for marine macrophytes exposed to increased salinities, both in macroalgae and seagrasses (Garrote-Moreno et al., 2015; Rodríguez-Rojas et al., 2020). Possibly, plant cells could be trying to compensate this decline in photosynthetic activity by increasing α_{ETR} and decreasing Ek_{ETR}. Fernández-Torquemada et al. (2005a, 2005b) found this effect when exposing the seagrass *Halophila johnsonii* to 40 psu (i.e. +10 psu above natural salinity) during 15 days, and Sandoval-Gil et al. (2012a) in *Cymodocea nodosa* exposed to salinity increments (+2 and + 4 psu) for 47 days. This alterations in the normal photosystem functioning caused by salinity are related to the increment in ion permeability of thylakoid membranes and its subsequent disruption of the electron transport chain (Touchette, 2007; Xia et al., 2004).

Considering this stress source, plants would possibly try to cope with it by releasing energy excess via NPQ_{max}. The significantly higher values of NPQ_{max} at 40 psu on the 3rd day, coinciding with the lowest ETR_{max} and Ek_{ETR} values, suggests an activation of the xanthophyll cycle, with the aim of releasing the energy excess unharvestable by a damaged photosynthetic apparatus. This short-term response has also been reported in *Thalassia testudinum* exposed to a salinity increment of +15 psu for 7 days (Trevathan et al., 2011), in *C. nodosa* at +12.5 psu and *P. oceanica* at +8 psu for the same period (Garrote-Moreno et al., 2015). This mechanism avoids free electrons to join ubiquitous oxygen and the subsequent formation of ROS (Marín-Guirao et al., 2013a, 2013b). The latter reduction of this parameter on day 10 at 40 psu could indicate a saturation or depletion of energy dissipation capacity of *Z. chilensis* cells to deal with higher salinity values in the long-term.

Thylakoid membrane damage leads to an electron transport chain malfunction. If the energy is not harnessed or dissipated, electrons can meet oxygen molecules and form free radicals, and therefore, ROS excess (Bartosz,

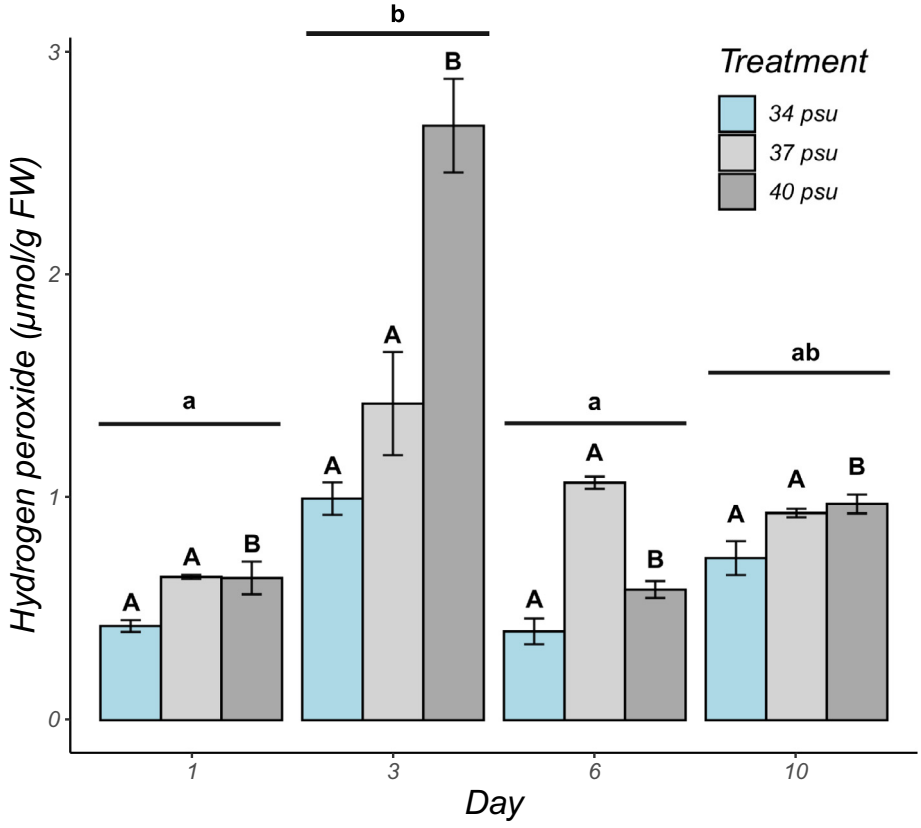

Fig. 4. Hydrogen peroxide content in *Z. chilensis* samples under controlled conditions by treatment (a) and day (b). Barplots represent the mean of each variable and error bars show the standard error. Upper case letters represent significant differences at 95 % confidence interval ($p < .05$) between treatments (34 psu, 37 psu, 40 psu). Lower case letter represents significant differences between days (1, 3, 6, 10).

1997; Yang and Guo, 2018). In fact, previous studies have shown that salinity changes are a cause of ROS over-production in marine macrophytes (Kumar et al., 2014; Lu et al., 2006), which coincides with the H_2O_2 levels detected in this study. Trevathan et al. (2011) found a significant increment of H_2O_2 in *T. testudinum* after 7 days of salinity increment (+15 psu), supporting the idea of this ROS to be a relevant indicator of salinity derived oxidative stress in the short term.

The significant decline in ETR_{max} and Ek_{ETR}, as well as the increment of H_2O_2 on the 3rd day in all treatments could be related with the mesocosm conditions, although affection was still greater under hypersalinity treatments. The posterior stabilization of those parameters might be related with the acclimation capacity of this plant to disturbances. ROS generation triggers the consumption of ASC, being oxidized to DHA thus decreasing the ASC/DHA ratio (Asada, 1992; Sáez et al., 2015). In fact, H_2O_2 production and ASC consumption as a response to increased salinities have already been reported both in macroalgae and plants (Luo and Liu, 2011; Sofo et al., 2015). In this work, 40 psu over natural 34 psu induced higher H_2O_2 content in *Z. chilensis*, even though no differences were detected in ASC or DHA. These results may indicate that under increased salinities ASC is being synthesized and consumed (in addition with other antioxidants),

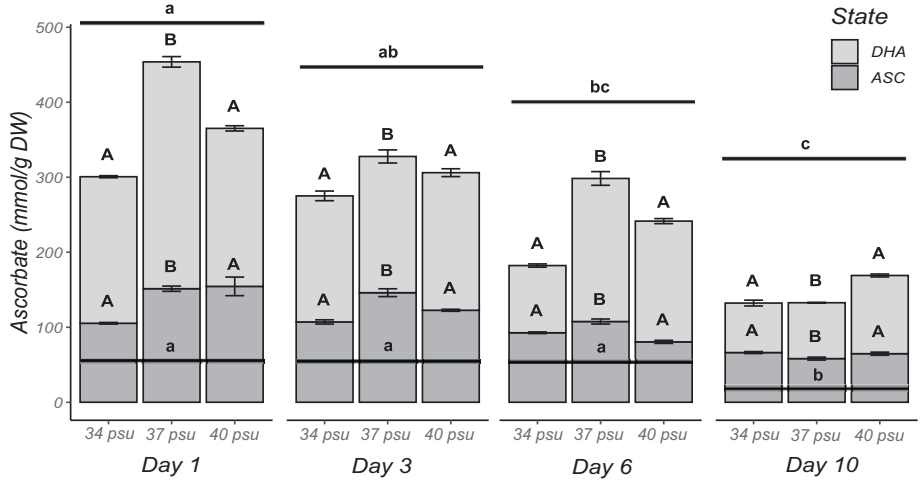

Fig. 5. Ascorbate (ASC) and Dehydroascorbate (DHA) content in *Z. chilensis* samples under controlled conditions for 10 days. Barplots represent the mean of each variable and error bars show the standard error. Upper case letters represent significant differences at 95 % confidence interval ($p < .05$) between treatments (34 psu, 37 psu, 40 psu). Lower case letter represents significant differences between days (1, 3, 6, 10).

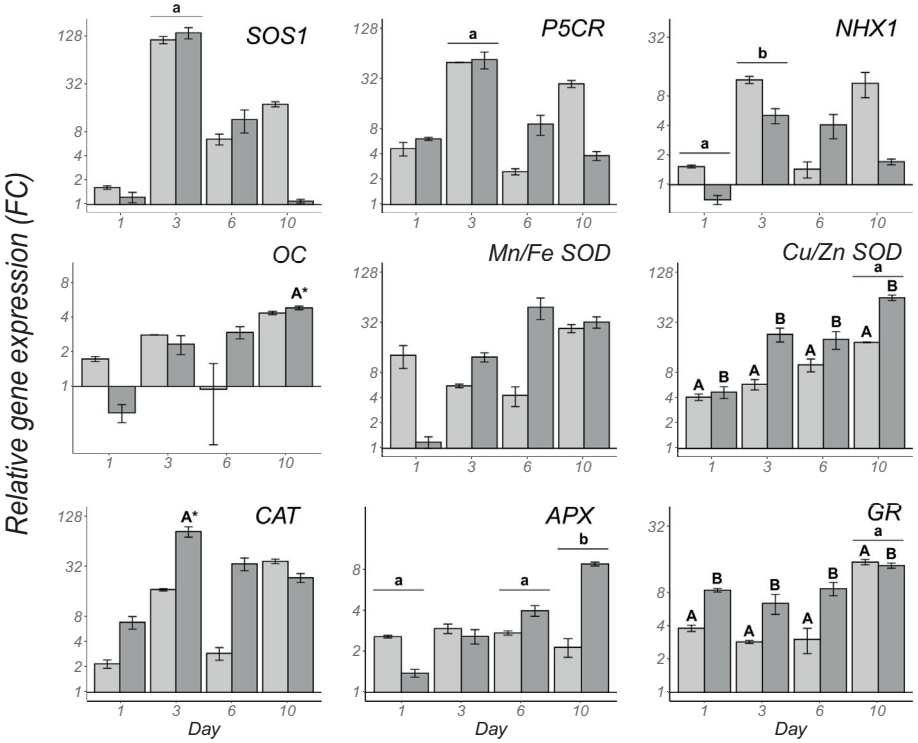

Fig. 6. Relative expression of hypersalinity and oxidative stress-related genes in *Z. chilensis* samples under two different salinity treatments: 37 (light grey) and 40 (dark grey) psu. Upper case letters represent significant differences at 95 % confidence interval ($p < .05$) between treatments (34 psu, 37 psu, 40 psu). Lower case letter represents significant differences between days (1, 3, 6, 10). Asterisk (*) show significant differences of a group in the post-hoc test when factor interaction was significant.

possibly keeping H_2O_2 levels closer to baseline levels. Also, both ASC and DHA decrease at day 10 could be an indicator of either acclimatation to the environmental conditions or metabolic depletion caused by salinities over the plant tolerance threshold. The latter is unlikely considering the rest of biological responses recorded in this study.

Also, plant cells respond to osmotic stress modifying gene expression triggered by specific intracellular signalling. That is the case of the salt overly sensitive (SOS) pathway, which maintains the cell homeostasis by, among other responses, triggering the synthesis of Na^+/H^+ antiporter proteins in the plasma and vacuole membranes (Ji et al., 2013; Yang and Guo, 2018). Osmotic regulation in plant cells include the extrusion of toxic ions like Na^+ or its accumulation in vacuoles and the synthesis of organic osmolytes (Yang and Guo, 2018). *SOS1* and *NHX1* codify for Na^+/H^+ transport proteins in the plasmatic membrane and the vacuole respectively. Their overexpression confirms that Na^+ extrusion is an essential mechanism in *Z. chilensis* to cope with hypersaline stress, as has been reported for other seagrass species, keeping intracellular concentrations even lower than in other halophytes (Touchette, 2007; Ye and Zhao, 2003). *Zostera marina* also has reported Na^+ exclusion mechanisms coupled with proton pumps on the plasma membrane, suggesting a pH-dependent osmoregulatory mechanism that may be also present in *Z. chilensis* (Fernández et al., 1999). Both P5CR and OC are enzymes related to proline biosynthesis and their up-regulation indicates the production of organic osmolytes to compensate osmotic unbalance (Delauney and Verma, 1993; Marín-Guirao et al., 2017; Touchette, 2007). Lv et al. (2018) found that hypersalinity triggered the overexpression of genes related to nitrogen metabolism in *Z. marina;* indeed, our results reveal some of these mechanisms, such as proline synthesis both from ornithine and pyrroline-5-carboxilic acid, thus affecting the glutamate metabolism, core of the nitrogen assimilation pathways in plants (Majumdar et al., 2015). These alterations on nitrogen metabolism could compromise normal growth and development in the long term and; certainly, growth reduction as a consequence of hypersaline stress is one of the most reported seagrass responses (Cambridge et al., 2017; Fernández-Torquemada and Sánchez-Lizaso, 2006, 2013).

Besides osmotic regulation, hypersalinity also triggers the up-regulation of genes related to oxidative stress and ROS scavenging. *CAT* up-regulation reached its maximum values on the 3rd day for the 40 psu treatment, corresponding with the same trend of H_2O_2 levels. The reduction of H_2O_2 on days 6 and 10 despite the overexpression of *SOD* might indicate the effectiveness of H_2O_2 metabolism by *Z. chilensis*. Overexpression of *SOD* and *CAT* is a common response to hypersaline stress in marine macrophytes, which is usually coupled with the activation of the ascorbate-glutathione cycle via APX and GR activities (Luo and Liu, 2011; Sofo et al., 2015; Sung et al., 2009). Sung et al. (2009) found an initial H_2O_2 scavenging carried out by APX rather than CAT in the macroalgae *Ulva fasciata* under severe hypersaline conditions (90 psu) in the short term (1–12 h). These results may indicate a mayor role of CAT in ROS regulation, even in the short term.

Most of the controlled experiments regarding seagrass tolerance to salinity increments have been made using seagrass species of the Posidoniaceae and Cymodoceae families (Sandoval-Gil et al., 2022). However, these seagrass families have evolved separately from Zosteraceae and experience different return-to-the-sea events (Les et al., 1997). Genomic studies have already stated different adaptations to the marine underwater life (Chen et al., 2022), shedding some lights on hypersaline responses of the *Zostera* genus (Zosteraceae), and specifically on the most geographically isolated species of this family. There were no differences between both hypersalinity treatments for genes codifying for proteins related to osmotic regulation (*SOS1*, *NHX1*, *P5CR* and *OC*). Therefore, above 3 psu over natural salinities triggers tolerance mechanisms in *Z. chilensis*, pattern that maintains at higher salinities. This may imply that a 3 psu salinity increment already activates the maximum capacity for salinity protection via osmotic regulation. In contrast, genes related to oxidative stress (*Cu/Zn SOD*, *GR, CAT*) were mostly up regulated in the 40 psu treatment, suggesting that oxidative stress starts when osmotic regulation gets overcome by excess salinities. Differences between days in gene expression also indicates an initial role of osmotic regulation mechanisms (*SOS1, P5CR, NHX1*) and a subsequent activation of genes related to ROM at day 10 (*APX, GR, Cu/Zn SOD*). The fact that 4 genes (*SOS1, P5CR, NHX1, CAT*) together with NPQ_{max} showed a decline at 40 psu treatment on the 10th day

compared to the 37 psu, may be indicating that tolerance capacity is near to be overwhelmed. This metabolic depletion reveals an initial development of tolerance mechanisms against osmotic stress which might fail if the stress is constant in time (Marín-Guirao et al., 2013b).

This multi-scale approach on *Z. chilensis* subject to hypersaline stress reveals several physiological and metabolic tolerance mechanisms. Among those, ETR_{max}, Ek_{ETR} and NPQ_{max} seem to be reliable photochemical parameters to address osmotic stress, as well as H_2O_2 levels; thus, becoming potential indicators to assess the effect of brine discharges on *Z. chilensis* meadows. In contrast, *Df/Fm′*, *Fv/Fm*, and α_{ETR} measurements did not reflect important modifications upon excess salinity. Regarding gene expression, the 9 studied genes presented up-regulation in both hypersalinity treatments, but the sensitivity of *CAT, Mn/Fe SOD, Cu/Zn SOD, SOS1* and *P5CR* highlight them as more suitable transcriptomic biomarkers to follow salinity increments. These parameters have the potential to be used as early-warning indicators to prevent seagrass degradation caused by desalination brine discharges.

Although environmental monitoring plans and their requirements related to brine discharges have improved in the last few years in Chile, there are still knowledge gaps on the effects of this activity on coastal ecosystems (Sola et al., 2019). Certainly, considering the potential future growth of desalination in the north and central regions of Chile (Herrera-León et al., 2021; Odell, 2021), the vulnerability assessment of marine ecosystems such as the secluded seagrass *Z. chilensis* is essential for a sustainable future development of the industry.

5. Conclusions

Zostera chilensis has shown photochemical and metabolic stress caused by hypersalinity, although demonstrating tolerance responses and viability within the salinities and time-frame of experiments. A decline in ETR_{max} and Ek_{ETR} as well as an increment in α_{ETR} in hypersalinity treatments indicate a negative effect of hypersalinity on photosynthetic normal functioning, while NPQ_{max} increased at 40 psu initially but declined at the end of the experiments. The higher production of H_2O_2 (+91.1 % at 40 psu) together with the up regulation of genes such as *CAT, Mn/Fe SOD, Cu/Zn SOD, APX* and *GR* (even 100 times over baseline levels) reveal that these levels of hypersalinity can cause oxidative stress in *Z. chilensis*.

Tolerance mechanisms and oxidative responses appear to better operate at 37 psu compared to 40 psu, indicating that the salinity may be above the physiological tolerance threshold of *Z. chilensis* in the long term; thus, direct brine discharges are not recommended on the species. ETR_{max}, Ek_{ETR} and NPQ_{max}, H_2O_2 levels and regulation of *CAT, Mn/Fe SOD, Cu/Zn SOD, SOS1* and *P5CR* might be reliable biomonitoring tools to follow the extent of desalination impacts with *Z. chilensis*.

CRediT authorship contribution statement

Fabio Blanco-Murillo: Conceptualization, Investigation, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **María José Díaz:** Investigation, Data curation, Formal analysis. **Fernanda Rodríguez-Rojas:** Investigation, Formal analysis, Data curation, Writing – review & editing. **Camilo Navarrete:** Investigation, Formal analysis. **Paula S.M. Celis-Plá:** Formal analysis, Data curation, Writing – review & editing. **José Luis Sánchez-Lizaso:** Conceptualization, Writing – review & editing. **Claudio A. Sáez:** Conceptualization, Investigation, Writing – original draft, Writing – review & editing.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Claudio Saez reports financial support was provided by European Commission. Fabio Blanco Murillo reports a relationship with University of Alicante that includes: funding grants.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.163538.

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