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Response of salt marsh plants to heavy metals in the Tagus estuary

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RESUMO

Há muito que a orla costeira, com os seus estuários e sapais, atrai a população para as suas margens, devido principalmente à sua localização privilegiada e de grande riqueza biológica. Consequentemente, a acção antropogénica desempenhada sobre estas regiões, que actuam frequentemente como reservatório de poluentes e nutrientes, tem vindo a exercer um impacto negativo sobre todos os ecossistemas costeiros. São vários os tipos de poluentes quer orgânicos quer inorgânicos, associados a estas regiões, como por exemplo, os metais pesados. Estes constituem uma séria problemática resultante, quer da sua elevada toxicidade quer da sua persistência. Contudo, vários estudos têm vindo a demonstrar a importância da vegetação existente nos sapais, como fitoremediadora. As plantas de sapal detêm um papel ecológico fulcral, não só devido à sua elevada produtividade, mas também à sua capacidade depurativa de retenção de metais pesados nos seus tecidos. Uma vez que os sapais são ecossistemas dinâmicos, sujeitos diariamente ao regime das marés e consequentemente a inundações periódicas, os halófitos que os colonizam estão continuamente expostos a poluentes vindos de várias fontes.

Um desses poluentes é o metal pesado zinco, muitas vezes proveniente da metalúrgica e de indústrias recicladoras de chumbo. Em baixas concentrações o Zn é um micronutriente essencial, uma vez que participa em várias funções vitais do metabolismo das plantas, nomeadamente no sistema de defesa celular contra espécies reactivas de oxigénio, como estabilizador de proteínas, enzimas e membranas contra o dano oxidativo e peroxidativo. Em concentrações elevadas é responsável por gerar espécies reactivas de oxigénio nas células vegetais, que causam por vezes danos irremediáveis ao nível dos ácidos nucleicos, peroxidação lipídica, oxidação de proteínas e redução da actividade fotossintética.

O principal objectivo deste estudo foi averiguar as respostas ecofisiológicas de *Halimione portulacoides* L. Aellen, uma halófita abundante nos sapais, perante uma gama de concentrações de Zn, que variou entre os 0 e os 400 µM, e várias salinidades (20, 30, 40 e 50 PSU). Em particular, pretende-se estudar o efeito Zn a diferentes salinidades, tanto ao nível do stress oxidativo como ao nível da resposta fotossintética. Para se atingirem estes objectivos efectuaram-se ensaios utilizando plantas de *H. portulacoides*, que foram sujeitas a diferentes tratamentos. De modo a avaliar a resposta da planta a estas diferentes condições experimentais determinaram-se as actividades enzimáticas, produtos da peroxidação lipídica e quantidade de fenóis e flavonóides. Adicionalmente, procedeu-se à medição da variação de pigmentos fotossintéticos, determinação da concentração total de vários elementos e análise da fluorescência clorofilina, parâmetros fundamentais no diagnóstico do estado fisiológico das células vegetais.

Os resultados obtidos para a actividade enzimática da superóxido dismutase, revelaram diferenças significativas ($p < 0.001$) perante as várias salinidades impostas. Embora a actividade desta enzima tenha sofrido ligeiras flutuações, ocorreu um aumento da mesma até ao tratamento de 30 PSU e 200 µM Zn, sofrendo para salinidades mais elevadas uma diminuição acentuada, indicando que a sua função antioxidante estaria comprometida. As plantas possuem um elevado número de enzimas que regulam os níveis intracelulares de H_2O_2, um dos produtos de reacção da superóxido dismutase, sendo as mais relevantes as catalases e peroxidases. Neste estudo, as actividades para a catalase e ascorbato peroxidase foram semelhantes, embora dentro de uma gama de valores muito baixa, mas estatisticamente significativas ($p < 0.05$), apresentando maior actividade para o tratamento 20 PSU e 400 µM Zn. Nos restantes tratamentos decresceram para valores muito próximos de zero revelando estarem próximas da inactivação. Contudo, existe uma relação entre as actividades de SOD, CAT e APX, uma vez que enquanto os níveis de SOD permanecem elevados, os níveis de CAT e APX revelam igualmente os maiores níveis de actividade, pois estas enzimas removem o H_2O_2 produzido nas reacções de SOD. A actividade da guaiacol peroxidase encontra-se extremamente diminuída, revelando valores muito próximos de zero. Embora os resultados de GPX não sejam estatisticamente significativos ($p > 0.05$), estes apontam que em *H. portulacoides* esta enzima actue como uma linha de defesa secundária contra o stress oxidativo. No que concerne aos níveis de malondialdeído, produto da peroxidação lipídica, ocorreu um aumento significativo ($p < 0.05$) para os tratamentos de salinidades mais baixas (20 e 30 PSU), verificando-se para salinidades mais elevadas um decréscimo abrupto. Todavia, nos tratamentos em que os níveis de MDA se apresentam mais baixos, tanto os flavonóides como fenóis e o nível de proteínas aumentaram. Os referidos aumentos sugerem que estes mecanismos não enzimáticos contribuíram imenso no combate contra os stresses impostos.

No que diz respeito aos parâmetros inerentes ao diagnóstico do estado fisiológico das células vegetais, os resultados exibem um acréscimo generalizado tanto para a clorofila a e clorofila b como para os carotenóides ($p < 0.05$), verificando-se contudo nesse aumento algumas flutuações. Todos os pigmentos registaram valores mais elevados para a concentração de Zn a 400 µM, e para a salinidade mais baixa, 20 PSU. Os resultados indicam que somente concentrações de Zn a 400 µM são tóxicas e que baixas salinidades também representam um stress ambiental para *H. portulacoides*. A análise da fluorescência clorofilina revelou um decréscimo geral nos níveis de fluorescência basal, tanto para as plantas adaptadas à luz como ao escuro, ou seja, estariam menos centros de reacção abertos, levando consequentemente a um decréscimo na capacidade fotoquímica. A fluorescência máxima apresentou-se constante em plantas adaptadas à luz. Já em plantas adaptadas ao escuro ocorreram alguns decréscimos acentuados. O parâmetro, F_v/F_m, que

estima a eficiência máxima da fotoquimica do fotosistema II, permaneceu idêntico quer em plantas adaptadas à luz ou ao escuro, sofrendo contudo algumas oscilações no seu conteúdo. Todos os aumentos que ocorreram para F_v/F_m foram, não devido a diminuições na fluorescência máxima como seria esperado, mas sim por decréscimos na fluorescência basal, uma vez que esta apresenta maiores taxas quando comparadas com as descritas para a fluorescência máxima. Os resultados obtidos mostraram que a fluorescência fotossintética não será um bom biomarcador para *H. portulacoides*, uma vez que permanece praticamente inalterada ($p > 0.05$), possivelmente devido à atenuação por parte dos mecanismos de defesa.

Por se saber que tanto o stress salino como o provocado por metais pesados interfere com o conteúdo iónico, foram analisadas as concentrações totais de sódio, potássio, cálcio e zinco. Uma vez que a absorção de Na causa despolarização na membrana plasmática com perda de K, sugere-se neste trabalho uma homeostase extremamente eficiente por parte do halófito em estudo, já que os resultados revelam que na parte aérea as concentrações de K e Ca permanecem praticamente inalteradas. Por outro lado a raiz, apresenta elevadas concentrações de Na acompanhadas de aumentos na concentração de Ca, indicando que neste caso o Ca desempenha uma função reguladora como mensageiro secundário. Quanto às concentrações totais de Zn, acompanham o progressivo aumento externo do mesmo metal, apresentando como esperado, maiores concentrações na raiz. Os elevados valores presentes na raiz poderão ser explicados devido à maior capacidade acumuladora de metais efectiva por parte de *H. portulacoides*, quando comparado com outras halófitas.

Em suma, as respostas desta halófita às várias concentrações de Zn e NaCl, aparentemente são expressas através do aumento da capacidade antioxidante por mecanismos enzimáticos e não enzimáticos, nomeadamente actividade de SOD e fenóis, pigmentos fotossintéticos e homeostase iónica.

O conhecimento das respostas fisiológicas de *H. portulacoides* aos stresses impostos, será um dado fulcral na percepção da capacidade que apresenta em habitar locais contaminados por metais, nomeadamente o Zn.

Palavras-chave: *Halimione portulacoides*, Zinco, Salinidade, Fotossíntese, Enzimas antioxidantes.

ABSTRACT

Due to their localization salt marsh plants are exposed to several pollution types, being sinks of pollutants, namely heavy metals, which are known by its ability to interfere with plant metabolism. In this work, *Halimione portulacoides* was subject to different salinities and Zn concentrations, ranging from 20 - 50 PSU and 0 - 400 µM, respectively.

Antioxidant enzymes activities, lipid peroxidation, phenols content, chlorophyll fluorescence and photosynthetic pigments were measured. The results showed that the superoxide dismutase activity suffered several oscillations in its content and catalase, ascorbate and guaiacol peroxidase were found to be inactive. As a lipid peroxidation parameter, MDA content greatly increased with lower salinities, decreasing abruptly for higher ones. Yet, it was interesting that the lowest values for the MDA content appeared just in the treatments were the content for flavonoids and phenols were highest. Furthermore, the protein content also suffered a peak just when the MDA content decreased. Leaves showed an overall increase in either chlorophyll a and b and total carotenoid content.

The chlorophyll fluorescence technique, revealed that the maximum quantum efficiency of PSII photochemistry, was identical either for light or dark-adapted leaves, yet suffering some oscilations. All the increases reported in the F_v/F_m, were caused not by lower F_m values but for F_0 decreases.

The determination of K, Ca and Na total concentration for stems and leaves, revealed almost unchangeable concentrations, suggesting a remarkably efficient homeostasis. The root huge accumulations in total calcium concentration indicate that in this case calcium plays a regulating role functioning as a secondary messenger.

Based on these results, it was concluded that *H. portulacoides* responses to Zn under different salinities appear largely to depend on changes in the antioxidant mechanisms, and in its pigments concentrations rather than affecting the PSII.

Keywords: *Halimione portulacoides*, Zinc, Salinity, Photosynthesis, Antioxidant enzymes.

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ABBREVIATIONS

ANOVA	Analysis of variance
APX	Ascorbate peroxidase
CAT	Catalase
Ca	Calcium
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Cl	Chloride
CO_2	Carbon dioxide
Cx+c	Carotenoids
DW	Dry weight
E.C	Enzyme Commission
F_0	Basal fluorescence level in the dark-adapted state
F_0'	Basal fluorescence level in light-adapted state
F_M	Maximum fluorescence level in the dark-adapted state
F_M'	Maximum fluorescence level in light-adapted state
F_v	Variable fluorescence
F_v/F_m	Maximum quantum efficiency of PSII photochemistry
FW	Fresh weight
G.A	Gallic acid
GPX	Guaiacol peroxidase
H_2O_2	Hydrogen peroxide
K	Potassium
MDA	Malondialdehyde
Na	Sodium
O_2	Oxygen
PS	Photosystem
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloro acetic acid
Zn	Zinc

CHAPTER 1

General Introduction

GENERAL INTRODUTION

Estuaries and salt marshes have attracted the populations to their margins as a result of their privileged localization, unique characteristics and natural richness. Consequently, these regions have suffered the development and negative impact brought through the anthropogenic action. By this reason these places are often sinks of pollutants and nutrients, namely dissolved organic matter and heavy metals (Almeida *et al*., 2008; Caçador *et al*., 1996; Reboreda and Caçador 2007). However, salt marsh vegetation influences all services of the estuary, retaining metals in their tissues, having this way an important ecological role. This recognized importance is due not only to their high productivity, but also due to their depuration ability (Magalhães *et al*., 2002), reducing the toxicity to benthic organisms (Almeida *et al*., 2009). By all these reasons, halophytes have been pointed out as suitable for several phytoremediation processes.

Halimione portulacoides L. Aellen is a perennial, shrubby C_3 halophyte (salt tolerant) with a dispersion limited to coastal salt marshes, and therefore highly influenced by both flooding stresses and salinity, (hipo- or hipersalinities) (Redondo-Gómez *et al*., 2007 and 2010). Moreover, as a halophyte, *H. portulacoides* has several adaptation mechanisms to the osmotic and ionic challenges of saline environments, since sediment salinity is one of the major abiotic stresses that adversely affect plants, caused by the excess of chloride and sodium ions. Previous experimental studies showed that *H. portulacoides* can maintain growth over a high range of salinities, mainly under high nitrate availability (Jensen, 1985). Additionally, other studies also reveal that this halophyte, it's an effective accumulator of metals compared to other vegetal species (Caçador *et al.,* 2000). More recently Sousa *et al*. (2008) had demonstrated that this halophyte mostly retaining metals in the cell walls of their roots. Therefore, it's reasonable to hypothesize that is expectable to found in roots high levels of the study metal.

Plants are able to uptake metals from the sediment throughout their roots, where the major accumulation occurs, translocating a small part to the stems and leaves (Caçador *et al*., 2000). In fact, halophyte plants have different uptake of metals depending on their mobility and availability in sediments. These factors are intrinsically associated with the physical and chemical characteristics of the sediment, such as pH, salinity, redox potential, organic matter content and grain size, characteristics that plant activity can also modify (Alloway, 1990 in Reboreda and Caçador 2007; Almeida *et al.,* 2008).

Being surrounded by highly industrialized and urbanized areas, salt marshes are often sinks of contaminants that in sum will affect plant metabolism. One of these pollutants is the heavy metal Zn. Zn is an essential micronutrient, with well establish vital functions in plant metabolism, that turns out to be toxic when its concentration increases (Zhang *et al.,* 2007).

Along with other roles, Zn plays a critical role in the defense system of cells against reactive oxygen species. Besides that, Zn has been known to have further functions. Additionally, it also has the ability to act as an important protective agent, stabilizing proteins, enzymes, membranes against oxidative and peroxidative damage, chlorophyll and DNA-binding proteins (Zn-fingers) (Aravind and Prasad 2003). Still it's important to have in consideration that the relative toxicity of heavy metals depends on their availability, and consequently on sediment property and plant species. Another common type of pollution is the water salinity level changing, usually due to industrial waste and domestic sewage. When the salinity undergoes a change, the osmotic pressure of plant cells suffers an imbalance that may lead to tragically damages. Thus, in this study salt stress was also taken in consideration because it has been revealed was an emerging problem.

The main goal of this study was to investigate the ecophysiological responses of *H. portulacoides*, over a wide range of experimental Zn concentrations under different external salinity supplies, 0 to 400 µM and 0 to 50 PSU, respectively. It is known that salt stresses aggravate ROS mainly in the chloroplastic compartments of the cells (Jithesh *et al*., 2006). By this reason and based on other salt and metal tolerance studies, a part of the investigation plan was directed to the leaves antioxidant status. By this propose several biochemical, cellular and physiological parameters were measured, namely the antioxidative mechanisms and the photosynthetic response. The knowledge of the physiological consequences of heavy metal and high salinities exposure, as well as an insight on the underling mechanisms of defense may bring a new understanding on the capacity of *H. portulacoides* to adapt and colonize contaminated sites of the Tagus estuary.

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CHAPTER 2

Antioxidative mechanisms

ABSTRACT

The effect of multiple Zn (0, 100, 200, 400 µM) and NaCl (20, 30, 40, 50 PSU) concentrations on the activity of antioxidant enzymes, lipid peroxidation, flavonoids, phenols and protein content were studied in *Halimione portulacoides* leaves. The main purpose of this study was to in light the defense mechanisms that underlining the oxidative stress. The results showed that the superoxide dismutase (SOD, E.C. 1.15.1.1) activity, while with slight fluctuations, increased until the 30 PSU and 200 µM Zn treatment. For higher salinities SOD activity decreased sharply, indicating that the oxygen scavenging function of SOD was impaired. Catalase (CAT, E.C. 1.11.1.6) and ascorbate peroxidase (APX, E.C. 1.11.1.1) activity showed similar behaves, with a major activity at 20 PSU and 400 µM Zn and then decreasing its activity for minimal values. Previous studies have been reported a large increase in APX activity after salinity stress. Nevertheless, in this study the SOD activity wasn´t upregulated, so the H_2O_2 levels normally generated from this reaction remained low, thus probably justifying the inactivation of CAT and APX activities. The guaiacol peroxidase (GPX, E.C. 1.11.1.7) was found to be inactive, assuming that in *H. portulacoides* GPX may act as a secondary line in stress defense.

As a lipid peroxidation parameter, MDA content greatly increased with lower salinities, decreasing abruptly for higher ones. Yet, it was interesting that the lowest values for the MDA content appeared just in the treatments were the content of flavonoids and phenols were highest. Furthermore, the protein content also suffered a peak just when the MDA content decreased, presuming that this raise served as a protective mechanism against the given treatment.

Broadly, this study shows an overall increased in the antioxidant defense system, a crucial step in the detoxification mechanism.

Keywords: Antioxidant enzyme activities, Lipid peroxidation, Flavonoids, Phenols.

1. INTRODUCTION

One of the major consequences of heavy metal toxic levels is not only the reactive oxygen species production, but also the free radicals increment. ROS are normally produced in plant cells as a result of mitochondrial, peroxissomal and chloroplastic activities. However, under normal circumstances the concentration of oxygen radicals remains low, due to the intrinsic detoxification mechanisms (Jithesh *et al.,* 2006).

In order to overcome oxidative stress situations, plants have developed an efficient and well organized antioxidant defense system, composed by enzymatic and non-enzymatic mechanisms, that in fact, eliminate or reduce the damaging effects (Assada, 1992; Foyer, 1993; Li *et al.*, 2008). Some of the most relevant enzymes when evaluating these mechanisms are superoxide dismutase (SOD, E.C. 1.15.1.1), catalase (CAT, E.C. 1.11.1.6), ascorbate peroxidase (APX, E.C. 1.11.1.11) and guaiacol peroxidase (GPX, E.C. 1.11.1.7), among others. The mechanism of action of these enzymes is, at least in part, thought to be associated, once that SOD catalyzes the detoxification of the free radical $O_2^{\bullet-}$ into O_2 and H_2O_2, while CAT and several classes of peroxidases like APX and GPX, scavenge the H_2O_2 into H_2O and O_2 (Aravind and Prasad, 2003). The non-enzymatic mechanisms include antioxidants, such as phenolic compounds and flavonoids that play a key role in delaying and/or preventing oxidative reactions catalyzed by free radicals. Additionally, the lipid peroxidation products increasing are also believed to be an indicator of free radical production and tissue damage. Actually, lipid peroxidation alters membrane proprieties such as fluidity and permeability and thereby the membrane functions. In the reaction of peroxidation, the membrane suffers a chain reaction in which unsaturated fatty acids are converted into different small hydrocarbon fragments such as malondialdehyde (Prasad, 2004). The measurement of MDA is this way considered to be an excellent parameter to measure membrane damage (Ohkawa *et al.,* 1979 in Zhang *et al.,* 2007).

As a sink of several contaminants, that will affect plant metabolism, salt marshes are often polluted with several heavy metals. Zn is an essential micronutrient included in the category of essential heavy metals. Presenting a defense function, it is also known that Zn has the ability to stabilize several proteins, enzymes and biomembranes against oxidative and peroxidative damage (Bettger *et al.,* 1981), thus revealing an antioxidant activity (Aravind and Prasad, 2003). Moreover, under deficient supply, the organism shows signals of poor yield and growth and when in elevated concentrations it causes several disruptions, indicating is toxicity, affecting a multiplicity of cellular components, and therefore interfering with normal metabolic functions and consequently growth (Kamal *et al.*, 2004).

The Tagus estuary salt marshes are abundantly colonized by the halophyte *Halimione portulacoides* L. Aellen (Chenopodeaceae) a common perennial herb and one of the pioneer species in many European salt marshes (Carvalho *et al.,* 2003). In the present work, *H. portulacoides* was used as an experimental material in order to determine the activity of SOD, CAT, APX and GPX, as well as MDA, phenolic and flavonoid content. To achieve these goals *H. portulacoides* was subject to increasing salinities and different Zn concentrations. It is known that halophytes are able to accumulate a large amount of heavy metals in their cells (Caçador *et al.*, 2000; Reboreda and Caçador, 2007), however the effects of high Zn concentrations under high salinities and its consequences in the integrity of cells membranes, enzymatic activities and the photosynthetic apparatus functioning, are unknown. In this way, the aim of this work is to understand how Zn under different NaCl concentrations may affect several biochemical, cellular and ecophysiological parameters in *H. portulacoides*.

2. MATERIAL AND METHODS

2.1. Sample collection and general considerations

H. portulacoides samples were collected at the northern margin of the Tagus estuary, near the Expo 98 exhibition site, between November 2009 and January 2010, during the low tide. The plants were brought to the Marine Botany laboratory, at the Center of Oceanography– Faculty of Sciences, University of Lisbon.

To make the props, the roots and a small part of the stem were cut, leaving at least two nods in the stem below the lowest branch, in order to gain grafts. The props were placed in a greenhouse, in dark-walled vases and filled with modified ¼ Hoagland nutrient solution (Hoagland and Arnon, 1950), for approximately one and a half month to allow new root biomass growth. During the experiments, plants were subjected to natural day night regime, with natural sunlight.

The concentrations for Zn and salinity chosen to achieve this aim of this work were based on values found in nature for those items, namely, 30 PSU for salinity and 3.75 mM for Zn sediment concentration. These concentrations were established according to the calculations of previous works and considering only the Zn available fraction described in field studies (Duarte *et al.,* 2008 and 2010).

2.2. Zn exposure

After the growing period the Hoagland solution was replaced by a new nutritive solution, of similar composition but without FeNaEDTA, removed in order to prevent possible chelating reactions with the metal in study. The plants were washed and placed in groups of five, in a total of sixteen vases, with several Zn and NaCl concentrations (Table 1, *in Appendix*). This new solution was renewed every 3 days. The pH of the solutions was kept at 5.5 in order to avoid Zn complexes. Samples from the different treatments were collected at day 0 and 7. All samples collected for the ecophysiological determinations were instantly frozen in liquid nitrogen and stored at -80ºC. All the values, from those determinations, below the detection limit were considered zero.

2.3. Estimation of Lipid Peroxides

According to Heath and Packer, 1968, the leaf samples were homogenized in 0.5% TBA containing 20% TCA (100:1 FW m/v acid) with a glass rod. The homogenate was extracted

at 95ºC for 30 minutes and subsequently the reaction was immediately stopped in ice and centrifuged at 3000 x g for 5 minutes at 4 ºC. The absorbance of the supernatant was read at 532 and 600 nm in a Shimadzu UV-1603 spectrophotometer. The non-specific absorbance at 600 nm was subtracted to the 532 nm absorbance. The concentration of malondialdehyde (MDA) was calculated using the molar extinction coefficient, 155 mM^{-1} cm^{-1}.

2.4. Enzymatic assays

All enzymatic analyses were performed at 4º C and according to Tiryakioglu *et al.,* 2006. A total of 500 mg of fresh leaves were extracted in 8 ml of 50 mM sodium phosphate buffer (pH 7.6) supplemented with 0.1 mM Na-EDTA.

The homogenate was centrifuged at 14000 x g for 20 minutes at 4 ºC, and the supernatant was used for the enzymatic assays. The enzymatic activities were determined in a period no longer than 1 hour. All of the spectrophotometric measurements were performed using quartz cuvettes, in a Shimadzu UV-1603 spectrophotometer.

Protein content was determined according to Bradford (1976), using bovine serum albumin as the standard, and the absorbance was measured at 595 nm in a TECAN Absorbance Microplate Reader (SPECTRA Rainbow).

2.4.1 Catalase (CAT, E.C. 1.11.1.6) activity was measured according to the method of Teranishi *et al.* (1974), by monitoring the consumption of H_2O_2, and consequent decrease in absorbance at 240 nm during 60 sec. (molar extinction coefficient of 39.4 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, and 100 mM of H_2O_2. The reaction was initiated with the addition of 100 µL of enzyme extract.

2.4.2 Ascorbate peroxidase (APX, E.C. 1.11.1.11) was assayed according to Tiryakioglu *et al.* (2006). The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.0), 12 mM of H_2O_2, 0.25 mM L-ascorbate. The reaction was initiated with the addition of 100 µL of enzyme extract. The activity was recorded as the decrease in absorbance at 290 nm for 1 minute, and the amount of ascorbate oxidized was calculated from the molar extinction coefficient of 2.8 mM^{-1} cm^{-1}.

2.4.3 Guaiacol peroxidase (GPX, E.C. 1.11.1.7) was measured by the method of Bergmeyer (1974) with a reaction mixture consisting of 50 mM of sodium phosphate buffer (pH 7.0), 2 mM of H_2O_2, and 20 mM of guaiacol. The reaction was initiated with the addition of 100 µL of enzyme extract. The enzymatic activity

was measured by monitoring the increase in absorbance at 470 nm during 1 min. (molar extinction coefficient of 26.6 mM^{-1} cm^{-1}).

2.4.4 Superoxide dismutase (SOD E.C. 1.15.1.1) activity was assayed according to Marklund (1974) by monitoring the reduction reaction of pyrogallol at 325 nm. The reaction mixture contained 50 mM of sodium phosphate buffer (pH 7.6), 0.1 mM of Na-EDTA, 3 mM of pyrogallol, Mili-Q water. The reaction was initiated with the addition of 100 µL of enzyme extract. The reaction was monitored during 1 min. Control assays were done in the absence of substrate in order to evaluate the auto-oxidation of the substrates. All enzymatic activities were expressed as U/ (min^{-1} µg of protein).

2.5. Quantification of Flavonoids and Phenolics

For the extraction of phenolics and flavonoids the fresh material was homogenized in methanol in the proportion of 0.01:1 (FW: m/v) and centrifuged at 3000 g for 10 minutes at 4ºC.

The quantification of the flavonoids, was based in the method described by Kumazawa *et al.* (2004). 1 ml of methanolic extract was added with 1 ml of ethanolic solution aluminum chloride 2 % (m/v). The mixture was kept for 1h in darkness, at room temperature, and then the readings were performed at 420 nm and compared with the calibration curve for quercitin. The flavonoid content was expressed as quercitin milli -equivalents per gram of fresh weight.

Phenolics were quantified by the method of Folin-Ciocalteu (1927), using a reaction mixture consisting of 1 ml of methanolic extract, 5 ml of diluted Folin-Ciocalteu´s phenol reagent (1:10 v/v) and 4 ml of sodium carbonate 7.5 % (m/v). The mixture was also kept for 1h in darkness at room temperature, and then the absorbance readings were performed at 750 nm and compared with the calibration curve for gallic acid (GA). Phenolic content was expressed as GA milli-equivalents per gram of fresh weight.

2.6. Statistical analysis

Statistical analysis was performed using Statistic Software version 9.0 from Statasoft Inc.

Because we compared more than two independent samples and the null hypothesis for normality was rejected, non parametric Kruskal-Wallis test was used.

Five replicates were always considered and in all of the comparisons, the significance level to reject the null hypothesis was 5%.

The study population was divided into two separate groups; the control group and the experimental group that was submitted to the several different treatments. In order to search for the existence of significant differences between the parameters measured in all groups, it was necessary to verify first if all variables were normally distributed, in order to choose the statistical test to apply. Normality was analyzed using the Kolmogorov-Smirnov test. Since almost all variables presented unequally distribution, we opted to apply the non parametric test Kruskal-Wallis to all groups, given the fact that there were more than two independent variables, instead of apply the parametric test ANOVA to the ones that were normally distributed and the non parametric to the others.

3. RESULTS

3.1. Antioxidant enzymatic activity

The superoxide dismutase, catalase, ascorbate and guaiacol peroxidase antioxidant activities responses to Zn and NaCl in *H. portulacoides* leaves were shown in Fig.1A-D.

The superoxide dismutase activity (Fig. 1A), revealed that salinities treatments significant affected this parameter ($p < 0.001$), increased is content for the 20 PSU and 30 PSU treatments, where the major peaks of activity were detected. On the other hand, SOD activity have suffered an overall decreased in is content for the 40 and 50 PSU treatment when compared to the control group.

The analysis of catalase activity (Fig. 1B) showed higher activity for the 20 PSU and 400 µM Zn treatment. The activities in treatments 20 PSU with 0 and 100 µM Zn and 40 PSU with 200 µM Zn were slightly higher than the control group, being this differences statistically significant ($p < 0.05$).

Resembling the CAT activity the APX activity (Fig. 1C) presented a higher concentration at the 20 PSU with 400 µM Zn treatment. Besides its activity have been increased with 20 PSU and 0 µM Zn, in all other treatments it was observed that ascorbate activity stayed significantly quite low ($p < 0.001$).

The results obtained for GPX activity (Fig. 1D), showed constants ups and downs, presenting always values near zero. The results weren´t statistically significant ($p > 0.05$).

Note that almost all enzymatic activities revealed a large standard deviation.

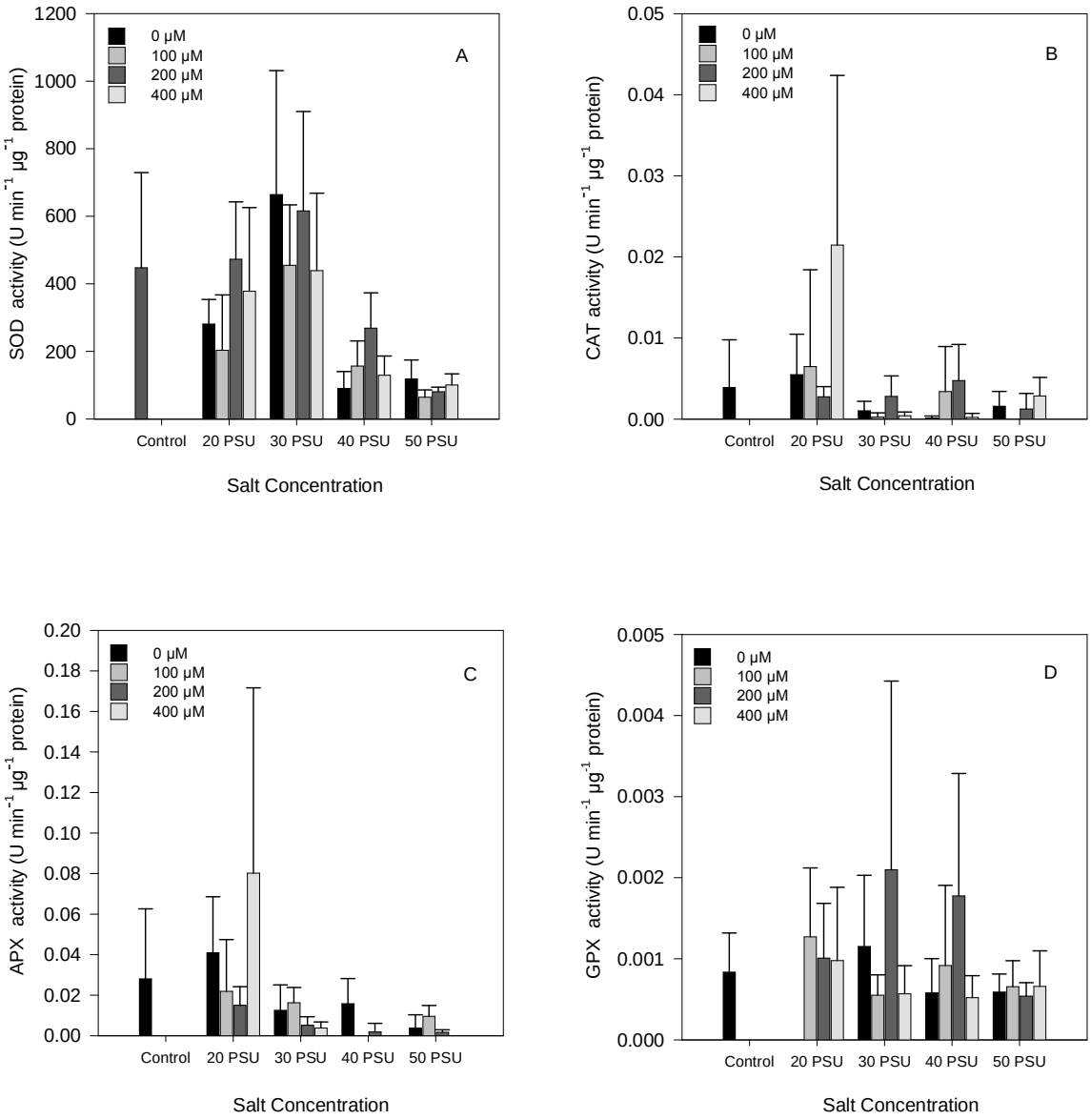

Fig. 1. Change in the SOD (A), CAT (B), APX (C) and GPX (D) activity on *H. portulacoides* leaves, under different NaCl and Zn concentrations. (Data presented as means ± S.D., n=5).

3.2. Lipid Peroxidation Products

Changes in the membrane lipid peroxidation content detected in leaves are shown in Fig. 2. The MDA content showed its maximum when treated with 20 PSU and 0 µM Zn. This parameter strongly increased in the 20 and 30 PSU treatments compared with the control (plants at day zero), having a subsequent abrupt descending in leaves treated with 40 and 50 PSU. The comparison between the different treatments showed statistically significant differences among the different salt treatments ($p < 0.001$) in the amount of MDA founded.

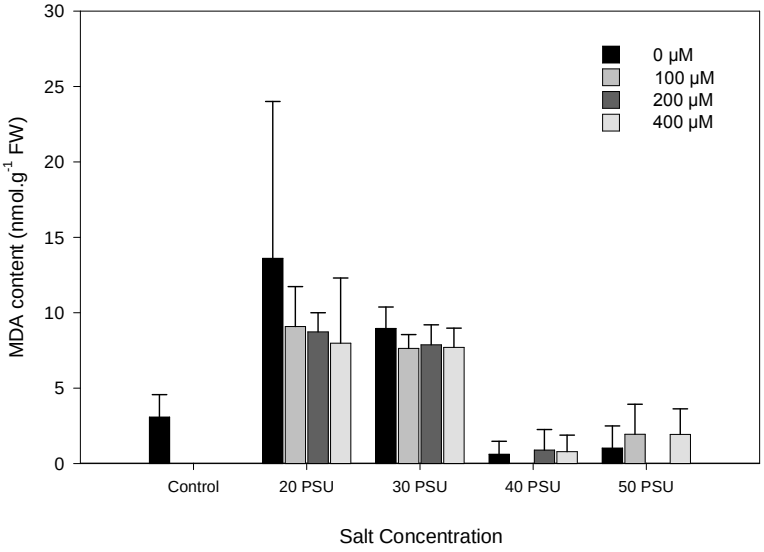

Fig. 2. Change of the MDA content in *H. portulacoides* leaves, under different NaCl and Zn concentrations. (Data presented as means ± S.D., n=5).

3.3. Flavonoid and Phenolic content

Zn and salinity exposure significantly affected the flavonoid content on *H. portulacoides* ($p < 0.05$) (Fig.3A). Plants treated with 20 PSU and 200 and 400 µM Zn, 30 PSU and 200 µM Zn, 40 PSU with 400 µM Zn and 50 PSU with 200 µM Zn were the less affected, revealing the lowest values. The remaining treatments were all higher than the control level, having two peaks at 40 PSU with 0 µM Zn and 50 PSU with 100 µM Zn.

In all the treatments, preceding the 40 PSU with 200 µM Zn the phenolic content (Fig. 3B) was, similar to control levels (zero). The 40 PSU with 200 µM Zn treatment leaded to a significant severe increase ($p < 0.001$) on this parameter, in mean 21.6 times higher than the previous ones.

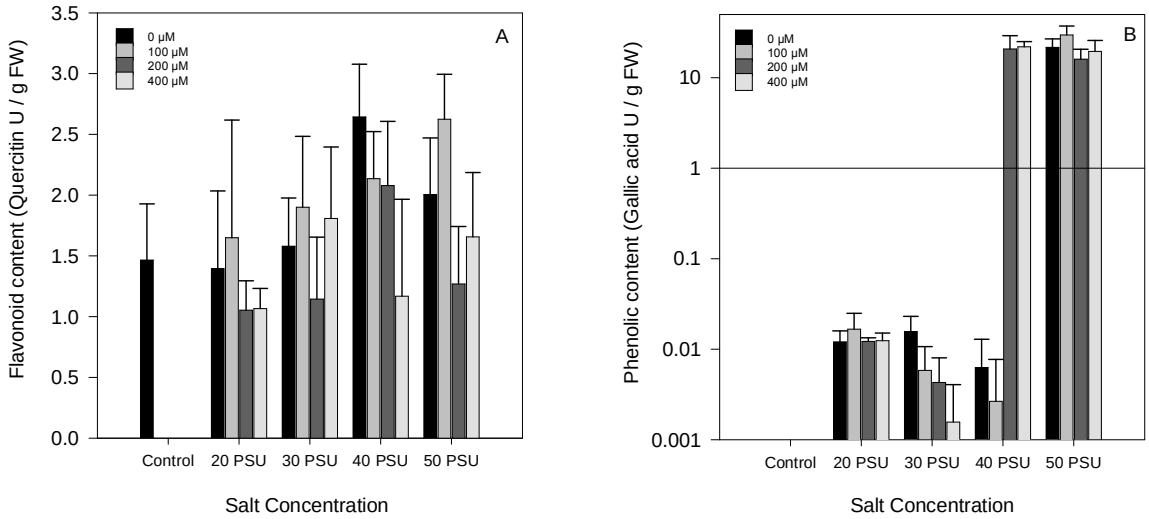

Fig. 3. Change of the Flavonoid (A) and Phenolic (B) content in *H. portulacoides* leaves, under different NaCl and Zn concentrations. (Data presented as means ± S.D., n=5). The Phenolic content was Log (common) transformed.

3.4. Protein content

The Zn and salinity treatments did not significantly affected protein content on *H. portulacoides* leaves ($p > 0.05$) (Fig. 4). Besides this parameter had suffered slightly fluctuations, with a peak for the 40 PSU with 0 µM Zn treatment, which presented however a large standard deviation, the values were all around the same range.

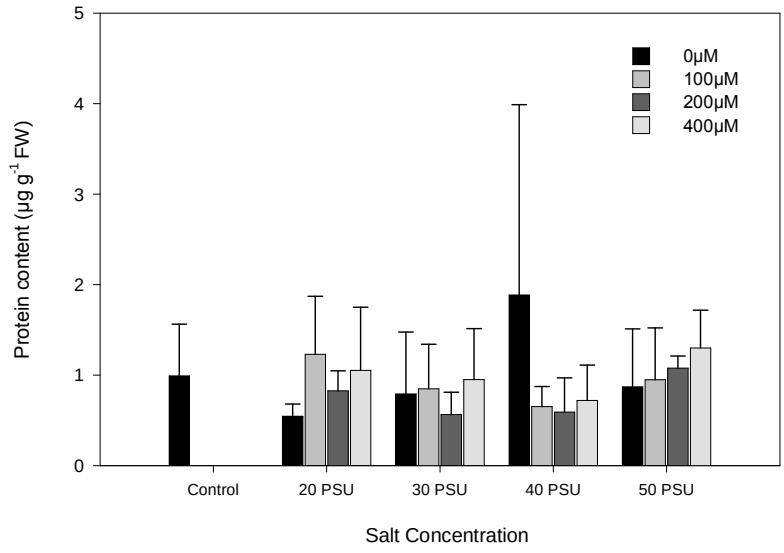

Fig. 4. Change of the Protein content in *H. portulacoides* leaves, under different NaCl and Zn concentrations. (Data presented as means ± S.D., n=5).

4. DISCUSSION

As previous described, there are several environmental stresses that can cause the accumulation of reactive oxygen species in tissues and their detoxification is the major goal of plant cell metabolism. In fact, ROS are routinely generated during normal cell metabolic processes. However, various environmental stresses can cause ROS accumulation, namely the superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and singlet oxygen ($^1O_2$) (Devi and Prasad, 1998). ROS can rapidly attack important cellular components, leading to cell death. By this way plants have developed an effective antioxidative mechanism, with enzymatic and non-enzymatic components (Jithesh *et al.,* 2006).

The general aim of this study was to investigate the ecophysiological responses of *H. portulacoides*, over a wide range of experimental salinity and Zn concentrations. In order to achieve this goal *H. portulacoides* plants were subject to sixteen different treatments, and essayed for biochemical, cellular and physiological parameters, including the antioxidative mechanisms.

The results showed that SOD activity in leaves was significantly affected by salinity treatments. At highest salinities concentrations, this enzyme activity decreased sharply, indicating that the oxygen scavenging function of SOD was impaired. These data is in agreement with the results from *Kandelia candel* and *Bruguiera gymnorrhiza* (Zhang *et al*., 2007). Interestingly, several studies have reported the increase in total SOD activity, during salt stress (Cherian *et al.,* 1999; Parida *et al.,* 2004; Takemura *et al.,* 2000), result that wasn´t accomplished in this study, perhaps due to the short experimental time (only seven days). One of the reaction products of SOD activity is H_2O_2. Plants possess an elevated number of enzymes that regulate the H_2O_2 intracellular level, among which the most important ones are catalases and peroxidases. In this study, both CAT and APX activities were similar and statistically affected by the given treatments, although presenting very low concentrations. In fact, CAT and APX are antioxidants enzymes involved in the same general functions, but with different reducing agents. Parida *et al*. (2004) showed that when plants were subject to NaCl stress conditions, a decreased in total catalase activity was also observed. On the other hand, several studies have reported a large increase in APX activity after salinity stress. Nevertheless, in this study the SOD activity was impaired, so the H_2O_2 levels normally generated from this reaction remained low. On the other hand the $O_2^{\bullet-}$ levels scavenged with this impaired enzyme probably increased, thus justifying the inactivation of APX and CAT activities, once that its known that CAT is sensitive to $O_2^{\bullet-}$ and can actually be inactivated by its increasing levels (Cakmak, 2000). The GPX activity (Fig. 1D) exhibited extremely low values, point out that this enzyme should also be near inactivation. Based on

this we can assume that probably this enzyme acts as a secondary line in stress defense in *H. portulacoides* (Aravind and Prasad 2003). All the enzymatic activities values reach in this study were comparatively much lower than the ones found in Li *et al*. (2008) and Zhang *et al*. (2007).

MDA is a decomposition product of polyunsaturated fatty acids hydroperoxides (Li *et al*., 2008), commonly used as an index of lipid peroxidation under stress conditions (Zhang *et al*., 2007), a consequence of oxidative damage. MDA content (Fig. 2) was significantly affected by external salinity, increasing until the 30 PSU treatment and decreasing abruptly for higher salinities. These results are in disagreement with other previous studies that reported a reduction in the MDA content but, at the same time, an increased in antioxidant enzyme activities (Li *et al*., 2008; Zhang *et al.,* 2007). Yet, it was interesting that the lowest values for the MDA content appeared only in the treatments were the content of flavonoids and phenols were highest (Fig. 3A and 3B, respectively). This data revealed that the antioxidant defense system in *H. portulacoides* was mainly leading by the non-enzymatic mechanisms in order to eliminate the damaging effects of the given treatments.

Additionally, the protein content was all in the same range values, although showing slights decreases and increases with the different treatments. The decreases on the protein content can probably be justified was the resulting inactivation of a variety of enzymes, since high concentrations of this heavy metal causes several disruptions, affecting a multiplicity of cellular components, including proteins (Kamal *et al.*, 2004), leading to his degradation (Palma *et al.,* 2002). The increases verified, could be indicative that this raise served as a protective mechanism against those treatments.

Note that, in some cases, namely SOD activity, flavonoids, proteins and MDA content, the control (day zero) exhibit an awkward high level. A possible explanation might be the fact that the props were growing in a greenhouse for one and a half month, being subject to different environmental conditions.

The results obtained in this study point out to a combined effect of different salinities and Zn concentrations in *H. portulacoides* leaves, enhanced mainly non-enzymatic mechanisms in the presence of higher salinities, where the antioxidant enzymes activities remained low. Plants treated with lower salinities, (20 and 30 PSU), showed high MDA content and high SOD antioxidant activity, leading to an increase in ROS scavenging ability. This skill can be crucial to *H. portulacoides* survival in its habitat were the salinity and heavy metal stress are increasingly as an emerging problem.

Additional studies should be performed in order to fully clarify the ecophysiological responses of *H. portulacoides* when subject to similar abiotic stresses. Nevertheless, it is worth to mention the complexity of the plant metabolic system that may not be explained only by

these parameters and that there are other factors contributing to the detoxification of ROS that weren´t taken in consideration in the present work.

Also in future studies, we should contemplate both the quantitative and qualitative proteomic analyses considering the possible proteome differences among *H. portulacoides* populations that colonize different estuaries in the Portuguese coast. Those analyses may clarify the action of several proteins, metabolic and cell signalization processes, parameters with an extremely importance to became possible the whole response understanding to these kind of treatments.

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CHAPTER 3

Photosynthetic Responses

ABSTRACT

This work investigates the photosynthetic responses to a range of Zn (0, 100, 200 and 400 µM) and salinities concentrations (20, 30, 40, 50 PSU) in *Halimione portulacoides*, a halophytic C3 shrub. Chlorophyll fluorescence, photosynthetic pigments, sodium, potassium, calcium, and Zn total concentrations were determined.

Leaves showed an overall increase either in chlorophyll a, chlorophyll b and total carotenoid content, with the higher pigment values appearing in the 400 µM Zn treatments, indicating that only Zn concentrations above 400 µM were found to be toxic to the plant. In the same way, at 20 PSU and 0 µM Zn the values of all photosynthetic pigments had suffered a huge increase, suggesting that in *H. portulacoides* low salinity might represent an environmental stress.

The chlorophyll fluorescence technique revealed that the maximum quantum efficiency of PSII photochemistry, was identical either for light or dark-adapted leaves, yet suffering some oscilations. All the increases reported in the F_v/F_m, were caused not by lower F_m values but for F_0 decreases.

The determination of K, Ca and Na total concentration for stems and leaves, revealed almost unchangeable concentrations, suggesting a remarkably efficient homeostasis in this halophyte. The root huge accumulations in total calcium concentration indicate that in this case calcium plays a regulating role functioning as a secondary messenger.

Based on these results, it was concluded that *H. portulacoides* responses to Zn appear largely to depend on changes in its pigments concentrations rather than in effects on PSII.

Keywords: Chlorophyll fluorescence, Photosynthesis, Photosystem II, Photosynthetic pigments.

1. INTRODUTION

Sediment salinity is one of the major abiotic stresses that adversely affect plants, caused by water evaporation that leads to an excess of Cl^- and Na^+ ions. Due to the periodic tidal flooding, only a few salt-tolerant species (halophytes) are able to survive in coastal salt marshes, supporting salinities two- to three-times higher than seawater, (Redondo-Gómez *et al*., 2006).

The Tagus estuary salt marshes are colonized by several halophytes, being the more abundant the *Halimione portulacoides, Spartina maritima* and some species of *Sarcocornia* (Duarte *et al.,* 2010). In these plants salt stress has been reported to affect mainly the CO_2 diffusion in leaves, throughout a decrease in stomatal and mesophyll conductance's, influencing thereby many different aspects of growth. Moreover, *H. portulacoides* studies have revealed that salt stimulates growth even at 200 mol m^{-3} NaCl and that some growth was observed even at higher salinities (Redondo-Gómez *et al*., 2007). It is also known that at cellular levels there are ionic effects imposed by salinity namely concerning Na^+ and K^+ ions. Internal excesses of particular ions may cause membrane damage (Volkmar *et al.,* 1998).

H. portulacoides is also known for its ability to scavenge and tolerate heavy metals (Caçador *et al.,* 2000; Reboreda and Caçador 2007; Sousa *et al.,* 2008). The heavy metals effects in plants depend on both physic and chemical properties of the sediment, which will affect their availability and mobility. One the other hand, plants also have an intrinsic genetic ability to react against those abiotic stresses. Furthermore, the metal characteristics will dictate the absortion, accumulation and translocation processes within the plant. The heavy metal tolerance mechanisms in vegetal cells are commonly associated with, retention of metals in the cell wall, restrictions on the uptake, cell exportation, bounding to proteins, lipids, DNA, polyphosphates, as well as chelation to metalloproteins, phytochelatins (Nalimova *et al.,* 2005) and also with organic acids (Duarte *et al.,* 2007).

Previous *in vitro* studies measured the damage caused by heavy metals in the photosynthetic apparatus and revealed that the PSII is one of the most sensitive spot to detect metals action. This is mainly a consequence of the fact that heavy metals can inhibit the electron chain reactions, blocking the production of ATP and $NADPH_2$ or directly by inactivating the reaction center of PSII, thereby interfering with the photosynthetic efficiency (Sigfridsson *et al.,* 2004). However, *in vitro* studies using isolated thylakoids not always reflects the photochemical activity found *in vivo*. It is well known that although the measured of gas exchange (CO_2 e O_2) is extremely important in order to determine photosynthetic rates, it might not be an efficient way to evaluate the adverse effects of several stresses in the thylakoids, as a result of some limitations inherently associated with the control of the

chamber temperature and evapotranspiration that restricts the natural conditions (Stuhlfauth *et al.,* 1988).

So, the photochemical efficiency of photosynthesis is thought to be the most reliable alternative method, measured throughout several parameters of chlorophyll a fluorescence. The chlorophyll a fluorescence efficiency reveals the energy excitement level on the pigment system that directs photosynthesis and also gives crucial information for estimating the inhibition or damage in the electron transference process from the PSII (Maxwell and Johnson, 2000). Interestingly, Redondo-Gómez *et al.* (2007) reported that for *H. portulacoides* the ranging salinity from 0 to 700 mol m^{-3} did not have any adverse effect on photosystem II, suggesting that there is no correlation between those factors.

The technique of fluorescence analysis is a non-invasive method used to study the photosynthetic performance, being a simple, quick and sensitive method to evaluate photoinhibition and pollutant effects on plants (Marwood *et al.,* 2001).

Chlorophyll fluorescence measurements give several sets of parameters with ecophysiological meaning: basal fluorescence (F_0), maximum fluorescence (F_m), variable fluorescence (F_v), maximum quantum yield of PSII (F_v/F_m), relative rate of electron transport (ETR), photochemical quenching (qP) and non-photochemical quenching (qN). The F_0 is the fluorescence with all reaction centers "open" and refers to the fluorescence emission by the molecules of chlorophyll a of PSII. The F_m is related to the complete reduction of quinone A (QA) driven by the incidence of a light pulse in the reaction center Q, generating fluorescence maximum. The difference between F_m and F_0 is called variable fluorescence (F_v). The maximum quantum yield is calculated as $F_v/F_m = (F_m-F_0) / F_m$. Actually, when the photosynthetic apparatus is intact, the F_v/F_m ratio should range between 0.75 and 0.85, while a decrease in this ratio reflects the photoinhibitory damage in PSII reaction centers (Linger *et al.,* 2005).

Some heavy metals are essential for organism life, exerting its function as cofactors of enzymes, involved in biochemical reactions or playing other metabolic roles, like Zn.

Zn is a component of all basic enzymes classes required for a normal metabolic performance, but in high concentrations becomes toxic (Nalimova *et al.,* 2005). Besides its key role in several cell components, Zn has been known to have additional functions in the defense system (Aravind and Prasad 2003).

The aim of this work is to report the photosynthetic response of *H. portulacoides* to a wide range of salinities and Zn concentrations. Therefore, plants were growth in modified Hoagland solution, with salinity treatments ranging from 20 to 50 PSU and Zn concentrations from 0 to 400 µM. Chlorophyll fluorescence, photosynthetic pigments and tissue concentration of sodium, potassium, calcium, nitrogen and total zinc content were also

determined in order to see the effects of the exposure to those stresses in the physiology of this halophyte.

2. MATERIAL AND METHODS

2.1. Sample collection and general considerations

H. portulacoides samples were collected at the northern margin of the Tagus estuary, near the Expo 98 exhibition site, between November 2009 and January 2010, during the low tide. The plants were brought to the Marine Botany laboratory, at the Center of Oceanography– Faculty of Sciences, University of Lisbon.

To make the props, the roots and a small part of the stem were cut, leaving at least two nods in the stem below the lowest branch, in order to gain grafts. The props were placed in a greenhouse, in dark-walled vases and filled with modified ¼ Hoagland nutrient solution (Hoagland and Arnon, 1950), for approximately one and a half month to allow new root biomass growth. During the experiments, plants were subjected to natural day night regime, with natural sunlight.

The concentrations for Zn and salinity chosen to achieve this aim of this work were based on values found in nature for those items, namely, 30 PSU for salinity and 3.75 mM for Zn sediment concentration. These concentrations were established according to the calculations of previous works and considering only the Zn available fraction described in field studies (Duarte *et al.,* 2008 and 2010).

2.2. Zn exposure

After the growing period the Hoagland solution was replaced by a new nutritive solution, of similar composition but without FeNaEDTA, removed in order to prevent possible chelating reactions with the metal in study. The plants were washed and placed in groups of five, in a total of sixteen vases, with several Zn and NaCl concentrations (Table 1, in Appendix). This new solution was renewed every 3 days. The pH of the solutions was kept at 5.5 in order to avoid Zn complexes. Samples from the different treatments were collected at day 0 and 7. All samples collected for the ecophysiological determinations were instantly frozen in liquid nitrogen and stored at -80ºC. All the values, from those determinations, below the detection limit were considered zero.

2.3. Photosynthetic Pigments

Photosynthetic pigments from five replicates of each treatment were extracted with 10 mL of pure methanol and kept overnight at 4 ºC. The extract was centrifuge at 4000 x g for 15 minutes at 4 ºC, and chlorophyll a, chlorophyll b and carotenoid concentration were

determined using a Shimadzu UV-1603 spectrophotometer at 665.2, 652.4 and 470 nm, according to the method of Lichtenthaler and Wellburn (1983).

2.4. Measurement of Chlorophyll Fluorescence

At the beginning (day 0) and at the end (day 7) of the experiments, the photosynthetic efficient was determined using a Diving-PAM (Pulse Amplitude Modulation) device.
For day 7, chlorophyll fluorescence was measured in five random leaves for each of the sixteen treatments using a portable Diving-PAM (Walz, Germany). Fluorescence parameters were measured in light and dark-adapted (for 30 minutes) leaves. Ground fluorescence (F_0) was measured by using a weak pulse-modulated red measuring light. The maximum fluorescence (F'_m) at 650 nm was then measured by applying a saturating pulse of actinic light (> 3000 µmol quanta.$m^{-2}s^{-1}$) for 7 ms. The distance between the fiber optic sensor and the upper surface of the leaf was kept constant (10 mm) using a leaf clip-holder.

2.5. Determination of sodium, potassium, calcium, and total zinc content

Plants were separated into aerial parts and roots and completely dried at 60ºC until constant weight. All the material was reduced to powder with liquid nitrogen. Approximately 100 mg of each sample was digested for 3 hours in a Teflon bomb, at 110 ºC, by adding 2 ml of HNO_3/$HCLO_4$ (7:1, v/v). After cooling overnight, the extracts were filtered through Whatman No. 42 (2.5 µm of pore diameter) filters, diluted with Mili-Q water to a final volume of 10 ml and analyzed by atomic absorption spectrometry (Perkin-Elmer A Analyst 100), using as reference plant material the *Olea europaea* BCR62, according to the method described in Duarte *et al.* (2007).

2.6. Statistical analysis

Statistical analysis was performed using Statistic Software version 9.0 from Statasoft Inc.
Because we compared more than two independent samples and the null hypothesis for normality was rejected, non parametric Kruskal-Wallis test was used.
Five replicates were always considered and in all of the comparisons, the significance level to reject the null hypothesis was 5%.

The study population was divided into two separate groups; the control group and the experimental group that was submitted to the several different treatments. In order to search

for the existence of significant differences between the parameters measured in all groups, it was necessary to verify first if all variables were normally distributed, in order to choose the statistical test to apply. Normality was analyzed using the Kolmogorov-Smirnov test. Since almost all variables presented unequally distribution, we opted to apply the non parametric test Kruskal-Wallis to all groups, given the fact that there were more than two independent variables, instead of apply the parametric test ANOVA to the ones that were normally distributed and the non parametric to the others.

3. RESULTS

3.1. Photosynthetic Pigment concentration

Besides pigment concentrations in leaf tissues had suffered several fluctuations, with decreases and increases along the chart line, all the treatments had values higher than the control group. Chl a and Chl b increase were only statistical significant when submitted to salinity treatments ($p < 0.05$). However, carotenoid pigment concentration was statistically significant ($p < 0.05$) for both treatment groups (salinity and Zn).

Regarding Chl a, Chl b and Cx+c charts (Fig. 5A-C) it was possible to observe a similar behavior among these parameters. Chl a and Cx+c exhibited four increasing concentration peaks at 20 PSU and 0 and 400 µM Zn, 40 PSU with 400 µM Zn and 50 PSU with 400 µM Zn. Chl b concentration showed a consistent trend when related with the other pigments concentration, except for the 50 PSU with 100 and 400 µM Zn treatment, fact that is reflected in the Chl a/b ratio (Fig. 5D). This ratio was consistent across all treatments, except for the last ones, mainly due to high differences in the Chl b values, reflected in high standard deviations patterns.

3.2. Chlorophyll Fluorescence

None of the treatments, measured whether in dark or light adapted leaves, significantly changed base or maximum fluorescence or the maximum quantum efficiency, ($p > 0.05$).

In the light-adapted conditions (Fig. 6), the base fluorescence level (F_0') (Fig. 6A) was always relatively lower than the control group, except for the 40 PSU with 0 µM Zn treatment. The values for maximum fluorescence level (F_m') (Fig.6B) were constants, although with minimal fluctuations, for the 20 and 30 PSU treatment with 400 µM Zn and for 50 PSU and 0 µM Zn.

For dark-adapted leaves, F_0 (Fig. 7A) was always lower than the control group, suffering high decreases in some treatments. The F_m (Fig.7B) parameter was either similar or relatively lower than the control, namely for the 30 PSU with 200 µM Zn and 50 PSU with 100 and 400 µM Zn treatment

Values of F_v/F_m both in light and dark-adapted plants (Fig. 6C and 7C, respectively), were similar among treatments with values varying around 0.68 and 0.71, respectively. The values were always higher than the control group (0.62 for dark-adapted plants and 0.64 for light adapted-plants), except in 40 PSU and 0 µM Zn treatment for light-adapted plants.

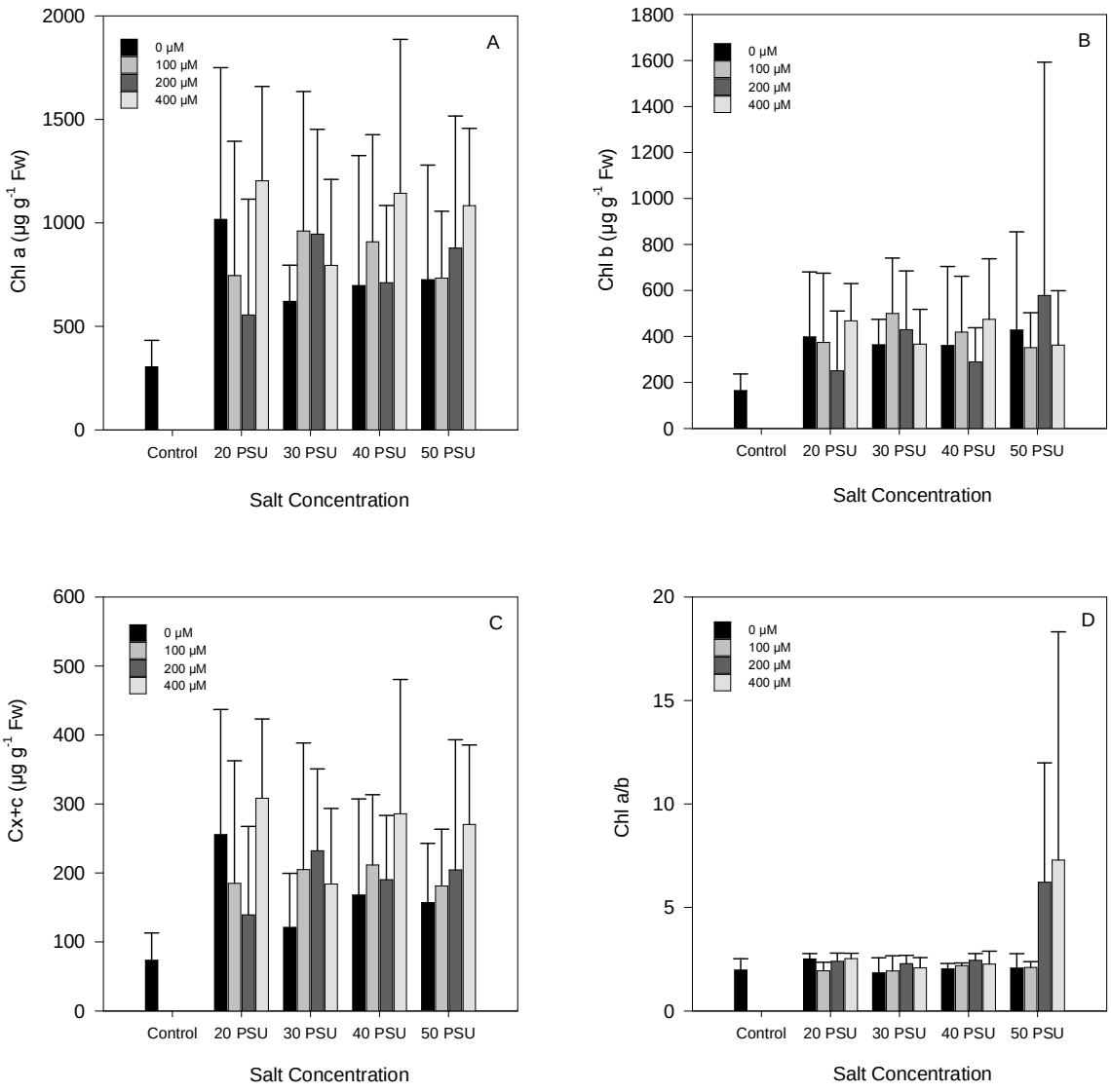

Fig. 5. Chlorophyll a (Chl a) (A), Chlorophyll b (Chl b) (B) and Carotenoid (Cx+c) (C) concentrations, and Chl a/b ratio (D) on *Halimione portulacoides* in response to a range of Zn and NaCl concentrations. Values presented as means ± S.D., n=5.

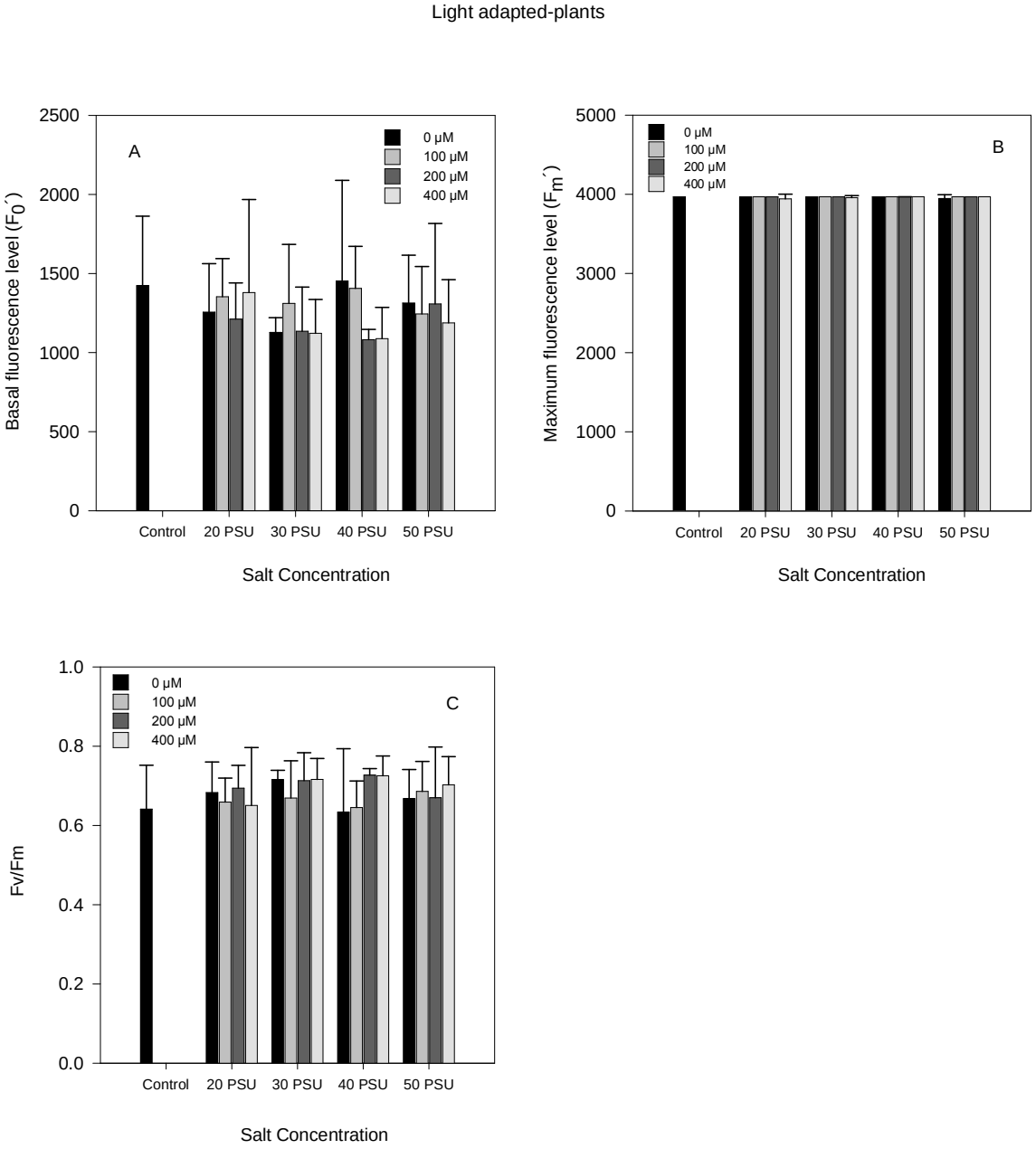

Fig. 6. Basal Fluorescence level ($F_0´$) (A), Maximum fluorescence level ($F_m´$) (B) and Maximum quantum efficiency of PSII photochemistry (F_v/F_m) (C), for light-adapted plants in randomly selected, fully expanded leaves of *Halimione portulacoides* in response to treatment with a range of Zn and NaCl concentrations. Values presented as means ± S.D., n=5.

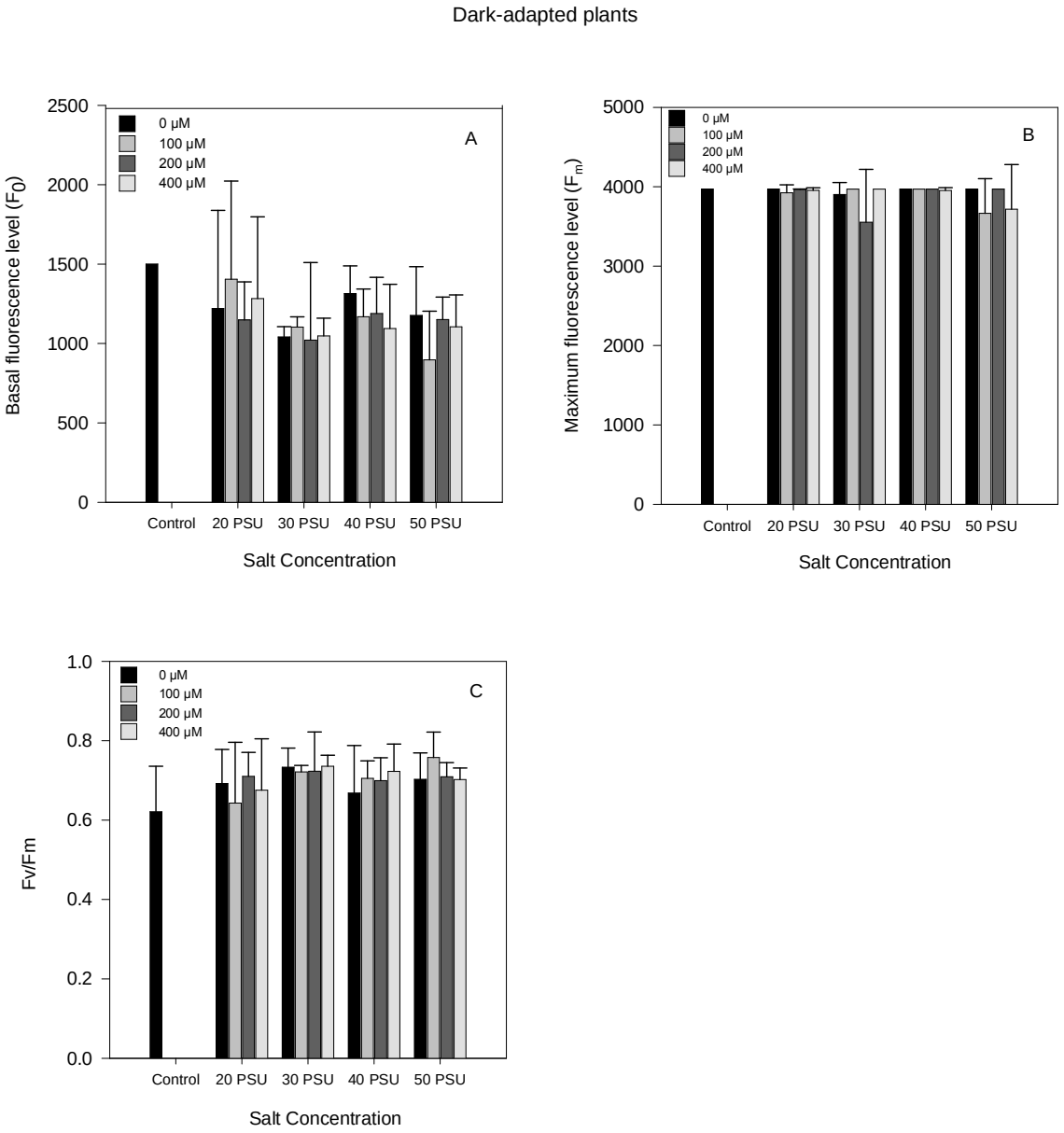

Fig. 7. Basal Fluorescence level (F_0) (A), Maximum fluorescence level (F_m) (B) and Maximum quantum efficiency of PSII photochemistry (F_v/F_m) (C), for dark-adapted plants in randomly selected, fully expanded leaves of *Halimione portulacoides* in response to treatment with a range of Zn and NaCl concentrations. Values presented as means ± S.D., n=5.

3.3. Sodium, potassium, calcium, and total zinc concentration

The total Zn concentration found either in the aerial part or in roots was significantly affected by external NaCl and Zn concentrations ($p < 0.05$). Consistently, it was possible to verify that the total Zn concentration found in stems and leaves (Fig. 8A), increased with increasing

external NaCl and Zn concentrations. Except for the 50 PSU treatment, all the other treatments presented similar values when regarding the same Zn given treatment.

There was a marked increase in total Zn accumulation determined in roots (Fig.9A), with increasing external NaCl and Zn concentrations, when compared with Zn concentration in stems and leaves.

Neither root or stems and leaves K concentrations were statistically affected by external NaCl and Zn concentrations ($p > 0.05$). According with Fig. 8B, the K concentration in stems and leaves was always similar to the control group, presenting only relatively lower values for the highest external salinities and Zn concentrations. Root tissue K concentrations (Fig. 9B) suffered a slight decreased when compared with the control group, presenting lower values for higher salinities. The responses to external salinity and Zn concentrations seen in tissue Ca concentrations show us that in stems and leaves this element concentration remained constant, once it didn´t suffered statistical differences concerning the applied treatments ($p > 0.05$). In roots, the Ca concentration (Fig. 9C), was greatly increased in every treatments compared with the control group (with significant differences for external salt concentrations $p < 0.05$), except for the higher salinity (50 PSU) were the concentration declined to a minimum.

Na concentration in stems and leaves (Fig. 8D), was constant along the treatments, although the slights verified differences were statistically significant ($p < 0.05$) either for external NaCl or Zn concentrations. Root tissue Na concentration (Fig. 9D), was also statistically significant affected ($p < 0.05$) both by external NaCl or Zn concentrations, but in this case with a more marked variation in its content.

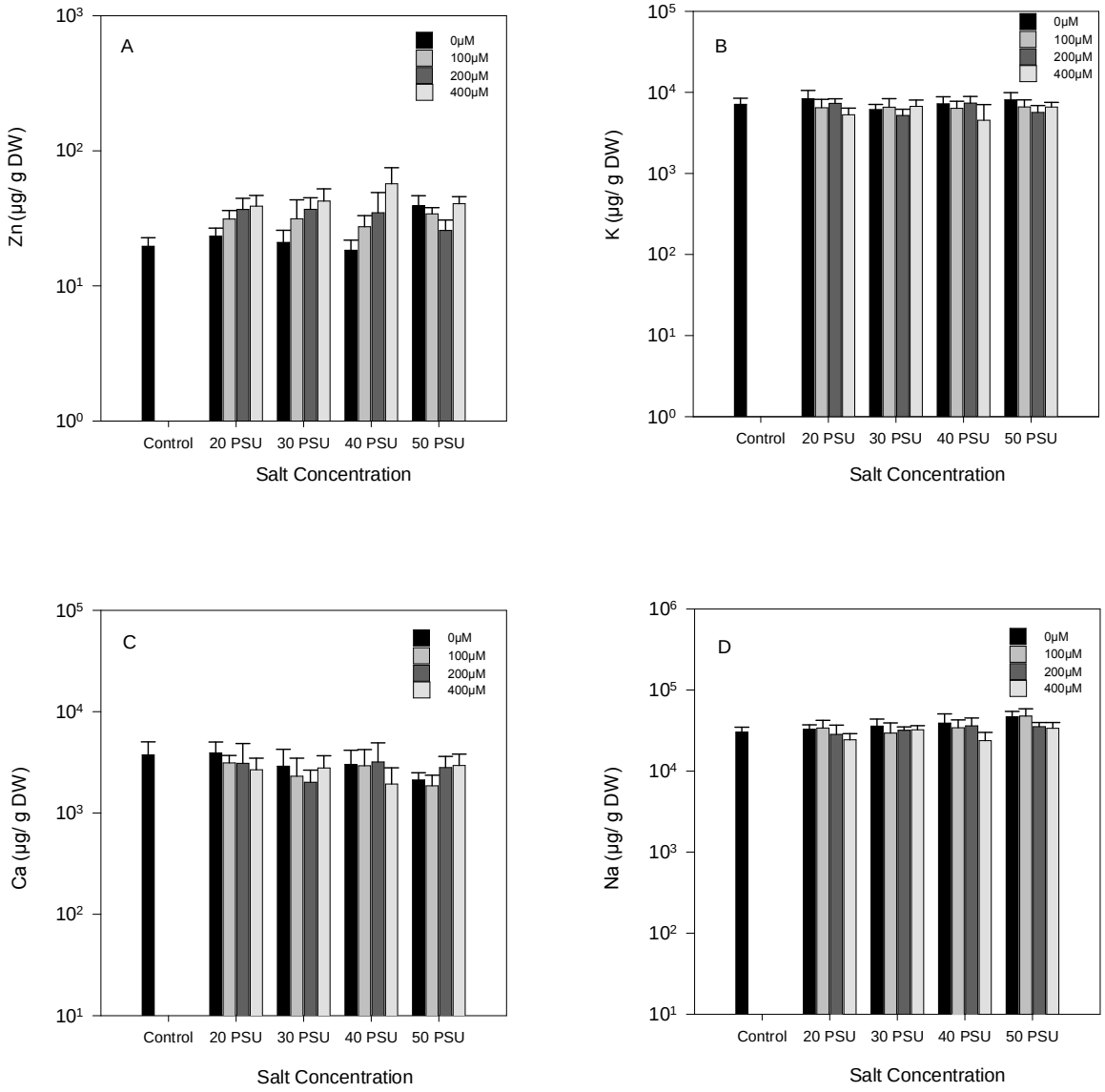

Fig. 8. Zinc (A), potassium (B), calcium (C) and sodium (D) concentrations for stems and leaves of *H. portulacoides,* in response to treatment with a range of Zn and NaCl concentrations. Values presented as means ± S.D., n =5**.** Scales were log (common) transformed.

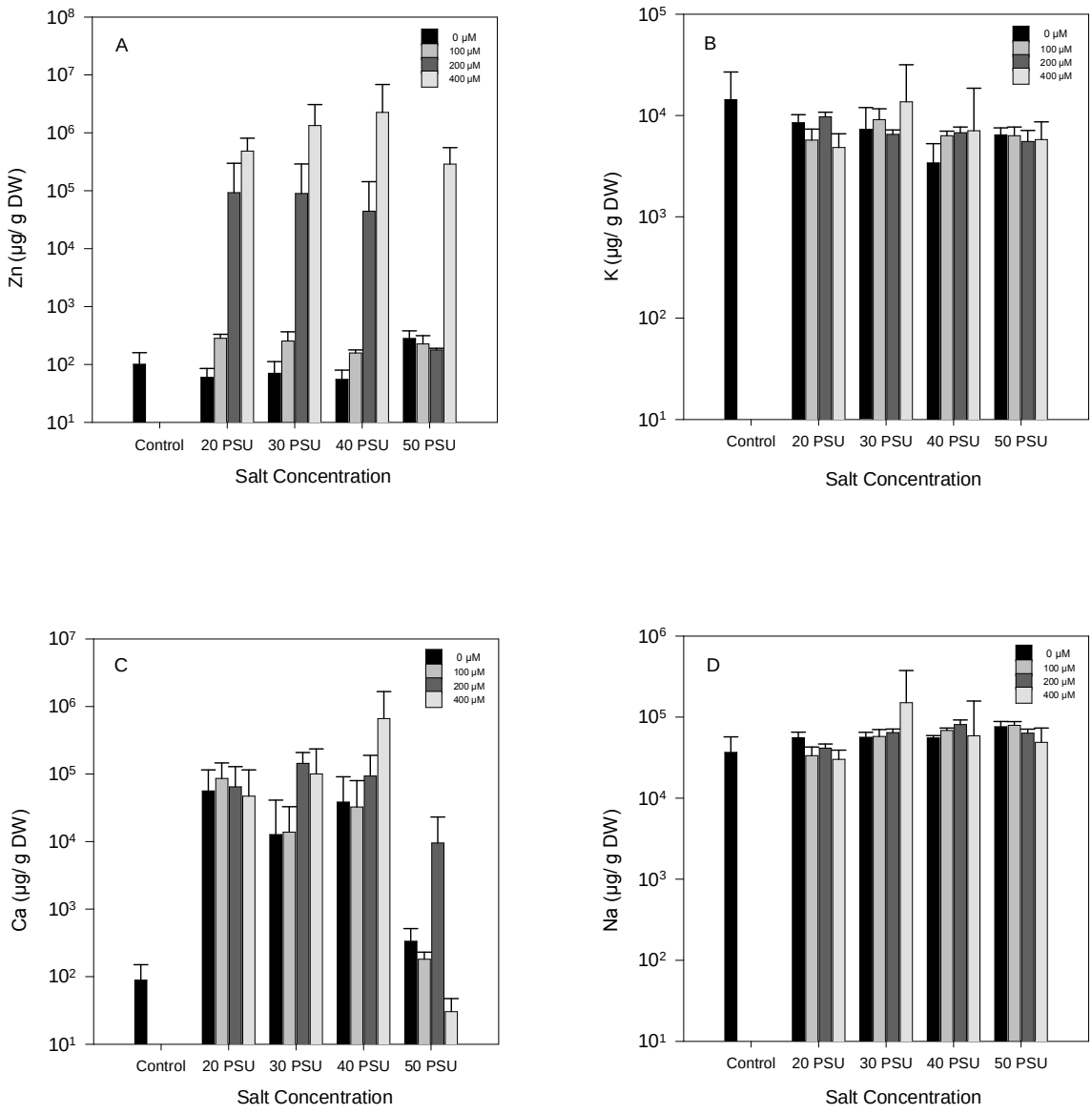

Fig. 9. Zinc (A), potassium (B), calcium (C) and sodium (D) concentrations for roots of *H. portulacoides,* in response to treatment with a range of Zn and NaCl concentrations. Values presented as means ± S.D., n =5**.** Scales were log (common) transformed.

4. DISCUSSION

In the present study, photosynthetic efficiency, leaf pigment concentration, metal and some element total concentration were measured to be used as tools for salinity and heavy metal plant stress. The results showed that *H. portulacoides* photosynthetic efficiency and leaf pigment concentration, weren´t significantly affected by none of the given treatments. Noteworthy to mention, that although the results aren´t statistical significant, in the biological context those differences might be relevant.

A recent review, Bertrand and Poirier (2005), stated that numerous heavy metal studies reported lower photosynthetic pigments contents after the exposure to those pollutants. On the other hand, other authors observed an increase in the amount of carotenoids (Malaga et al., 1997, Mallick and Ray 1999, Mallick, 2004), suggesting that chlorophyll pigments can act as antioxidants against oxidative damages. In the present study, leaf pigment composition showed that chlorophyll a, chlorophyll b and total carotenoid content had suffered an overall increase when compared with the control group (Fig. 5A-C). Furthermore, all the higher values appeared in the 400 µM Zn treatments, indicating that only Zn concentrations above 200 µM were found to be toxic to the plant (Aravind and Prasad 2003). However, at the 20 PSU and 0 µM Zn the values for all pigments had suffered a huge increase, when compared with the control, being possible to infer that in *H. portulacoides* low salinity might represent an environmental stress, assumption already shown by other authors (Redondo-Gómez *et al*., 2007 and 2010). The Chl a/b ratio (Fig. 5D) normally varied between 3 and 1 (Lichtenthaler *et al.,* 1983), still in this study that parameter stayed lower, around 2.18. The higher values reported were mainly due to lower values of Chl b, possible due to the fact that metal differentially affects the light-harvesting complex, LHC2 of PSII, where Chl b is located (Wisniewski and Dickinson 2003, Prasad, 2004).

Regarding the chlorophyll fluorescence it had become one of the most commonly used techniques to study the ecophysiological responses in plants. Photochemistry, chlorophyll fluorescence and non-photochemical quenching are competing processes. Therefore, a variation in any parameter is followed by a change in the other two (Maxwell and Johnson 2000).

A recent study, considering the photosynthetic responses of *H. portulacoides* when subject to a salinity concentration range, revealed that besides the possible osmotic shock at the beginning of the experience, salinity does not have any adverse effect on photosystem II (Redondo-Gómez *et al*., 2007). In this study, a decreased in base fluorescence levels is reported (Fig. 6A and 7A), whereas the maximum fluorescence level (Fig. 6B) was constant

for light-adapted plants, and suffered accentuated decreases for dark-adapted plants (Fig. 7B). The maximum quantum efficiency of PSII photochemistry (F_v/F_m), was identical either in light or dark-adapted leaves (Fig. 6C and 7C, respectively), yet suffering some oscilations, but always within the values proposed for this parameter by Li *et al*., 2004 (0.72). In a previous study, Redondo-Gómez *et al*. (2010) posted that whenever the maximum photochemical efficience shows a redution, is indicative of photoinhibition associated with an over-redution of PSII, a situation that mayght be occurred in this cases. Furthermore, none of the photosynthetic parameters was statistically affected by the given treatments. In this study all the increases reported in the F_v/F_m, were caused not by lower F_m values but for F_0 decreases, once that it ocorres in an higher rate compared with F_m. Similar results were achieved for *Antithamnion* (Küpper *et al.,* 2002). Interestingly, unlike the expected the three mainly decreases that ocorred in F_m did not affected the F_v/F_m, once that this parameter didn´t suffered any decreased at the correspondent treatments, mainly due to simultaneous decreases in the F_0. Also, the increase in Chl a content could have caused a higher proportion of open reaction centers, an consequently atenuated the F_v/F_m redutions (Redondo-Gómez *et al*., 2007).

It is known that sodium uptake causes plasma membrane depolarization, leading to activation of outward-rectifying K channels and a consequent K loss (Shabala *et al.,* 2003, 2005). As previously demonstrated, concomitant decreases in K and Ca concentrations appeared in the presence of external sodium supply, being the reduction in K attributed to the displacement by Na. Furthermore, the K leakage can occur as a result of Ca displacement by Na (Khan *et al.,* 2000; Redondo-Gómez *et al.,* 2007, 2010). Nevertheless, in this work, K, Ca and Na concentrations for stems and leaves were almost unchangeable suggesting a remarkably efficient homeostasis in *H. portulacoides*, also verified in others studies (Chen *et al.,* 2007). Moreover the results reported that the increasing external Zn and NaCl supply wasn´t accompanied with a progressive expected accumulation of tissue Na and Zn concentrations. These reasons may have led to the K and Ca concentrations previous described, justified probably by the presence of an alternative osmolyte.

On the other hand, root K concentrations suffered slightly oscillations along the given treatments, reducing is content. The root huge accumulations in total Ca concentration as well as an increase in Na concentration indicate that in this case calcium plays a regulating role functioning as a secondary messenger. This secondary messenger assists several plant functions, from nutrient uptake to changes in cell status in order to help plant reacting to the impact of environmental stresses (Bush, 1995).

In agreement with previous studies (Caçador *et al.,* 2000; Sousa *et al.,* 2008), the roots total Zn concentration was greatly enhanced when compared with the values found for stems and

leaves. The same study shows that *H. portulacoides* is a more effective accumulator of metals when compared with others halophytes, reason that might explain the elevated metal concentrations found for this specie.

As other heavy metals, Zn can actually replace the Mg within chlorophyll (Küpper *et al.,* 1996, Solymosi *et al.,* 2004), which will lead to a breakdown in photosynthesis. This breakdown is predominantly due to disturbances in the PSII, once that this substitution occurs on the photolysis site, inhibiting oxygen emission (Rhalp and Burchett 1998). However, in this study we didn´t verified significant alterations in the chlorophyll fluorescence, and consequently accepted that the PSII remaining intact, assuming once more that in lower concentrations Zn acts an essential micronutrient. Zn has an important role within the cell, stabilizing several proteins, enzymes and biomembranes, being able to increase the biosynthesis of antioxidant enzymes and therefore interfering with normal metabolic functions (Cakmak 2000). Yet, in higher concentrations this metal can induce several disruptions, as verified in this study accompanied by external salt supply. Although, Zn and NaCl are known to damage photosynthetic apparatus in several plant species, in halophytes, such as *H. portulacoides*, its effects aren´t severe.

Based on these results, it was concluded that *H. portulacoides* responses to Zn appear largely to depend on changes in its pigments concentrations rather than in effects on PSII.

In the future, these studies should be combined with other techniques, in particular, gas exchange measurements, using infrared gas analyzers, to obtain a full picture of the plant response to those environmental stresses.

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CHAPTER 4

Conclusions

CONCLUSIONS

1. In general, Zn and salinity exposure caused an overall stress in *H. portulacoides,* demonstrated through a huge malondialdehyde increase accomplished with a decreased in basal fluorescence and consequently in the photochemical efficiency of photosynthesis. However, it is clear that the given treatments did not have any overall adverse effect on the photosystem II apparatus once that the maximum quantum efficiency of PSII photochemistry remained stable.
2. This halophyte was able to adapt physiologically to the different imposed situations, creating responses, involving ionic homeostasis as well as cell detoxification with antioxidant defense mechanisms and photosynthetic pigments.
3. The results for the antioxidant mechanisms, revealed a reactive oxygen species impaired enzymatic scavenging, only with the superoxide dismutase activity actively contributing for this function. The catalase, ascorbate and guaiacol peroxidase activities were near inactivation.
4. Moreover, the non-enzymatic mechanisms skills were enhanced mainly due to a highly phenol content. This ability has been shown to be crucial in determining the success of the plant study, in surviving to stress conditions, once that it is also critical in the detoxification process, eliminating reactive oxygen species.
5. In stems and leaves, potassium, calcium and sodium were almost unchangeable suggesting a remarkably efficient homeostasis, while in roots the calcium suffers a huge accumulation revealing a functioning regulating role as a secondary messenger.
6. Although several mechanisms had been activated due to the zinc presence, only the 400 µM Zn concentration was found to be the most induce oxidative stress treatment, that was reinforced in the presence of salinity.
7. Taking into consideration all the obtained data, there are chronic effects resulting from the zinc exposure, which can probably be used as a tool to evaluate the health of salt marshes in field.

APPENDIXS

APPENDIX A

Table 1. Experimental design of the concentrations of Zn and NaCl used in this work. The 20 PSU NaCl worked was zero.

NaCl(PSU)	Zn (µM)	NaCl(PSU)	Zn (µM)