Cerium uptake, translocation and toxicity in the salt marsh halophyte *Halimione portulacoides* (L.), Aellen

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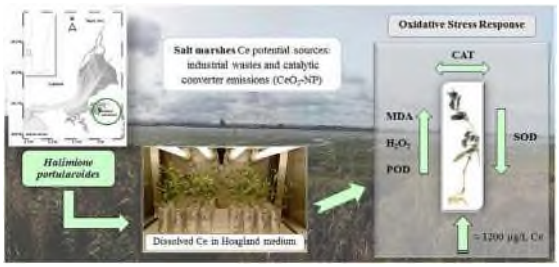
HIGHLIGHTS

- Cerium (Ce) toxicity was tested on a salt marsh halophyte, *Halimione portulacoides*.
- The rare earth element Ce may induces biphasic response in *H. portulacoides*.
- Ce accumulation in *H. portulacoides* organs followed metal's increase in the medium.
- Five-days exposure to 1200 μg Ce/L triggered shoots antioxidant enzymatic response.
- *H. portucaloides* may be a promising salt marsh Ce hyperaccumulator.

GRAPHICAL ABSTRACT



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ABSTRACT

Halimione portulacoides plants were exposed to dissolved cerium (Ce) in a hydroponic medium for five days. Ce accumulation in plants followed the metal's increase in the medium although with a very low translocation factor (TF < 0.01) between roots and shoots. Ce median concentrations in roots were 586, 988 and 1103 μg/g (dry wt.),while in shoots the median values reached 1.9, 3.5 and 10.0 μg/g (dry wt.), for plants exposed to 300, 600 and 1200 μg/L of Ce, respectively. No significant differences occurred in the length of roots and shoots among treatment groups, albeit plants exposed to the highest Ce concentration showed a clear loss of turgor pressure on the fifth day. An increase of hydrogen peroxide and malondialdehyde levels were observed in the plant shoots at1200 μg/L of Ce.The highest concentration also triggered an answer by the shoots' antioxidant enzymes with a decrease in the activity of superoxide dismutase and an increase in peroxidase. However, no significant change in catalase activity was observed, compared to the control group, which may indicate that peroxidase played a more crucial role against the oxidative stress than catalase. Combined results indicate that *H. portulacoides* was actively responding to a toxic effect imposed by this higher Ce concentration. Nevertheless, changes in normal environmental conditions, may increase the bioavailability of Ce, while in areas where acid mine drainage may occur, the highest Ce concentration tested in this study may be largely exceeded, placing the sustainability of halophytes and estuarine marshes at risk.

1. Introduction

Rare earth elements (REE) are widely used in a large range of applications: in the development of new materials and technologies, as a raw material in a variety of industrial processes, agriculture, medicine, etc. (Du and Graedel, 2013; Goonan, 2011; Haque et al., 2014). Cerium oxide (CeO_2) is one of the most common REE compounds, and CeO_2 nanoparticles (CeO_2-NP) are the principal form used in commercial and industrial products, such as, in catalysts, fuel cells, ceramic and glass applications or as diesel fuel and nano-agrochemicals additive (e.g. Baalousha et al., 2010; Dahle and Arai, 2015; Ermolin et al., 2019; Marucci et al., 2019; Peralta-Videa et al., 2011). The increasing production and consumption of these engineered nanomaterials, coupled with the enormous diversity of particle type and size, has raised concerns about its emissions into the environment, fate and effects in the ecosystem (Cassee et al., 2011; Dahle and Arai, 2015; Du et al., 2017; Gottschalk and Nowack, 2011; Tripathi et al., 2017). These emissions occur mainly due to the natural ageing (e.g., catalysts) or washing out (e.g., sunscreen) of products, the intensive use of agrochemicals (e.g., nanopesticides) and fertilisers and inadequate or accidental disposal of various residues (Mu, 2019 and references therein). The estimated CeO_2-NP global flows in landfills, soil, air and water, in 2010, were 8200, 1400, 100 and 300 tons/year, respectively (Keller et al., 2013).

When studying the effects of CeO_2-NP on organisms in different environments, it is important to take into account the different physical, chemical and biochemical interactions, such as, NP dissolution, ion adsorption, cell adhesion, uptake by organisms and the combinations of them (Baalousha et al., 2010; Cross et al., 2019; Dahle and Arai, 2015; Handy et al., 2012; Schwabe et al, 2013, 2014, 2015). CeO_2-NP were initially considered stable and insoluble (Brunner et al., 2006; Mädler et al., 2002) and have been frequently used in plant uptake studies in aqueous suspensions or hydroponic media (Miralles et al., 2012; Schwabe et al., 2015). Thus, in most of these studies, solubility and biotransformation of CeO_2-NP were not considered (Du et al., 2017 and references therein). Zhang et al. (2012) observed that even highly stable CeO_2-NP can undergo biotransformation in plants by reducing biogenic substances and organic acids, as well as phosphate ($PO^3{}_4^-$). Recently, some studies have demonstrated a higher solubility of CeO_2-NP in nutritive solutions, such as the Hoagland

medium, with dissolved Ce reaching values up to 680 µg/L (Schwabe et al, 2014, 2015). Those studies have suggested greater Ce mobility from CeO_2-NP that may lead to higher metal bioavailability in the aquatic environment, posing major environmental concerns. Another potential anthropogenic enrichment of REE in estuarine environments is acid mine drainage (AMD) that results from mining activity, and which is characterised by low pH values and high concentrations of various metals, including REE (e.g. Delgado et al., 2012; Fernández-Caliani et al., 2009; Pérez-López et al., 2010). Several studies have shown that in estuaries affected by AMD, dissolved ΣREE levels can reach values above 11,000 µg/L, with dissolved Ce reaching concentrations of 4480 µg/L (Lecomte et al., 2017).

Estuaries are usually located near industrial and highly populated areas, while salt marsh ecosystems are known to act as important sinks and sources for several contaminants, including different NP (e.g. Duarte et al., 2019; Fernandes et al., 2017). Halophyte plants are recognised as heavy metal accumulators and have been the subject of several studies in salt marshes (Caçador et al., 2000; Castro et al., 2009; Duarte et al., 2010; Monteiro et al., 2016; Valega et al., 2008a, 2008b). *Halimione portulacoides* (L.), Aellen, is one abundant halophyte species in the Tagus estuary salt marshes and is described as a highly productive accumulator species (Cabrita et al., 2019; Duarte et al., 2012 and references therein). Although it is a species that has a good tolerance when exposed to high concentrations of various metals (Anjum et al., 2016; Cabrita et al., 2019; Duarte et al., 2012), its physiological/biochemical strategies and adaptation/tolerance to environmental high Ce concentrations is still unknown. Cerium is a redox-active metal as it is capable of inducing the production of reactive oxygen species (ROS) through a Fenton-like reaction (Heckert et al., 2008). ROS can oxidise various cellular components that can lead to oxidative cell destruction (Mittler, 2002 and references therein). In addition to ROS produced in plant cells as a result of metabolic reactions, such as photosynthesis and respiration, other forms result from abiotic factors, such as periods of prolonged flooding or extreme drought, salinity, extreme temperatures, radiation, exposure to contaminants, mechanical stress, nutrient deprivation and pathogen attacks (Mittler, 2002). Excessive production of ROS in abiotic stress situations can pose a threat to cells, but may also act as a trigger to activate stress response and defence pathways (Knight and Knight, 2001). In fact, plants have developed a complex regulatory chain to mediate the stress responses based on the ROS synthesis, scavenging and signalling (Huang et al., 2019). To protect from ROS cytotoxicity, plants synthesise a set of key enzymes such as superoxidase dismutase (SOD), catalase (CAT) and peroxidase (POD) that integrate an effective ROS-scavenging system in different cellular compartments. SOD acts as the first line of defence against ROS by promoting the dismutation of O^{-}_2 to O_2 and H_2O_2. CAT breaks down H_2O_2 directly to form O_2 and H_2O, whereas POD catalyses H_2O_2-dependent oxidation of the substrate (Janku et al., 2019).

Several studies have reported that *H. portulacoides* has different enzymatic responses to different abiotic factors. Kalir et al. (1984) showed that low concentrations of sodium chloride stimulated CAT activity, while higher concentrations inhibited them but had a stimulation effect on POD. Duarte et al. (2012) found no inactivation of the enzymatic defences in *H. portulacoides* after exposure to high chromium (Cr) concentrations and accumulation of this metal in its roots, with SOD proving to be an excellent dose-related biomarker of stress induced by Cr. When *H. portulacoides* was exposed to an atmospheric CO_2-enriched environment, Duarte et al. (2014) observed a significant decrease in its antioxidant enzymatic defence system, whereas a significant increase in total soluble protein content was observed in the leaves. In a temperature stress study on this plant, it was noticed an increase in CAT and SOD activity in leaves of cold treated plants, while heat exposed plants showed a significant increase in SOD activity (Duarte et al., 2015). Anjum et al. (2014) observed a reduction in the *H. portulacoides* enzymatic activity, namely CAT, when exposed to high concentrations of environmental mercury.

Thus, considering the scarce knowledge about Ce effects on the mechanisms of antioxidation, as well as the potential phytotoxicity of this metal in one of the most common halophyte species of estuarine marshes and an important phytoremediation agent of contaminated areas, the main objectives of this work were: (1) to study the metal's accumulation and translocation in *H. portulacoides* submitted to hydroponic experimental treatments of dissolved Ce between 0 and 1200 µg/L; (2) to evaluate the plant response to the potential oxidative stress induced by Ce; and, (3) to evaluate the potential risk of toxicity of Ce.

2. Material and methods

2.1. Collection and treatments of H. portulacoides

Specimens of *H. portulacoides* were collected in the Rosario salt marsh (38° 40.161′ N, 9° 00.198' W; see Fig. S1 in Supplementary Material), in the southern margin of the Tagus estuary (Portugal, SW Europe), during low tide, at the beginning of the growing season (early March 2019). Plants were stored in clean plastic bags and quickly transported to the laboratory in refrigerated conditions, where they were carefully washed with ultra-pure water (18.2 MΩ cm^2, Merck Millipore) to remove any sediment and dust. To allow the development of new roots in a controlled environmental chamber (Fitoclima S600, Aralab, Portugal), the roots and a small part of the stems were cut, leaving at least two nodes in the stem below the lower branch. Cuttings were placed in dark-walled 100 mL polypropylene vases with modified 1/4 Hoagland nutrient solution (Hoagland and Arnon, 1950) and kept in the environmental chamber under controlled conditions (Table 1) for approximately two months to allow the growth of new roots.

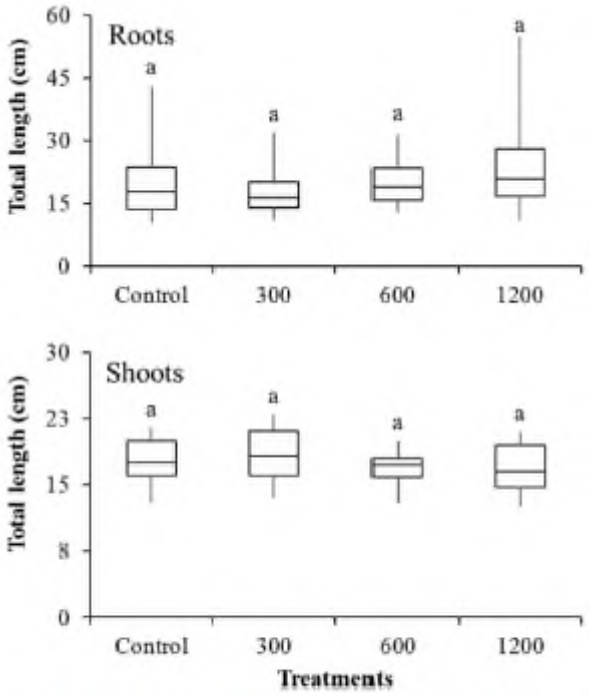

Fig. 1. *Halimione portulacoides* roots and shoots length after 120 h exposure to different dissolved Ce treatments (n = 20); Control, 300, 600 and 1200 µg/L. *Post hoc* test results between Ce treatments are given by different lower-case letters for $p \leq 0.05$.

Table 1
Halimione portulacoides conditions during growth and exposure to dissolved Ce in the environmental chamber. Temperature (T), relative humidity (H) and photosynthetic photon flow density (PPFD).

	Day (12 h)	Night (12 h)
T (°C)	23	20
H (% rH)	40	60
PPFD (µmol/m²/s)	250	0

2.2. *Cerium exposure experiments*

After the growing period, plants were placed in separate darkwalled 50 mL polypropylene tubes and distributed into four treatment groups: a control group (in 1/4 Hoagland's solution) and three treatment groups exposed to 300, 600 and 1200 µg/L of Ce prepared from a Ce(IV) oxide certified reference standard solution (1000 mg Ce/L in 5% nitric acid, prepared with high purity CeO_2, Sigma-Aldrich). Each treatment group consisted of four biological replicates (R1-R4) with 5 individuals each, for a total of 20 plants per group. The concentrations used were chosen according to the values found in the literature for Ce dissolved from CeO_2-NP in Hoagland medium (Schwabe et al, 2014, 2015). These values are significantly lower when compared with the AMD reported cases, in which dissolved Ce concentrations were much higher than the highest concentration chosen for this study (Lecomte et al., 2017). The exposure trial was performed during a period of 120 h, in the controlled environmental chamber under the previous environmental conditions (Table 1). All treatment group solutions (including the control group) were totally renewed every 24 h to ensure that initial concentrations were maintained throughout the exposure period. To estimate the amount of dissolved Ce uptake by plants, an aliquot from each group's solution was collected (composite sample of the 5 plant vials) every 24 h before renewal, filtered through a 0.45 µm filter and acidified with nitric acid (2% v/ v) for dissolved Ce quantification.

2.3. *Samples preparation for Ce and biochemical analysis*

To evaluate the concentration of dissolved Ce in the Hoagland solution of each treatment group, a 5 mL aliquot from each tube was taken into a composite sample every 24 h before renewal. The composite samples (four replicates per treatment group) were filtered through a 0.45 µm filter and acidified with nitric acid (2% v/ v) before analysis.

Plants from each treatment group were harvested at the end of the exposure period and individual biometric data were collected (total length of roots and shoots). The roots and shoots (stems plus leaves) were then ground under liquid nitrogen in a steel mortar and pestle before long-term storage in liquid nitrogen (Arpege 40, Air Liquide).

The quantification of Ce concentration in the *H. portulacoides* roots and shoots was determined in aliquots previously oven-dried at 40 °C and digested according to Brito et al. (2020). Briefly, about 100 mg of dry plant tissues were digested in a microwave (CEM, MARS 5), with nitric acid, at 160 °C, for 30 min. Digested samples were analysed by ICP-MS (Perkin-Elmer NexION 2000C) equipped with a cyclonic spray chamber, a concentric Meinhard nebulizer and a dual detector. A 6-point calibration curve was used to quantify each element, using a commercial solution of indium (^{115}In) as internal standard (Alfa Aesar, Plasma Standard Solution, Specpure®). Reagent blanks, random sample duplicates and certified reference materials (CRM; SLRS-6 from the National Research Council of Canada, for water; GSB-11 Citrus leaves, from China National Analysis Center for Iron and Steel, China, and BCR® e 670, from the Institute for Reference Material and Measurements e IRMM, European Commission, for aquatic plants) were processed in the same way and for each batch of water/plant organs samples, and analysed every fifteen samples as an analytical quality control procedure to evaluate the accuracy and precision. Reagent blanks always accounted for less than 1% of total metal contents in samples and the duplicates coefficient of variation were ≤10%. Contents of major and REE elements in CRM were not statistically different from certified values (t-student; $\alpha = 0.05$).

2.4. Biochemical analysis

All material was previously autoclaved at 121 °C for 15 min. All chemical reagents were of high chemical grade and ultra-pure water (18.2 MΩ cm^2, Merck Millipore) was always used. Due to the reduced amount of roots' biomass obtained at the end of the exposure period, it was not possible to carry out biochemical analyses on this plant organ. Thus, only *H. portulacoides* shoots (stems plus leaves) were used. All assays were done in triplicate for each biological replicate (n = 12).

2.4.1. Protein crude extract preparation

H. portulacoides shoots crude extracts were prepared as previously described by Ferreira et al. (2014). Briefly, 8 mL of an extraction buffer (50 mM phosphate buffer, pH 7.5, containing 10 mM KCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 5 mM dithiothreitol, 0.5 mM Pefabloc and 25%(w/w) polyvinylpolypyrrolidone) was added to approximately 1 g of ground tissue. The suspension was centrifuged (4000 rpm for 20 min at 4 °C) and the collected supernatant was submitted to a second centrifugation (24,000 rpm, for 90 min, at 4 °C). The soluble protein extract (resulting supernatant) was filtered, desalted (PD-10 columns, GE Healthcare) and stored at -80 °C in aliquots, prior to analysis. Total protein concentration was determined using the Pierce® BCA protein test kit and using Bovine Serum Albumin as standard.

2.4.2. Hydrogen peroxide content

The determination of H_2O_2 levels was accomplished as described by Bouazizi et al. (2010). Briefly, 0.3 g of the ground tissues (preserved in liquid nitrogen) were homogenised on an ice bath with 1.5 mL of 0.1% (w/v) trichloroacetic acid solution. The homogenate was centrifuged (12,000 g, for 15 min, at 4 °C) and 0.5 mL of the resulting supernatant was added to 0.5 mL potassium phosphate buffer (10 mM, pH 7.0) and 1 mL potassium iodide (1 M). The absorbance of the mixture was measured at 390 nm and the content of H_2O_2 was estimated by reference to a standard curve. The results are expressed in micromoles per gram offresh weight of ground tissues (μmol/g FW).

2.4.3. Lipid peroxidation

The degree of lipid peroxidation in *H. portulacoides* shoots was determined using the thiobarbituric acid-reactive-substances assay, which measures the content of the aldehyde malonaldehyde (MDA; an end product formed from the breakdown of lipid peroxides). The MDA content was determined in an extract of 0.1 g of macerated tissue in 1 mL of 1% (w/v) thiobarbituric acid, as described in Velikova and Loreto (2005). The results are expressed in nanomoles of MDA per gram of fresh weight of ground tissues (nmol/g FW).

2.4.4. Enzymatic activity

Enzymatic reactions were performed at 25 °C and monitored using a spectrophotometer equipped with a temperature- controlled microplate reader (Cary 50 Bio, Varian).

SOD activity was determined by a modification of Carias et al. (2008) method. The reaction mixture, containing 170 μL of potassium phosphate buffer (50 mM, pH 8), 30 μL xanthine (3 mM),10 μL EDTA (3 mM), 30 μL nitro blue tetrazolium (NBT; 1.2 mM), 30 μL xanthine oxidase (60 mU/mL) and 30 μL of crude extract, was incubated at 25 °C in the dark for 30 min, with slight agitation. Blank 1 (reaction mixture without crude extract), blank 2 (reaction mixture without xanthine oxidase) and blank 3 (reaction mixture without crude extract and xanthine oxidase) were also prepared using ultra-pure water (18.2 MΩ cm^2, Merck Millipore) as replacement of the mixture components, in a volume of 300 μL, and incubated as before. The enzyme activity was determined by measuring the absorbance of the reaction mixtures at 560 nm. One unit of SOD activity was defined as the amount required for inhibiting photo reduction of NBT by 50% at 560 nm.

CAT activity was determined according to Davies and Swanson et al. (2001), with slight modifications. Briefly, the reaction mixture containing 60 μL of Tris-acetate buffer (500 mM, pH 7), 60 μL H_2O_2 (12.0 mM) and 10 mg of protein, in a volume of 300 μL, was monitored at 340 nm for 100 s. One unit (U) of CAT activity was defined as the μmol of H_2O_2 consumed per minute (ε_{240} = 43.6 M/ cm; Rad et al., 2007).

POD activity was assessed as reported in Ferreira and Martins- Dias (2016).

Specific activities were expressed as units of activity (mmol of substrate converted per minute) per milligram ofsoluble protein in the crude extract (U/mg).

2.5. Statistical analysis

For statistical analysis, PAST 4.0 software (Hammer et al., 2001) was used. The data were tested for normality using the Shapiro-Wilk test, which revealed a non-normal distribution of results, except for the Ce concentration in the hydroponic medium within each treatment group during the trial (in this specific case, Student's paired *t*-test was used for statistical significance analysis). The Kruskal-Wallis non-parametric test was used to assess statistical significance differences in the studied variables among treatments, followed by the Mann-Whitney pairwise *post-hoc* test. A statistically significant difference was considered at the level of $p \leq 0.05$.

3. Results and discussion

3.1. Cerium influence on H. portulacoides growth

At the end of the exposure period, no statistically significant differences ($p > 0.05$) were found in the root's and shoot's lengths between the control group and the plants treated with Ce (Fig. 1), with comparable values among all the treatment groups.

Our results showed no significant effects ($p > 0.05$) on the *H. portulacoides* growth when treated with different Ce concentrations. However, a clear wilting was observed by the end of the exposure period in the treatment group with the highest Ce concentration, suggesting a loss of turgor pressure (Fig. 2). Although Ce has been widely used in China since the 1970s to improve plant growth and yield (Hu et al., 2004), the effects on plants' growth have been quite controversial among the scientific community, with contradictory results reported (Hu et al., 2002 and references therein). The reduction in plants growth has been observed in different species (e.g. Andersen et al., 2016; Hu et al., 2002; López-Moreno et al., 2010; Ma et al., 2013; Morales et al., 2013; Priester et al., 2012; Shyam and Aery, 2012). However, this adverse effect on plant's vegetative growth was mainly reported when exposed to some critical Ce concentrations, while at lower concentrations the

Fig. 2. *H. portulacoides* status after 120 h of exposure to different Ce treatments (300, 600 and 1200 µg/L). The R1 to R4 represent the four biological replicates for each treatment group.

effects were in general beneficial both for roots and shoots growth (for a review see Hu et al., 2004). On the other hand, the meaning of a low or high concentration, responsible for this biphasic dose response to an environmental agent (hormesis), is quite subjective and varies substantially (by a 10^4 of magnitude variation range) between studies and tested species (Andersen et al., 2016; Hu et al., 2002; López-Moreno et al., 2010; Ma et al., 2013; Morales et al., 2013; Priester et al., 2012; Shyam and Aery, 2012).

To the authors' knowledge, no studies on *H. portulacoides* growth effects caused by Ce or any other REE exposure have been reported previously. Yet, Andrades-Moreno et al. (2013) found that the relative growth rate of *H. portulacoides* stem cuttings cultivated in sediments highly contaminated with heavy metals revealed a decrease of approximately 26 and 32% in the dry mass of shoots and roots, respectively, relative to a control group.

The loss of turgor observed in the *H. portulacoides* exposed to the highest Ce concentration have been reported in previous studies and can occur in plants subjected to osmotic stress induced by an excess of metal ions in the tissues (Rucińska-Sobkowiak, 2016 and references therein). The adverse effect observed in the physical structure of the plant may have resulted from its loss of capacity to produce specific chelating agents (e.g. phytochelatins) to retain metal ions and reduce their toxic potential in the cells (e.g. Babula et al., 2015; Parrotta et al., 2015). In this study, the Ce retained in *H. portulacoides* cells exposed to 1200 mg Ce/L may have exceeded the plant's capacity to neutralize it. Therefore, the continuous exposure of plants to Ce may have led this metal to interact with negatively charged molecules (for example, carboxyl and sulfate groups), modifying the cell wall resistance to turgor pressure and weakening the cells.

3.2. Cerium uptake by H. portulacoides

The daily variation of Ce concentrations in each treatment group solution throughout the entire exposure period is shown in Fig. 3. Dissolved Ce concentration in the control group was always below the method's limit of quantification (LQ = 0.02 µg/L), thus, not included in Fig. 3. The mean concentration of dissolved Ce in the Hoagland solutions showed a clear decrease in the first 24 h in all treatments. This reduction in the Ce nominal concentration represented about 51% in the treatment group exposed to 300 µg/L (154 ± 26 µg/L), being higher compared to the treatment groups subjected to 600 and 1200 µg/L, with decreases of about 24% (456 ± 60 µg/L) and 20% (965 ± 62 µg/L), respectively. After the first 24 h, Ce concentration in solution remained stable, with the exception of the 1200 µg/L treatment group, which showed a slight decrease after 120 h of exposure, accounting for about 7% of the nominal value of Ce (1112 ± 14 µg/L).

It should be noted that these concentrations were obtained daily and just before the solution renewal. These data, when compared with the results obtained from a previous Ce stabilisation test in Hoagland's medium (Fig. S.2 in Supplementary Material), showed

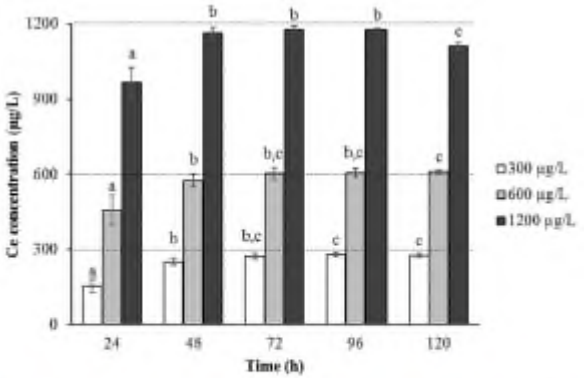

Fig. 3. Dissolved Ce concentration in the hydroponic medium (mean ± SD), in each treatment group (300, 600 and 1200 µg/L), measured daily just before the medium renewal. The dashed lines represent the three nominal concentrations in each Ce treatment group. The dissolved Ce concentrations in the control group (¼ Hoagland's solution) were always below the method's limit of quantification (LQ = 0.02 µg/L). Within the treatment group, values with the same lower-case letters are not significantly different ($p < 0.05$, $n = 4$).

that metal loss observed in this exposure trial was mainly due to the Ce removed by the plant and not to metal's adsorption to the tube walls or precipitation resulting from the oxidation of dissolve Ce^{3+} in Ce^{4+}. Thus, the higher dissolved Ce reduction in the medium in the first 24 h, compared to the rest of the exposure period, suggests the existence of a plant defence mechanism, such as iron plaque formation, capable of blocking the entry of this metal in response to the increase of Ce within the plant. The formation of iron plaques on the surface of plant roots has been reported by several authors, mainly in the rice plant *Oryza sativa* L. (e.g. Cao et al., 2018; Hu et al., 2014; Lai et al., 2012; Xu et al., 2018) including studies with Ce (e.g. Bao et al., 2020, 2019), but also in halophyte species, such as *H. portulacoides* (e.g. Caetano and Vale, 2002; Godinho et al., 2014; Sundby et al., 1998; Vale et al., 1990). This mechanism consists of iron co-precipitation with other metals forming small placoid structures that line the outer layers of the roots, even forming small tubes around the underground organs (rhizoconcretions). This protection mechanism significantly reduces the uptake and accumulation of toxic metals in plant tissues, allowing the passage of elements with a physiological role in plants. The results of this study show, however, that an eventual formation of iron plaques does not completely block the Ce uptake, as evidenced by the reduction in the metal's concentration observed at the end of the test (120 h) for the treatment group of 1200 µg/L. On the other hand, plants may be also translocating the Ce to the aerial part that are no longer capable of accumulating in the roots. An estimated Ce removal from the medium (see Fig. S.3 in Supplementary Material) by the plants was calculated using the daily Ce concentration variation determined in the hydroponic medium (presented in Fig. 3). In the first 24 h of exposure the Ce removal reached its maximum (7.3 ± 1.3, 7.2 ± 3.0 and 11.7 ± 3.1 µg of Ce in the plants exposed to 300, 600 and 1200 µg/L, respectively), with no statistically significant difference between each treatment group ($p > 0.05$). During the following three days of trial the metal removal varied between 0.5 and 2.5 µg of Ce. Only at the end of the exposure period, and for the maximum Ce concentration treatment, the metal removal increased (4.4 ± 0.7 µg of Ce) indicating a possible new plant-response step.

The Ce accumulated by the *H. portulacoides* organs clearly followed the metal's concentrations increase in the hydroponic medium (Fig. 4). The median concentrations of Ce accumulated in the roots showed a statistically significant increase ($p < 0.05$) in all the treatment groups exposed to the metal compared to the control group (2.8 µg/g). Cerium median concentrations in the belowground organ increased to 586, 988 and 1103 µg/g, in the plants exposed to 300, 600 and 1200 µg/L of Ce, respectively. The difference between the two highest concentrations was not statistically significant ($p > 0.05$) which seems to suggest that plants were no longer able to accumulate more Ce in the tissues even though roots continued to uptake it from the hydroponic medium, as evidenced by the metal's decrease in the 1200 µg/L treatment group at 120 h (Fig. 3).

Concerning the Ce concentrations accumulated in the *H. portulacoides* aerial organs, there was a statistically significant increase ($p < 0.05$) between the control group, with a median concentration of 0.51 µg/g and the plants exposed to 300, 600 and 1200 µg/L, with values of 1.9, 3.5 and 10.0 µg/g of Ce, respectively, and among each treated group. The concentrations of Ce in *H. portulacoides* shoots were one to two orders of magnitude lower than the values observed in the roots, which is in line with other previous works revealing higher accumulation of REE in the underground tissues compared to the shoots (Tyler, 2004 and references therein). In a previous study, Brito et al. (2020) observed that *H. portulacoides* collected in the Rosario's salt marsh (Tagus estuary) had accumulated higher Ce concentrations in the roots compared to the stems and leaves. Similar results have been observed for other metals (e.g. Caçador et al., 2009; Canario et al., 2010; Duarte et al., 2010; Milić et al., 2012; Pedro et al., 2015; Reboreda and Caçador, 2007). Regardless of the Ce concentration used, the translocation factor, calculated as the ratio of the concentration of Ce in the aerial part of the plant to the root concentration in dry

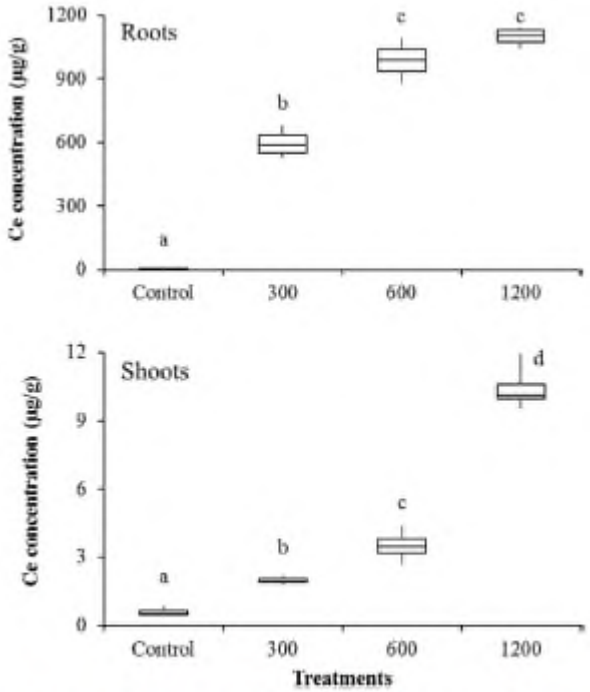

Fig 4. Cerium concentrations in *H. portulacoides* roots and shoots (n = 4) after 120 h of metal exposure. *Post hoc* test results are given by different lower-case letters for $p < 0.05$.

biomass, was <0.01. This low translocation from roots to the aerial organs could be explained by the metal binding to organic ligands, although rates seem to change considerably between plant species and REE (Tyler, 2004).

3.3. Anti-oxidative response of H. portulacoides to Ce

3.3.1. Protein content

The protein content in shoots of *H. portulacoides* exposed to 300 and 600 µg/L of Ce showed no significant differences ($p > 0.05$) when compared to the control group (see Fig. 5a). On the other hand, plants exposed to a Ce concentration of 1200 µg/L reached a significantly higher ($p < 0.05$) content relative to all the other treatment groups.

The obtained results seem to indicate a trigger of soluble protein synthesis, due to Ce increase in the plant's tissues, as a plant response mechanism to the oxidative stress imposed by the production of ROS. Xie et al. (2015) found a Ce dose-dependent stimulus in protein synthesis in *Cyclocarya paliurus* seedlings. Moreover, Wang et al. (2007) observed a gradual decrease in the protein content in the submerged plant *Hydrilla verticillata* with the increase in Ce concentration (from 0 to 100 µM). These authors suggested that the protein content reduction probably occurred due to the ROS toxic effects, resulting in protein degeneration, but also to the greater activity of proteases activated under metallic stress.

3.3.2. Endogenous H_2O_2 content

The endogenous H_2O_2 in the *H. portulacoides* shoots showed a significant increase ($p < 0.01$) compared to the control group only

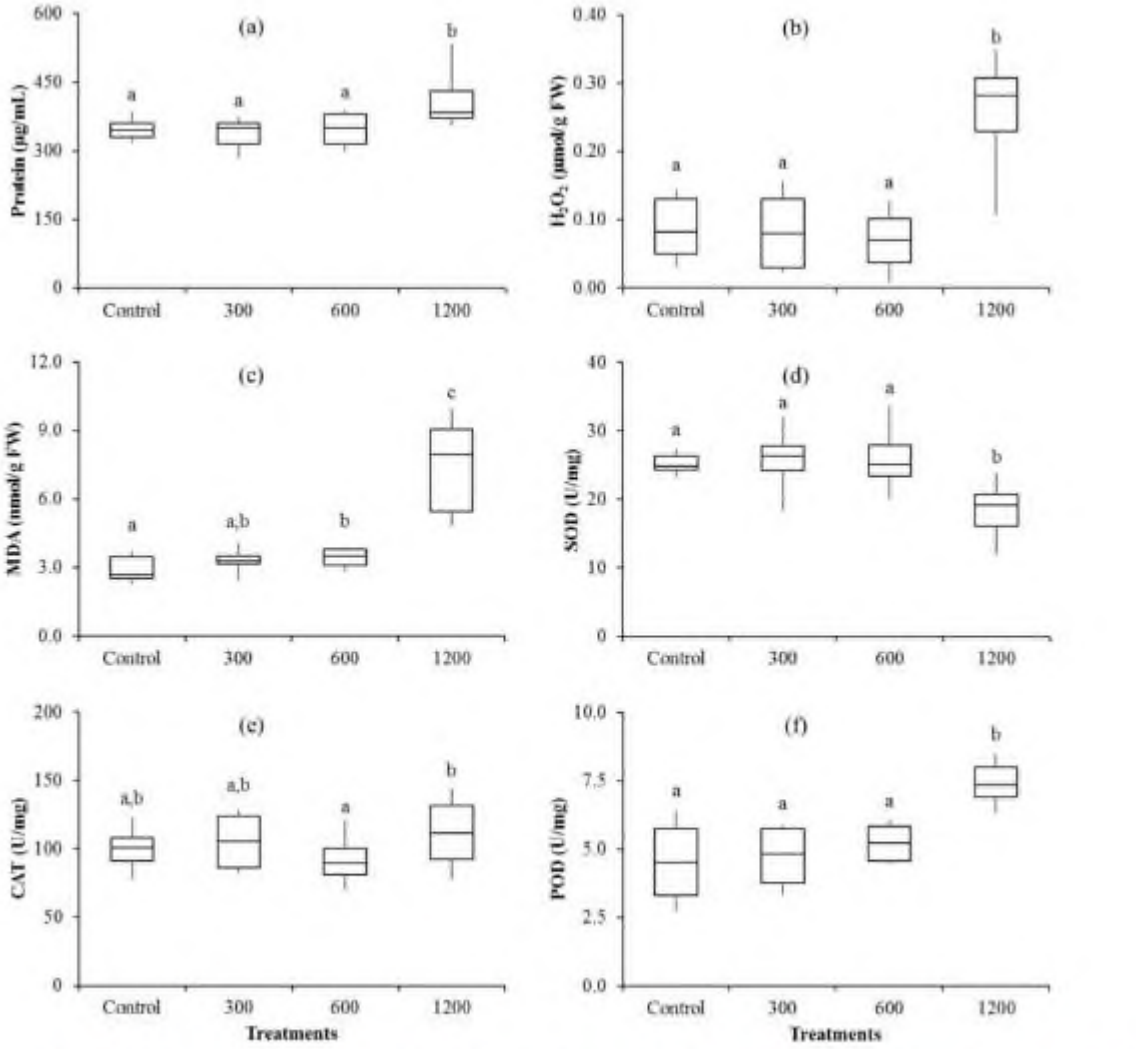

Fig. 5. Contents of (a) protein, (b) hydrogen peroxide (H_2O_2), (c) malondialdehyde (MDA), and antioxidant enzymatic activities of (d) superoxide dismutase (SOD), (e) catalase (CAT) and (f) peroxidase (POD) in *H. portulacoides* shoots after 120 h exposure to different dissolved Ce treatments: Control, 300, 600 and 1200 µg/L. Three determinations of each parameter per biological replicate, and per treatment group, were performed. Within a given parameter, the significant differences between treatment groups are indicated by different lower-case letters ($p < 0.05$, $n = 12$).

for the treatment of 1200 µg/L, while the treatments of 300 and 600 µg/L did not show any significant variation to the control endogenous H_2O_2 content (see Fig. 5b). The results obtained in this study revealed that, after 120 h of exposure, *H. portulacoides* was able to cope with the lowest concentrations of Ce (300 and 600 µg/L) without altering its H_2O_2 contents. However, and for the same period, when exposed to the highest concentration, the H_2O_2 content increased substantially compared to the other treatments, suggesting a disturbance in the ROS-scavenging system.

Lanthanides have many physical and chemical characteristics common to calcium (Ca), in particular the ionic radius. This feature can prevent the influx of Ca, competing for available binding sites in cell membranes and affecting many Ca-dependent processes (e.g. Wang et al., 2007). Wang et al. (2007) observed that H_2O_2 content in *Hydrilla verticillata* plants increased progressively with the increase in Ce concentrations from 0 to 100 µM. These authors also suggested that this augment in H_2O_2 could be attributed to a Ca deficiency in the plant cells.

3.3.3. Lipid peroxidation

Regarding the MDA content in *H. portulacoides* shoots (Fig. 5c), the treatment group exposed to the 300 µg/L Ce did not show any significant variation ($p > 0.05$) comparatively to the control group. However, the plants exposed to the Ce concentration of 600 µg/L showed a significant increase ($p < 0.05$) compared to the control group, which is even more pronounced for plants exposed to the 1200 µg/L Ce concentration.

ROS are known to be chemically aggressive species that can oxidise polyunsaturated fatty acids and promote membrane lipid peroxidation (Mittler, 2002 and references therein). In the present work, an increase in MDA levels was observed with the rise in Ce concentration, suggesting that cell membranes might have been damaged following the metal's concentration increase. MDA content was also positively correlated with the increase of Ce concentrations in other plant species (e.g. Morales et al., 2013; Tripathi et al., 2017; Wang et al., 2007). Delgado (2007) also observed the lipid peroxidation in *H. portulacoides* leaves induced by 1, 2 and 4 µM of copper during 15 days of exposition, while Duarte et al. (2012) found no evident membrane damage in the leaf tissues of this species when exposed to 15 and 30 mg/L of Cr during 7 days.

3.3.4. Enzymatic response

Enzymatic assays showed that SOD activity (Fig. 5d) remained constant in the lower concentrations relative to the control group. Albeit at 1200 µg/L a significant ($p < 0.05$) decrease in SOD activity was denoted. These results show that SOD activity was effective in dealing with the ROS generated at the lowest Ce concentrations, but in the presence of higher concentration an inactivation of this enzyme was observed. Duarte et al. (2012) in the leaves of *H. portulacoides* exposed to different concentrations of Cr also observed a reduction of SOD activity. Thus, with the inactivation of SOD, the risk of membrane lipid peroxidation is greater (e.g. Monk et al., 1989; Zhang et al., 2004), as noticed with the significant increase of MDA (Fig. 4c).

CAT activity (Fig. 4e) showed a statistically significant difference ($p < 0.05$) between the treatment groups of 600 and 1200 µg/L of Ce but no significant differences ($p > 0.05$) were found between these two treatment groups and the control and the 300 µg/L. Therefore, the overall results suggest that there was no relevant Ce effect on the activity of this enzyme since the control is statistically similar to all the other treatment groups. However, similar studies have reported a decrease in CAT activity on *H. portulacoides* leaves with the metal concentrations increase (e.g. Anjum et al., 2014; Duarte et al., 2012), suggesting a metal inhibitory effect on this protein synthesis pathway.

The POD activity in the shoots (Fig. 5f) was only significant ($p < 0.05$) in the 1200 µg/L treatment, when compared to the control and the other

treatment groups. With these results, POD appears to play an important role in catalysing H_2O_2 and hydroperoxide functional group (ROOH) to H_2O and hydroxyl functional group (ReOH), contributing more to the protection of cell damage, compared to CAT. Wang et al. (2007) observed a significant increase in POD activity in *Hydrilla verticillata* plants exposed to increasing concentrations of Ce compared to the control group. In fact, POD activity increased with the decrease of CAT activity, indicating that in *Hydrilla verticillata* POD also played a more crucial role against the oxidative stress imposed by the Ce than CAT.

Increases in enzyme activities in plants have been reported for low REE concentrations but when concentrations exceed certain values significant decreases are observed (Hu et al., 2004). Therefore, REE may inhibit the enzymes activities when metals' concentrations exceed a critical value. However, it is difficult to compare our results with those previously published in the literature. If low REE concentrations stimulate the growth of certain plants, critical concentrations seem to inhibit their growth (Kovarikova et al., 2019 and references therein). The use of these metals as micro-fertilizers may have a beneficial effect, improving the growth and development of certain plants when supplied in an appropriate concentration, while an excess can cause toxicity, depending on both the plant species and the substrate and the dose. Kovarikova et al. (2019) mentioned there are two opposing opinions about REE toxicity within the scientific community. One states that REE are of limited toxicity and not considered to be extremely hazardous to the environment, while the other expresses that slow accumulation can become a serious environmental problem. However, any potential ecotoxicological effects, as well as the mechanisms of action of REE, are still poorly understood.

4. Conclusions

The results obtained in this study showed an adverse effect on *H. portulacoides* physiological state due to a plant response when exposed to the Ce concentration of 1200 μg/L during 120 h. The Ce uptake and accumulation in the plant roots and shoots followed the metal's increase in the medium. Although no significant differences in growth were observed between plants exposed to Ce and the control group, a clear loss of turgor pressure was observed in the highest concentration treatment group. This group also revealed high levels of H_2O_2 and MDA, that can lead to membrane lipid peroxidation, indicating that the plant was actively responding to the toxic effect imposed by Ce when the plant shoots reached 10.0 μg of Ce per g DW of tissue (present at concentrations of 1200 μg/L of Ce in the medium). Furthermore, the highest Ce concentration triggered the ROS-scavenging action of antioxidant enzymes such as SOD and POD, contributing to the plant metal stress response. No significant change in CAT activity was observed, when compared to the control group.

The prediction of increased use of CeO_2-NP, changes in normal environmental conditions, such as a decrease in environmental pH, due to acidification of the medium or the presence of AMD in certain estuaries may result in an exponential rise in Ce availability in the estuarine environment, placing the sustainability of halophyte communities and estuarine marshes at risk.

Credit author statement

Pedro Brito: Conceptualisation, Validation, Formal Analysis, Investigation, Writing - Original Draft. Renata A. Ferreira: Methodology, Formal Analysis, Visualisation, Writing - Review & Editing. Susete Martins-Dias: Resources, Writing & Review. Olga M. Azevedo: Investigation, Writing & Editing. Miguel Caetano: Project Administration, Resources, Writing & Review. Isabel Caçador: Resources, Supervison.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2020.128973.

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