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Effects of heavy metal pollution on germination and early seedling growth in native and invasive *Spartina* cordgrasses

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ABSTRACT

Seed germination and seedling establishment are the stages most sensitive to abiotic stress in the plant life cycle. We analyzed the effects of copper, zinc and nickel on seed germination and early seedling growth of native *Spartina maritima* and invasive *S. densiflora* from polluted and non-polluted estuaries. Germination percentages for either species were not affected by any metal at any tested concentration (up to 2000 μM). However, the increase in metal concentration had negative effects on *S. densiflora* seedlings. The primary effect was on radicle development, representing initial seedling emergence. *Spartina densiflora* seedlings emerging from seeds from Tinto Estuary, characterized by high bioavailable metal loads, showed higher tolerance to metals than those from less polluted Odiel and Piedras Estuaries. Comparing our results to metal concentrations in the field, we expect *S. densiflora* seedling development would be negatively impacted in the most metal-polluted areas in Odiel and Tinto Estuaries.

1. Introduction

Seed germination and seedling establishment are crucial in the life cycle of plants (Ungar, 2001). They are the most sensitive stages to abiotic stresses such as metal pollution because defence mechanisms have not fully developed (Liu et al., 2005; Ahsan et al., 2007). However some metals such as copper (Cu), manganese (Mn), cobalt (Co), chromium (Cr), nickel (Ni) and zinc (Zn) are essential elements for plant metabolism by participating in redox reactions and as integral part of several enzymes, and they can become toxic when they are bioavailable in high concentrations (Munzuroglu and Geckil, 2002; Nagajyoti et al., 2010). Thus, metals are major environmental pollutants and their toxicity has a high impact on plants and, consequently, on ecosystems (Nagajyoti et al., 2010). For example, metal exposure of growing plants may reduce or inhibit seed germination (Kranner and Colville, 2011; Sethy and Ghosh, 2013). Germination can be affected by metals in two ways: direct toxicity and/or water uptake inhibition (Kranner and Colville, 2011). In this context, one of the most used methods to test metal toxicity in plants is the seed germination test (Munzuroglu and Geckil, 2002). In this context, the aim of our study was to analyze the effects of increasing metal concentrations on the germination and establishment of two closely-related halophytes.

Globally, many estuarine wetlands have been degraded by pollution from urban, industrial and mining runoff that has led to concentrations of heavy metals in salt marsh sediments (Caffrey et al., 2007; Curado et al., 2010). Halophytes have mechanisms of adaptation that allow them to survive to environmental stresses, such as high salt concentrations and exposure to heavy metals (Van Oosten and Maggio, 2015). Thus, halophytes offer a great potential for phytoremediation and phytostabilization of heavy metal polluted soils since they have the ability to accumulate metals in its tissues (Manousaki and Kalogerakis, 2011; Curado et al., 2014; Van Oosten and Maggio, 2015). Some halophytes species are tolerant of metals during their adult life stage (Mateos-Naranjo et al., 2008a, 2008b; Manousaki and Kalogerakis, 2011; Redondo-Gómez et al., 2011; Van Oosten and Maggio, 2015). Many of these tolerant adult halophytes have a high capacity for metal accumulation in their roots, avoiding metal translocation to sensitive photosynthetic tissues (Redondo-Gómez, 2013). Plants develop through phenological life stages that often differ in environmental tolerances, and early life germination traits can affect the timing and duration of subsequent life stages (Burghardt et al., 2015). Functional and morphological plant trait-based approaches are frequently used to predict species abundance and responses to altered environmental conditions, yet quantitative information on the full life cycle of many plants is often

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lacking (Adler et al., 2014). For example, little is known of the effects of sediment metal pollution on early life stages of plants, including germination and seedling growth (Williams et al., 1994; Márquez-García et al., 2013).

Spartina species (cordgrasses) mostly inhabit salt marshes around the world, and many of them have successfully invaded many areas beyond their native ranges (Strong and Ayres, 2013; Ainouche and Gray, 2016). For this reason, cordgrasses are a good model group to study the responses of native and invasive halophyte species to altered environmental conditions such as estuarine sediments polluted with metals. In the case of invasive species (i.e. *S. densiflora*), for effective control it is important to understand mechanisms allowing their populations to grow and spread. In contrast, understanding how environmental pollution may impact critical life stages influencing population dynamics of native plant species such as *S. maritima* can improve conservation management.

In salt marshes along the Gulf of Cádiz (Southwest Iberian Peninsula), native *Spartina maritima* (Curtis) Fernald, the only native cordgrass in European marshes (Marchant and Goodman, 1969), co-occurs with invasive *Spartina densiflora* Brongn., introduced from South America centuries ago (Nieva et al., 2001). Seeds of some *Spartina* species, such as *S. densiflora*, are able to germinate in highly metal-polluted sediments (Curado et al., 2010; Mateos-Naranjo et al., 2011). In *Spartina alterniflora* Loisel., mercury (Hg), cadmium (Cd), lead (Pb) and Zn stimulated early germination at low salinities, whereas metals had negative effects on germination at high salinities (Mrozek Jr, 1980; Mrozek and Funicelli, 1982). In *S. densiflora*, Curado et al. (2010) analyzed the germination and establishment in acidic and metal-polluted sediments collected along the Tinto River (Southwest Iberian Peninsula), observing that final germination decreased with pH and higher aluminum (Al) and Cr concentrations, and that germination was the rapidest and growth decreased in the most polluted sediments. Mateos-Naranjo et al. (2011) analyzed the differences in metal tolerance between *S. densiflora* populations from polluted and unpolluted estuaries founding that seeds of all populations were able to germinate even in the most contaminated soils and that seedling growth and survival decreased in high metal concentrations. To our knowledge, there is no information in the literature about the effects of metals on seed germination and early seedling growth of native *S. maritima*, since until recently seed production in this species has been described as very low or nonexistent (Marchant and Goodman, 1969; Castellanos et al., 1994; Castillo et al., 2010), but in a recent study we recorded that *S. maritima* produced a moderate number of caryopses with high germination rates in Southwest Iberian Peninsula (Infante-Izquierdo et al., 2019a).

Our main goal was to analyze and compare the effects of metals on seed germination and early seedling growth of native *S. maritima and invasive S. densiflora*. Although the effects of metals on germination traits of invasive *S. densiflora* has previously been studied, past work has evaluated responses to composite field samples of sediments with multiple pollutants at varying concentrations. For this reason, our objective was to include both *S. maritima* and *S. densiflora* in this study to compare and contrast the germination and seedling trait responses of both congeners to Cu, Zn, and Ni individually, conducting tests with each of the three metals at a range of concentrations that reflect variation of pollution levels at field sites. In addition, we analyzed the effects of these metals on seeds of *S. densiflora* collected from both polluted and non-polluted estuaries. Both environment and genetic factors may play a role in plant response to metals. For example, the legacy metal concentrations on mountains nearby these marshes have led to speciation processes, such as the case of the endemic heather *Erica andevalensis* Cabezudo and J. Rivera (Márquez-García et al., 2009). Therefore, we hypothesized that early life stages of native *S. maritima* from Odiel and Tinto Estuaries would have a greater tolerance to metals than *S. densiflora*, since these estuaries have been affected by naturally high concentration of metals for at least 4500 years (Davis Jr et al., 2000; Leblanc et al., 2000; Pérez-López et al., 2011), and the native cordgrass is the long-term resident species.

2. Materials and methods

2.1. Study species

Native *S. maritima* (2n = 6× = 60) and invasive *S. densiflora* (2n = 7× = 70) are both rhizomatous perennial cordgrasses. *Spartina maritima* has a sparsely caespitose growth form, whereas *S. densiflora* is densely caespitose. Both species present racemes with spikes bearing unifloral spikelets with 3 stamens (Infante-Izquierdo et al., 2019b). Whereas *S. maritima* produces an average percentage of fruit set per spikelet of 13% (Infante-Izquierdo et al., 2019a), *S. densiflora* produces and disperses abundant viable caryopses that greatly increase its invasive potential (Nieva et al., 2001). *Spartina maritima* colonizes low intertidal elevations where it facilitates salt marsh ecological succession (Castellanos et al., 1994). In contrast, *S. densiflora* is able to colonize different habitats along the intertidal gradient from low-middle marshes to salt pans (Bortolus, 2006), due to high phenotypic plasticity levels (Castillo et al., 2014). *Spartina maritima* is considered as a threatened species in some European coastal regions, and in South Africa (Cabezudo et al., 2005; Cooper, 1993; Fish et al., 2006). In contrast, South American *S. densiflora* is highly invasive where naturalized in wetlands of North America, Europe and Africa (Bortolus, 2006).

2.2. Study sites and sediment pollutant loads

Our study sites were along the coast of the Gulf of Cádiz in Southwest Iberian Peninsula. The estuaries of Odiel and Tinto rivers are among the rivers of the world that are most highly polluted by heavy metals (Nelson and Lamothe, 1993; Davis Jr et al., 2000; Sainz et al., 2004). This extreme metal contamination is the result of mining pollution by acid mine drainage in the Iberian Pyrite Belt that is crossed by the drainage basins of Odiel and Tinto Rivers. Additive metal pollution also results from industrial discharges and urban effluents (Fernández-Caliani et al., 1997; Ruiz, 2001; Borrego et al., 2002; Galán et al., 2003; Sainz et al., 2004). Many studies have analyzed metal loads in sediments in Odiel and Tinto Marshes (Fernández-Caliani et al., 1997; Elbaz-Poulichet et al., 1999, 2001; Borrego et al., 2002; Achterberg et al., 2003; Braungardt et al., 2003; Galán et al., 2003; González-Pérez et al., 2008; Mateos-Naranjo et al., 2011). According to these studies, the maximum concentrations of metals registered in sediment solution were > 1000 µM iron (Fe) and Al; > 500 µM Zn and Cu; 50–500 µM Mn; < 50 µM arsenic (As), Co, Pb, Ni, Cd and Hg. These concentrations are higher for maximum allowable limits of heavy metals in soils established by the World Health Organization (Chiroma et al., 2014). The accumulation, mobility and bioavailability of metals depend on physical and chemical properties of soils, as texture, pH, salinity, redox potential, organic matter content, etc. (Williams et al., 1994; Gambrell, 1994). In this sense, some metals dissolved in the Estuaries of Odiel and Tinto precipitate as salinity increases, being trapped in the sediments, while others remain in the solution and, therefore, are more mobile, such as Cu, Mn and Zn (Elbaz-Poulichet et al., 2001; Borrego et al., 2002; Achterberg et al., 2003; Braungardt et al., 2003). In contrast, neighboring Piedras Estuary is much less polluted, with sediment metals concentrations < 400 ppm Ni, Zn, Cu, Cr, Pb, As, Cd and Co (Ruiz, 2001; Redondo-Gómez et al., 2009; Mateos-Naranjo et al., 2011).

2.3. Plant material

Fruiting inflorescences of invasive *S. densiflora* were collected from three middle marsh populations, from at least ten different tussocks per population, in November 2016. Each of these populations was located in different estuaries with different degree of pollution by metals (Odiel

Estuary (37.209102 N, −6.952582 W), Tinto Estuary (37.213830 N, −6.925824 W) and Piedras Estuary (37.213804 N, −7.174761 W)). Fruiting inflorescences were collected in August 2017, from at least ten different tussocks, in one population of native *Spartina maritima* colonizing a low salt marsh in the metal-polluted Odiel Estuary (37.174343 N, −6.931640 W). *Spartina maritima* flowers between May and July and *S. densiflora* between May and December in the study area (Gallego-Tévar et al., 2019), so harvesting times followed the different fruiting period of each species in order to always collect mature caryopses. Once in the laboratory, spikelets containing caryopses (simple one-seeded fruit of grasses) were randomly selected and stored in cold (+5 °C) conditions until use.

2.4. Germination experiments

In 2017, sequential germination experiments were carried out for *S. densiflora* and for *S. maritima*. To prevent fungal contamination, spikelets were surface-sterilized in 5% (*v*/v) sodium hypochlorite for 10 min and then rinsed with distilled water (Muñoz-Rodríguez et al., 2012; Infante-Izquierdo et al., 2019a). For each species and population, four replicates of 25 spikelets from different individuals were sown for each metal at tested concentrations on Petri dishes (9 cm diameter) with two layers of autoclaved filter paper adding different treatments solutions: distilled water (control), solution containing 100, 250, 500, 1000 and 2000 μM Cu (as $CuSO_45H_2O$), Zn (as $ZnSO_47H_2O$) or Ni (as $NiSO_46H_2O$). Metals and concentrations were chosen based on the metal composition and their levels registered in the waters and soils of the source estuaries reported above, and because of the impact of these contaminants in plants, and in the environment (Kranner and Colville, 2011; Nagajyoti et al., 2010). The metals tested in this study were dissolved in distilled water. We used sulphates because it is the most abundant chemical form in the source estuaries (Barba-Brioso et al., 2010), and because the toxicity of chloride ion might have inhibitory effects on seed germination (León et al., 2005). Petri dishes were sealed with adhesive tape (Parafilm™) to avoid desiccation. The experiments were done under controlled-environmental conditions at temperatures between +20–25 °C and a 12 h/12 h photoperiod. Radiation was provided by fluorescent lamps that produced a photosynthetic photon flux density of 60 μmol m^{-2} s^{-1}. Spikelets were exposed to treatments for 2 months, and germination was recorded every 3 or 4 days. A spikelet was considered germinated when the coleoptile emerged. Final germination percentage and days necessary to reach 50% of the final germination (T_{50}) were calculated for each dish. Germinated spikelets were kept in the Petri dishes to study initial seedling development.

2.5. Early seedling growth

To study the effects of metals on initial seedling growth, we measured cotyledon, first leaf and radicle length for 1–5 seedlings per Petri dish under a magnified glass using a ruler 15 days after germination (n = 4 Petri dishes per treatment). The radicle, cotyledon and first expanded leaf to appear from a germinating seed are highly sensitive to the germination environment, therefore their growth is a useful indicator of early seedling development in relation to environmental stresses such as high concentration levels of salinity and metals (Infante-Izquierdo et al., 2019c; Mabrouk et al., 2019).

2.6. Statistical analysis

Statistical analyses were carried out using STATISTICA 8.0 (StatSoft Inc., USA). Deviation to the mean was calculated as Standard Error (SE). Normality and homogeneity of variance of the data series were tested using Kolmogorov-Smirnov test and Levene test, respectively. When data or their transformations (using √x, 1/(x + 1), arcsine(x) and ln (x) functions) showed normal distribution and homogeneity of variance, one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference (HSD) test as post-hoc test were used to compare germination and seedling parameters among treatments. Otherwise, we used a non-parametric Kruskal-Wallis H-test and a Mann-Whitney *U* test as post-hoc tests. In all statistical analyses, we considered results significant when $p \leq 0.05$.

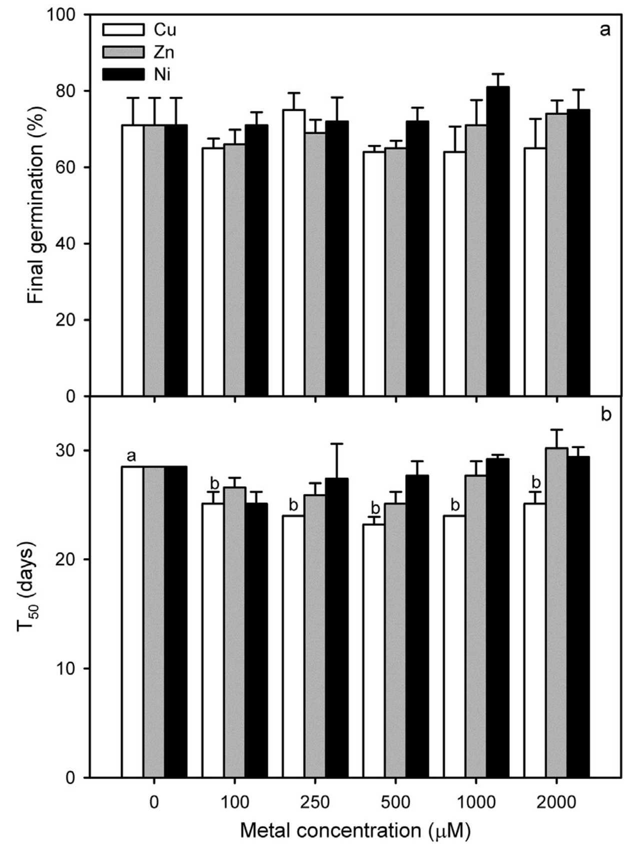

Fig. 1. Germination percentage (a) and days necessary to reach 50% of the final germination percentage (T_{50}) (b) for native *Spartina maritima* spikelets sown in different concentrations of copper (Cu), zinc (Zn), and nickel (Ni). Data shown are means ± SE (n = 4). Different letters indicate significant differences among heavy metal concentrations (Mann-Whitney *U* test, $p < 0.05$).

3. Results

3.1. Effects of metals on seed germination

Final germination percentage for *S. maritima* was c. 70% regardless of metal treatment or concentration level of the metals. Germination was significantly accelerated (lower T_{50}) in all Cu concentrations c. 3–5 days respect to the control treatment ($H_{5,24}$ = 14.77, $p < 0.05$; Man-Whitney *U* test, $p < 0.05$) (Fig. 1). Germination speed in all Zn and Ni concentrations (c. 27 days) did not show significant differences in comparison to the control (Fig. 1).

In *S. densiflora*, germination percentage was ca. 20% higher at 250 μM Ni than at 1000 and 2000 μM Ni for spikelets from Tinto Estuary (F = 4.56, df = 5, $p < 0.01$), and at 500 μM Zn than at 2000 μM Zn for spikelets from Piedras Estuary (F = 3.18, df = 5, $p < 0.05$) (Fig. 2). There were no significant differences in germination percentage and germination speed among spikelets sourced from Odiel, Tinto and Piedras estuaries. Moreover, T_{50} was independent of metal concentrations (Figs. 2 and 3).

3.2. Effects of metals on early seedling growth

In *S. maritima* seedlings, length of cotyledon and first leaf were not

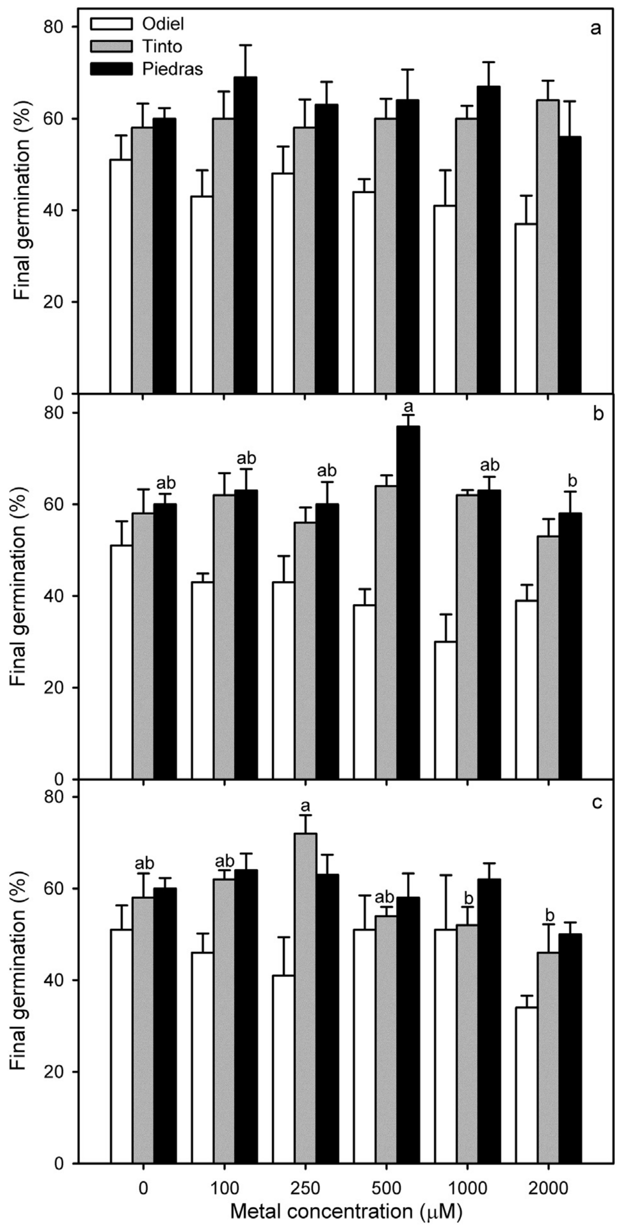

Fig. 2. Germination percentage for invasive *Spartina densiflora* spikelets from three estuaries (Odiel, Tinto and Piedras) sown in different concentrations of copper (Cu) (a), zinc (Zn) (b), and nickel (Ni) (c). Data shown are means ± SE (n = 4). Different letters indicate significant differences among concentrations of heavy metals and estuaries (Tukey's HSD test, $p < 0.05$). There were no significant differences among estuaries (ANOVA, $P > 0.05$).

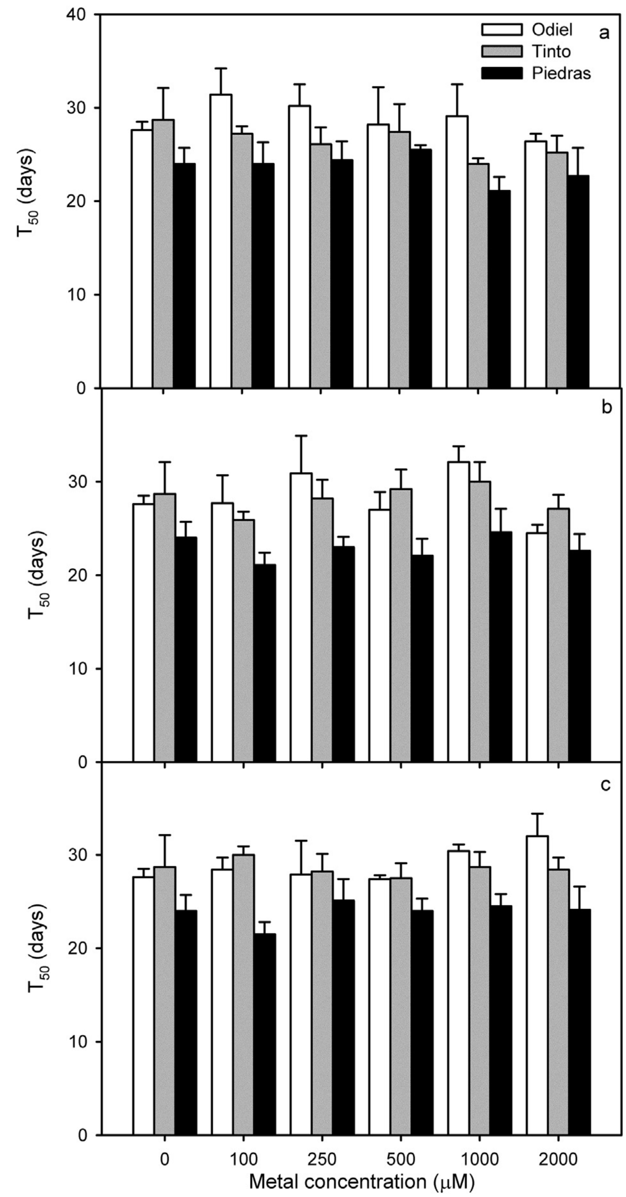

Fig. 3. Days necessary to reach 50% of the final germination percentage (T_{50}) for invasive *Spartina densiflora* spikelets from three estuaries (Odiel, Tinto and Piedras) sown in different concentrations of copper (Cu) (a), zinc (Zn) (b), and nickel (Ni) (c). Data shown are means ± SE (n = 4). There were no significant differences among metal concentrations and among (ANOVA, $P > 0.05$).

significantly affected by any individual metal or metal concentrations ($p > 0.05$) (Figs. 4a, 5a, 6a). *Spartina maritima* radicle length differed among tested Cu concentrations, with maximum length produced at 100 μM Cu ($H_{5,115} = 12.56$, $p = 0.03$; Man-Whitney *U* test, $p < 0.05$) (Fig. 4b).

In *S. densiflora*, cotyledon length was not diminished in any concentration of the three tested metals for any populations (Figs. 4, 5, 6c, e, g). Radicle length was significantly affected by all metals for spikelets sourced from all estuaries. Thus, a significant reduction on radicle length between 55 and 68% was recorded with increasing Cu concentration over 100 μM Cu for Odiel and Piedras populations, and over 250 μM Cu for Tinto population (Odiel: $H_{5,111} = 87.01$, $p < 0.0001$; Piedras: $H_{5,106} = 74.30$, $p < 0.0001$; Tinto: $H_{5,110} = 65.26$, $p < 0.0001$) (Fig. 4d, f, h). Moreover, radicle length decreased between 56 and 69% over 500 μM Zn (Odiel: $F = 17.86$, $df = 5$, $p < 0.0001$; Tinto: $F = 14.06$, $df = 5$, $p < 0.0001$; Piedras: $F = 16.35$, $df = 5$, $p < 0.0001$) (Fig. 5d, f, h), and between 69 and 83% over 100 μM Ni for the three studied populations (Odiel: $H_{5,101} = 58.83$, $p < 0.0001$; Tinto: $F = 17.76$, $df = 5$, $p < 0.0001$; Piedras: $H_{5,117} = 76.65$, $p < 0.0001$) (Fig. 5d, f, h). First leaf length was significantly shorter than the control only for seedlings from Odiel Estuary when exposed to 100 and 2000 μM Cu ($F = 3.13$, $df = 5$, $p = 0.01$) (Fig. 4c). In addition, first leaf length was reduced at concentrations higher than 500 μM Ni for seedlings from Odiel and Piedras (Odiel: $F = 11.03$, $df = 5$, $p < 0.0001$; Piedras: $H_{5,117} = 45.69$, $p < 0.0001$) (Fig. 6c, g) and at concentrations higher than 1000 μM Ni for seedlings sourced from Tinto Estuary ($H_{5,118} = 35.91$, $p < 0.0001$) (Fig. 6e). Exposure to Zn did not affect first leaf size for any population ($p > 0.05$) (Fig. 5c, e, g).

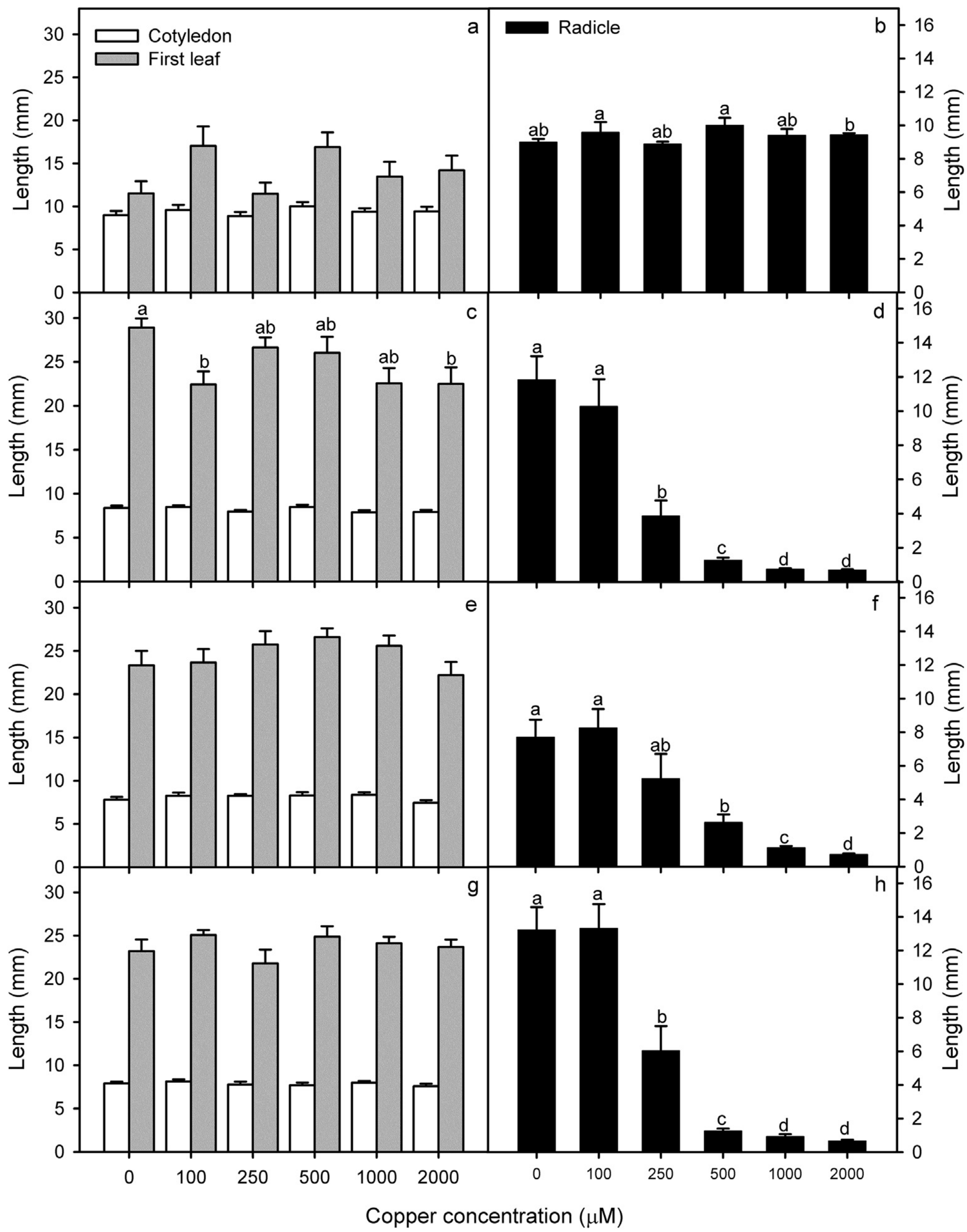

Fig. 4. Cotyledon length, first leaf length and radicle length for native *Spartina maritima* seedlings from Odiel Estuary (a, b), and invasive *S. densiflora* seedlings from Odiel (c, d), Tinto (e, f) and Piedras (g, h) Estuaries exposed to different concentrations of Copper. Data shown are means ± SE (n = 16–20). Different small letters above bars indicate significant differences among concentrations for each seedling parameter (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

4. Discussion

As we hypothesized, native *Spartina maritima* showed greater tolerance to metals than invasive *S. densiflora* in relation to early seedling growth, since neither *S. maritima* nor *S. densiflora* seed germination were affected by any of the tested metals. As in other studies, seedling phase was more sensitive to metal exposure than seed germination (Li et al., 2005; Ahsan et al., 2007; Curado et al., 2010).

Although seed coat may act as a barrier to metal uptake by other grasses and forbs (Munzuroglu and Geckil, 2002; Li et al., 2005; Kranner and Colville, 2011), seed germination and seedling growth can be diminished by high metal concentrations (Williams et al., 1994; Kranner and Colville, 2011). Per criteria by Kranner and Colville (2011), in our study, seeds from both *Spartina* species were metal-tolerant, since their germination percentage was not affected, even at high metal concentrations. Similar to our results, high concentrations of Cu and Zn (up to 2000 μM) did not affect seed germination in the halophytic herb *Salicornia ramosissima* J. Woods and in *Atriplex halimus* L. (Márquez-García et al., 2013). Curado et al. (2010) found that *S. densiflora* seeds germinated in high metal-polluted sediments even in acidic conditions (pH ca. 2), although final germination was reduced at the highest concentrations in fresh water conditions along the Tinto River. Mateos-Naranjo et al. (2011) found that *S. densiflora* seeds showed high germination percentages even in the most contaminated soils in the Odiel Estuary. As these studies and our own study show, germination of seeds from several halophyte species from different genera and families are highly tolerant of metals. Thus, many of the mechanisms that allow halophytes to deal with high salinity, including synthesis of organic solutes and efficient antioxidative systems, may confer tolerance to other abiotic stresses such as high concentrations of metals (Manousaki and Kalogerakis, 2011; Van Oosten and Maggio, 2015). Even so, germination of some halophytes can be sensitive to metal pollution. For

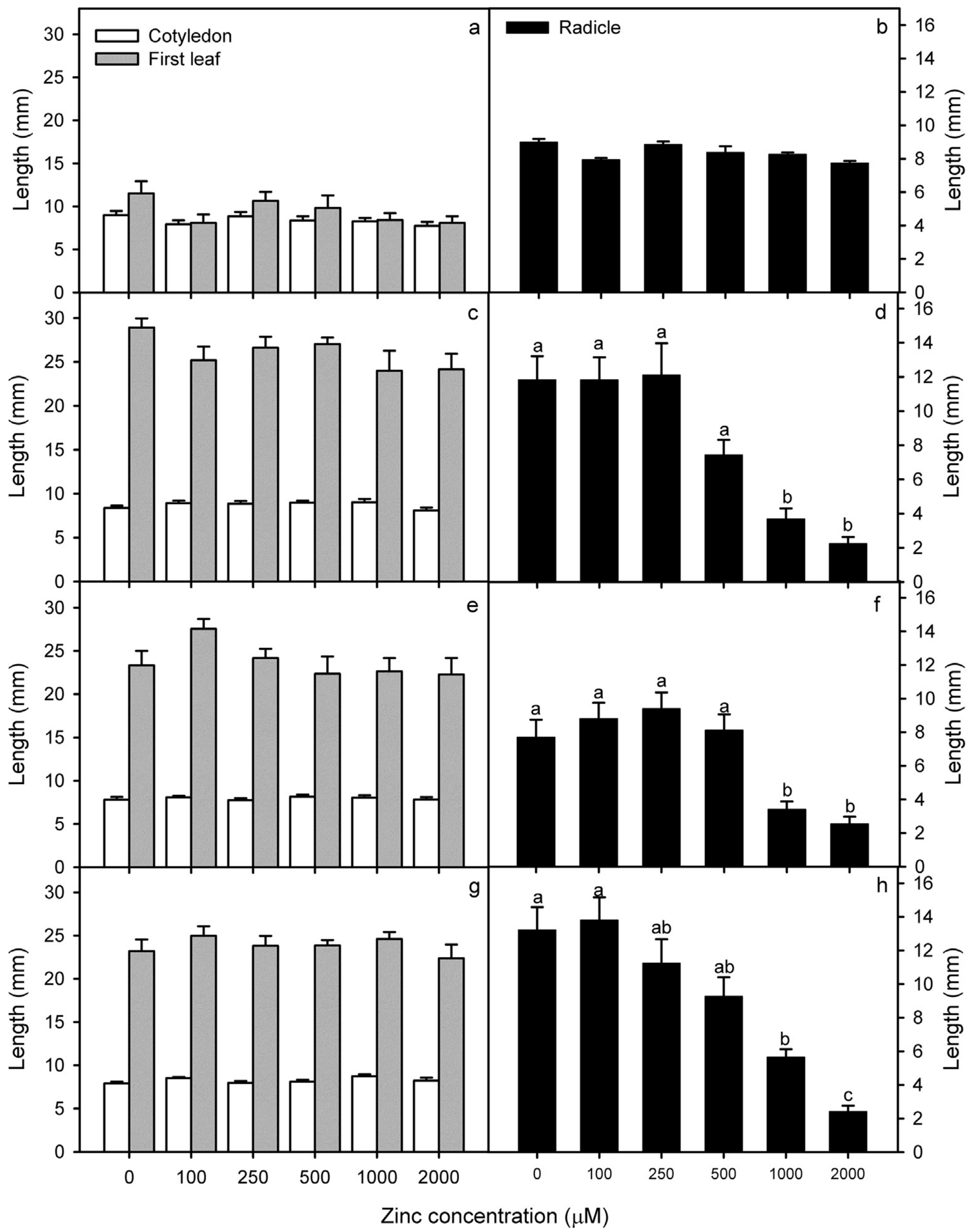

Fig. 5. Cotyledon length, first leaf length and radicle length of native *Spartina maritima* from Odiel Estuary (a, b) and invasive *S. densiflora* seedlings from Odiel (c, d), Tinto (e, f) and Piedras (g, h) Estuaries exposed to different concentrations of Zinc. Data shown are means ± SE (n = 16–20). Different small letters above bars indicate significant differences among concentrations for each seedling parameter (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

example, Márquez-García et al. (2013) found that Ni inhibited *Salicornia ramosissima* germination even at low Ni concentrations (10 μM Ni), and Sharma et al. (2011) observed *Salicornia brachiata* Miq. germination was reduced at 50 μM Ni and above.

Germination percentage and the speed of germination are not necessarily correlated. In our study, the final germination percentage of *S. maritima* did not change with increasing metal concentrations, though germination was significantly accelerated in the presence of Cu. In another example, germination acceleration occurred in *S. densiflora* seeds at metal-polluted and acidic conditions (Curado et al., 2010). In some species, Cu-enrichment during seed development accelerates germination, which could be attributed to an overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in plants exposed to metals, causing a slightly enhanced level of oxidative stress that stimulates germination (Kranner and Colville, 2011). But an excess of Cu may also cause osmotic stress, inhibiting water uptake by seeds and their germination (Ahsan et al., 2007; Kranner and Colville, 2011). For example, in *Oryza sativa* L., a glycophyte from the Poaceae family, Cu had an adverse effect on seed germination that was totally inhibited at 1500 μM Cu (Ahsan et al., 2007).

In our study, development of *S. maritima* seedlings was not affected by any of the heavy metals evaluated in our experiments. *Spartina maritima* seedlings produced very small radicles, that were even smaller than those of seedlings obtained from spikelets previously exposed to salt conditions (Infante-Izquierdo et al., 2019c). This reduced radicle development suggests a low capacity for metal uptake, that could reduce and thereby mitigate the potential toxic effects of greater metal uptake. In contrast, *S. densiflora* seedlings produced longer radicles, and their development was negatively affected by metals with the radicle being the most impacted organ. In this sense, most *Spartina* species reduce the degree of metal translocation to photosynthetic tissues by accumulating them in their belowground biomass (Redondo-Gómez,

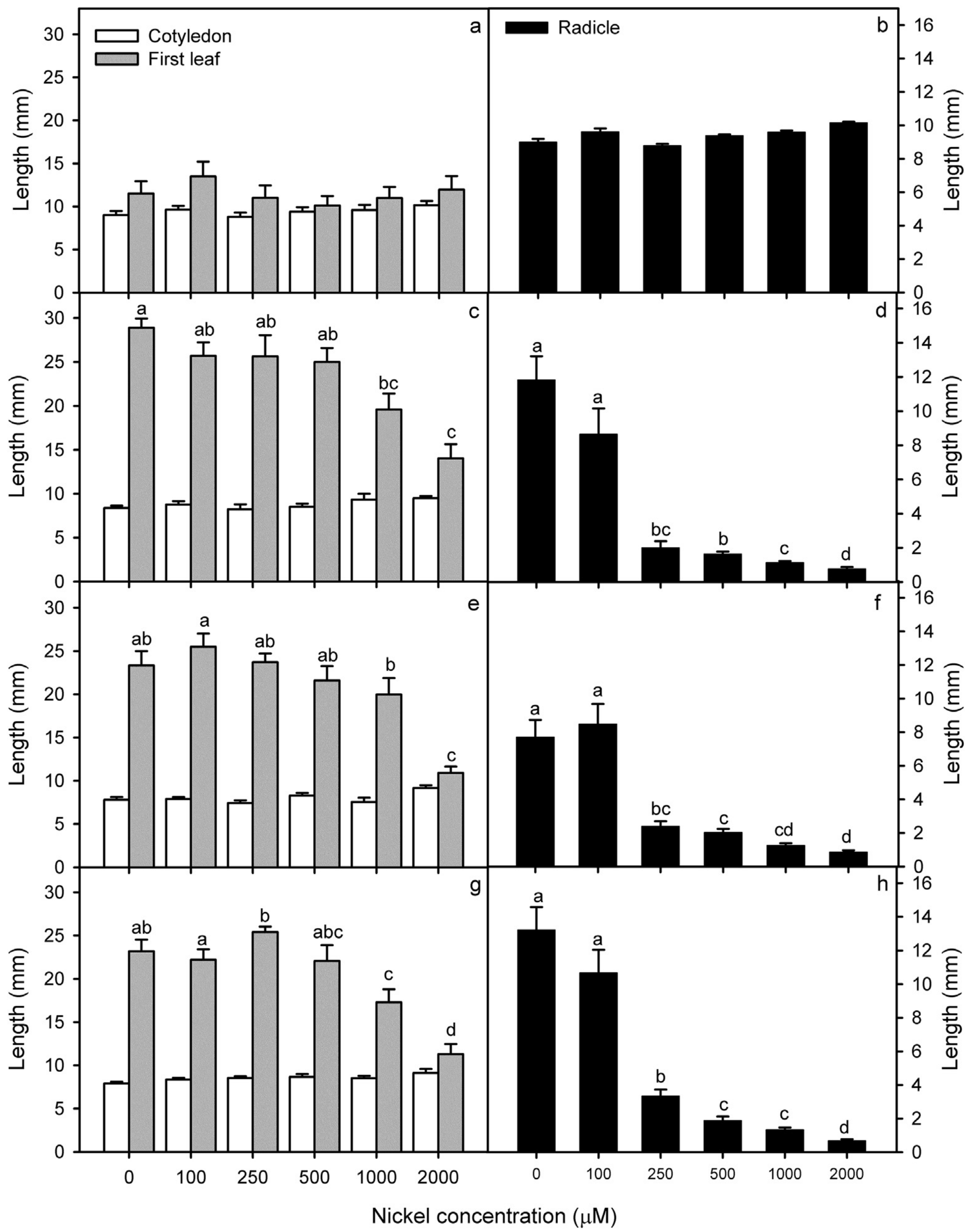

Fig. 6. Cotyledon length, first leaf length and radicle length of native *Spartina maritima* from Odiel Estuary (a, b) and invasive *S. densiflora* seedlings from Odiel (c, d), Tinto (e, f) and Piedras (g, h) Estuaries exposed to different concentrations of Nickel. Data shown are means ± SE (n = 10–20). Different small letters above bars indicate significant differences among concentrations for each seedling parameter (Mann-Whitney U test or Tukey's HSD test, $p < 0.05$).

2013). Curado et al. (2010) found that aerial and subterranean growth rate of *S. densiflora* seedlings decreased in more metal-polluted and acidic sediments from Tinto Estuary. The higher metal tolerance of native *S. maritima* seedlings in comparison to invasive *S. densiflora* may be explained by their contrasted habitats along the intertidal gradient since *S. maritima* is a colonizer of low tidal elevations, where metal pollution is higher than at medium elevations colonized by *S. densiflora* (Luque et al., 1999). In addition, *S. densiflora* is an invasive species native from South America that was introduced ca. 16th century to the Southwest Iberian Peninsula (Nieva et al., 2001; Strong and Ayres, 2013), coinciding with intercontinental coastal exploration by Europeans, and therefore has had less time than *S. maritima* to adapt to the polluted environment of Odiel and Tinto Estuaries.

The exposure of plants to excess Cu, Zn or Ni produces oxidative stress and inhibits metabolic functions such as photosynthesis, water uptake and pigment synthesis, and also damages membrane integrity and reduces plant growth (Fernandes and Henriques, 1991; Nagajyoti et al., 2010). Seed coat permeability to different metals depends on the physical and chemical properties of the metals (Kranner and Colville, 2011). There have been many reports on the toxic effects of Ni on germination and seedling growth in plants (Yusuf et al., 2011). For example, Sharma et al. (2011) observed that *Salicornia brachiata* shoot and root length decreased up to 400 μM Ni. In two rice cultivars, Maheshwari and Dubey (2008) found that the exposition of seeds up to 200 μM of Ni reduced root and shoot length. In contrast to other metals that are accumulated mainly in the seed coat cells, Ni can be translocate across the seed coat and accumulate in plant embryo cells and scutellum (Seregin and Kozhevnikova, 2005). In our experiments, this may explain why negative effects on seedling growth were noticed at lower concentrations of Ni (from 250 μM Ni for *S. densiflora* radicle), being the only metal associated with reduced length of the first leaf, and hence reduced photosynthetic tissue driving growth, for all *S. densiflora* populations. Our results were in accordance with Márquez-García et al. (2013) who recorded reduced growth of roots of *Atriplex halimus* and *Salicornia ramosissima* at lower concentrations of Ni (above 100 μM Ni) than Cu and Zn (at 250 and 1000 μM, respectively).

Comparing our results to metals concentration in the field, we expected invasive *S. densiflora* seedling development to be impacted in most metal-polluted areas in Odiel and Tinto Estuaries. The maximum concentrations of Cu registered in Odiel and Tinto Estuaries was 745 μM (Elbaz-Poulichet et al., 1999) and, in our study, the radicle length of *S. densiflora* was reduced at Cu concentration of 100 μM and above, and the first leaf was shortened over 100 μM Cu for seedlings from seed produced in the Odiel Estuary. Moreover, maximum Zn concentrations in Odiel and Tinto Estuaries are ca. 900 μM (Elbaz-Poulichet et al., 2001). We recorded shortened *S. densiflora* radicles at concentrations higher than 500 μM Zn, and *S. densiflora* radicle and first leaf length were reduced over 250 and 500 μM Ni, respectively. In contrast, the maximum concentration of Ni recorded in the Odiel and Tinto estuaries has been 3 μM Ni (Elbaz-Poulichet et al., 1999, 2001; Braungardt et al., 2003). In view of these results, the invasion of *S. densiflora* may be slowed down and diminished in the most polluted areas of Odiel and Tinto Estuaries.

Finally, it is interesting to point out that *S. densiflora* seedlings sourced from seeds produced in the Tinto Estuary showed higher tolerances to the three metals than those from Odiel and Piedras Estuaries. This higher tolerance was reflected in radicle length reduction over 100 μM Cu for Odiel and Piedras seedlings and over 250 μM Cu for Tinto seedlings, and first leaf length reduction over 500 μM Ni for Odiel and Piedras seedlings and over 1000 μM Ni for Tinto seedlings. These differences in the tolerance to metals may be because *S. densiflora* seedlings are exposed to higher metal concentrations that are more bioavailable (at lower pH) in Tinto than in Odiel and Piedras Estuaries (Elbaz-Poulichet et al., 1999; Mateos-Naranjo et al., 2011). The existence of *S. densiflora* ecotypes in relation to seedling metal tolerance is agreement with Waddell and Kraus (1990) who found different *S. alterniflora* seedling responses to Cu exposure between polluted and non-polluted estuaries.

5. Conclusions

We can conclude that seeds of native *S. maritima* and invasive *S. densiflora* tolerate very high concentrations of Cu, Zn and Ni during germination, and that native *S. maritima* seedlings are more tolerant of these three metals than invasive *S. densiflora*. Increasing concentrations of Cu, Zn and Ni were associated with negative effects on seedling growth of invasive *S. densiflora*, mainly in the radicle. Metal tolerance of the early seedling life stage of the *Spartina* species was spatially variable across estuaries in the Southwest Iberian Peninsula. Seedlings from seeds produced in the highly polluted Tinto Estuary were more tolerant to metals than those from Odiel and Piedras Estuaries, suggesting both environment and evolutionary processes have played a role in tolerance observed today. These results have broad implications, since cordgrasses are widely distributed in estuaries with varying degrees of metal contamination in sediments. In an applied context, management efforts for control of invasive *S. densiflora* should prioritize eradication of this invader in marshes with lower heavy metal concentrations where growth is not limited by metal pollution.

Credit authorship contribution statement

M. Dolores Infante-Izquierdo: Conceptualization, Methodology, Investigation, Writing - original draft. **Alejandro Polo-Ávila:** Investigation. **Israel Sanjosé:** Investigation. **Jesús M. Castillo:** Conceptualization, Writing - review & editing. **F. Javier J. Nieva:** Investigation. **Brenda J. Grewell:** Writing - review & editing. **Adolfo F. Muñoz-Rodríguez:** Conceptualization, Methodology, Investigation, Writing - original draft.

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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