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The aquatic macrophytes as bioindicators of heavy metal contamination in estuarine ecosystems

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Abstract: The use of aquatic plants as metal bioindicators may be a suitable tool for the management of aquatic ecosystems as they play an important role in the geochemical composition of water and sediments. This study evaluated the potential use of autochthonous aquatic macrophytes, *Veronica anagallis-aquatica* and *Cakile maritima*, as bioindicators of cadmium, chromium, lead, zinc, and copper contamination in wetlands and their role on metal cycling. The content and distribution of these elements in roots, stems and leaves were compared in specimens collected in two estuaries with different anthropogenic pressures (Douro and Ave) and in the corresponding water and sediments. Differences on metal content were found on both species and were dependent on the estuary. No positive correlation was found between plant and water metal concentration, but a positive correlation was observed for sediments, except for Cr and Cd. Bioaccumulation and translocation factors showed that both species can contribute to the immobilization of Pb and Cd in the Douro estuary, in contrast to the Ave estuary. These results demonstrate the influence of local environmental conditions in the bioaccumulation of *in situ* aquatic plants and suggest their potential use as bioindicators and for the management (phytoextraction and phytostabilization) of contaminated aquatic ecosystems.

Keywords: macrophyte; bioaccumulation; trace elements; metals; bioindicators; aquatic plants

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Introduction

Pollution by heavy metals (HMs) is a threat to the environment and a global concern due to HMs persistence, ability to accumulate and hazardous impacts in all life forms [1, 2]. Over the last decades, the concentration of HMs in the environment has increased because of human activities (e.g., industry, use of fertilizers and pesticides, mining, combustion of fossil fuels, improper waste disposal, etc.) [3-7]. Therefore, HMs are widespread in the environment, though aquatic ecosystems are among the most affected, as they are frequently the ultimate sink for many contaminants [4-6]. Aquatic macrophytes are widely distributed in aquatic and wetland ecosystems and are part of a healthy aquatic ecosystem, providing oxygen, food and habitat for many species. Aquatic vegetation plays an important role in water and sediment geochemistry, as it absorbs nutrients from sediments and water through its root/rhizome systems [3, 8, 9]. Its capability to accumulate contaminants makes it a good indicator of local contamination, as it is sessile and constantly exposed to *in situ* contaminants. On the other hand, contaminated aquatic vegetation may be a source of food for many organisms, leading to the possibility of bioaccumulation and biomagnification in higher trophic levels of the food chain. Thus, aquatic macrophytes are officially recognized under the Water Framework Directive of the European Union as essential elements for the biological assessment of a habitat quality [10].

Aquatic plant species have also been used to remove HMs from wastewater and for phytoremediation, as they accumulate variable amounts of diverse HMs [11]. Nevertheless, the potential uptake of HMs from water and sediments by aquatic macrophytes depends on biological factors (e.g., plant species, life cycle, age) and aquatic environment conditions (e.g., metal availability, season, temperature, salinity, pH, redox potential) [9, 12].

The concentration of metals in plants can be several times greater than that determined in the corresponding water and, therefore, aquatic plant species can be used as indicators of HMs contamination that might otherwise be difficult to detect [13, 14]. However, there is still a lack of information about the capability of various aquatic plants to accumulate HMs and the relationships between the plants and the metal concentrations in water and sediments. *Veronica anagallis-aquatica* and *Cakile maritima* are macrophyte species, widely distributed in coastal and estuarine habitats around the world and referred in the literature as potential accumulators of cadmium (Cd), nickel (Ni), copper (Cu), zinc (Zn), manganese (Mn), lead (Pb) and iron (Fe), among other metals [9, 15]. *V. anagallis-aquatica* is a perennial herb that shows a root-like subterranean stem, commonly horizontal in position. *C. maritima* is an annual species with a short life cycle and is a fast-growing halophyte that has been shown to colonize HMs-contaminated saline soils, suggesting its tolerance to these elements [15]. These two species are autochthonous species of coastal and estuarine areas of Portugal. Estuarine ecosystems are among the most vulnerable areas due to the high anthropogenic pressure and climate changes. Nevertheless, estuaries are the habitat for a wide variety of plant and animal life, especially water birds, and nursery for several species of fish and shellfish, and other aquatic organisms. Estuarine areas are also important for cycling nutrients and remove contaminants, including HMs, through absorption by plants. Therefore, this work aimed to evaluate the potential use of two autochthonous aquatic macrophytes, *V. anagallis-aquatica* and *C. maritima*, as biological indicators of HMs [Cd, chromium (Cr), Pb, Zn, and Cu] contamination and to investigate their utility for phytoextraction and phytostabilization in contaminated sites. For that, both plant species were collected in two estuaries with different anthropogenic pressures, the Douro River estuary and the Ave River estuary. The specific objectives were to: i) compare HMs bioaccumulation of both plant species collected in the two estuaries and in the corresponding water and sediments; ii) investigate the distribution of the selected elements in different organs (roots, stems and leaves); iii) determine the bioaccumulation and translocation factors and iv) discuss their potential use as bioindicators and for phytoextraction and phytostabilization of HMs contamination.

Materials and Methods

Study sites

The Douro River is one of the longest rivers of the Iberian Peninsula. It rises in Urbión (Spain) and then flows to its mouth at Porto, the second largest city of Portugal, and Vila Nova de Gaia, to meet the Atlantic Ocean (Fig. 1). The Douro River estuary is about 21.6 km long and its mouth is densely populated and highly impacted by anthropogenic activities, such as industry (e.g., metallurgic, textile, food and beverages, non-electrical machinery, wood and electrical machinery) and discharges from various wastewater treatment plants (WWTPs). Its average depth is about 8 m, with a natural semidiurnal tidal variation from 2 to 4 m. The water level in the estuary is highly variable depending on the climate situation, with the freshwater drainage pattern being controlled by the Crestuma-Lever dam and flood and ebb tides with a mean water flow of 450 $m^3 s^{-1}$.

The Ave River hydrological basin is located in the Northwest region of Portugal. General water quality was considered inadequate for numerous purposes due to high levels of nutrients and trace elements, but the construction of 12 WWTPs along the basin significantly improved water quality. Mean water flow is of 40 $m^3 s^{-1}$. Nevertheless, the basin still receives significant untreated wastewater discharges, mostly illegal discharges, and water quality is considered poor [5]. The Ave estuary is located at Vila do Conde (Fig. 1), where a natural ornithological reserve and the Regional Protected Landscape of Vila do Conde are located, a unique area in terms of biodiversity of flora, amphibians, birds, reptiles and mammals. The estuary is densely populated and mainly affected by agricultural practices.

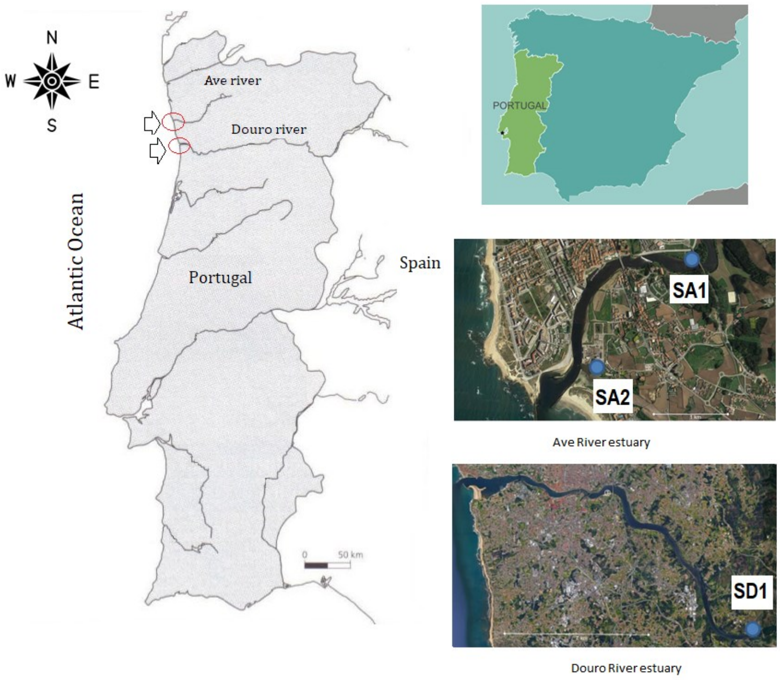

Figure 1. Map of Portugal showing the location of the Douro and the Ave rivers and the respective estuaries (red circles) and sampling points (blue circles).

Sample collection and metal analysis

Sample collection and analysis are described in previously published works [4, 5]. Various algae and plant species were collected. However, for this study, only specimens common to both estuaries were selected. Thus, two macrophyte species, *V. anagallis-aquatica* and *C. maritima*, were collected at low tide in spring (May 2013) in the Douro estuary (one sampling point SD1) and Ave estuary (two sampling points: SA1 for *V. anagallis-aquatica* and SA2 for *C. maritima*). These sampling points were selected according to previous studies and availability of flora. Three replicates were collected to integrate inter-population variability into the analysis. At the same sampling points, surface water samples (< 2 m) were collected in clean amber glasses, while sediment samples and plants were stored in polyethylene bags and transported to the laboratory in coolers. Water samples were then stored at 4 °C in the dark until analysis. Plants were washed with deionized water and dried in an oven at 60 °C to a constant weight, while sediments were frozen. Once dried, plants were dissected into roots, stems and leaves and each part ground into fine powder.

An iCAP™ Q ICP-MS from Thermo Fisher Scientific (Bremen, Germany) with a MicroMist™ nebulizer (Glass Expansion, Port Melbourne Vic, Australia), a Peltier-cooled baffled cyclonic spray chamber, a standard quartz torch and a two-cone interface design (nickel sample and skimmer cones) were used for Cd, Cr, Pb, Zn, and Cu determinations. High-purity argon (99.9997%; Gasin, Portugal) was used for nebulization and plasma. A microwave (Milestone MLS, Sorisole, Italy) was used for plant and sediment digestion.

The bioaccumulation factor (BAF) was also determined. The BAF is the ratio between the total concentration of the element in all parts of the plant and the total concentration of the element in the sediments. To assess the BAF of metals, the four-degree scale described in [16] was assumed. According to this scale, a BAF of < 0.01 means no accumulation; 0.01–0.1—low bioaccumulation; 0.1–1.0—medium bioaccumulation; and above 1.0—high bioaccumulation.

The translocation factor (TF), also called shoot-root quotient, explains the plant's ability to translocate metals from its roots to shoots and to leaves. Plants that have BAF and TF > 1 can be used as bioaccumulators [17]. Plants can be used as phytostabilizers if BAF > 1 and TF < 1 and as phytoextractors if BAF < 1 and TF > 1 [18].

Statistical analysis

Statistical analysis was performed using R Statistical Software version 4.0. Due to small sample size, a non-parametric distribution of data was assumed. Mean and interquartile ranges were estimated for metal concentration in plants. Inferential statistics were conducted using Mann-Whitney and Kruskal-Wallis tests, for factors with 2 and more than 2 levels, respectively. A $p < 0.05$ was considered statistically significant. Spearman's correlation coefficient was estimated to measure the correlations between plant metal concentrations and water and sediment metal concentrations. To further explore the impact of the water and sediments on plants, metal concentrations were dichotomized into low and high concentrations. Logistic regression models were subsequently fitted to study the associations between the water and sediments and metal concentrations in plants, except for Cd, which was not detected in one of the specimens and thus failed to meet the variance requirements for logistic regression.

Results

Heavy metal concentration in surface water and sediments

Concentrations of the HMs in surface waters and sediments in each estuary are summarized in Table 1 and compared to ecotoxicological threshold levels of water or sediments [19, 20]. Levels of HMs in surface waters were higher in the Ave River estuary for most of the elements, while in sediments most elements were found at higher concentration in the Douro River estuary. In the Douro estuary, the following descending order was observed: Pb>Zn>Cu>Cr>Cd, for both surface water and sediments. Pb, Cu and Zn exceed surface water threshold levels while, in sediments, only Pb and Cu exceed those values. Regarding the Ave River estuary, levels of HMs in surface waters at both sampling points followed the same order: Zn>Cu>Pb>Cr>Cd, while, in sediments, element concentration order was different among sampling points. The element order in sediments at sampling point of *V. anagallis-aquatica* (SA1) was Zn>Cu>Cd>Cr>Pb. At this sampling point, all elements except for Cd exceed surface water threshold and all elements exceed sediment safety threshold, except for Pb and Cr. At *C. maritima* sampling point (SA2), the order Zn>Cu>Cr>Pb>Cd was observed in sediments. At this sampling point (SA2), all elements surpassed the surface water threshold, while for sediments all element concentrations were lower than the respective threshold levels.

Table 1. HMs concentration in surface water (µg/L) and estuary river sediments (mg/kg) in the Douro River estuary and the Ave River estuary.

	Heavy metals				
	Cd	**Pb**	**Cu**	**Zn**	**Cr**
Douro River Estuary					
Surface water (SD1)	0.075	**16.6**	**6.53**	**12.9**	0.89
Sediments (SD1)	0.078	**135**	**66.0**	124	13.3
Ave River Estuary					
Surface water (SA1)	0.118	**4.91**	**10.7**	**43.3**	**4.14**
Sediments (SA1)	**29.0**	0.364	**40.4**	**220**	26.4
Surface water (SA2)	**1.59**	**27.7**	**47.4**	**89.0**	**5.21**
Sediments (SA2)	0.19	10.1	14.1	110	13.8
Ecotoxicological threshold for freshwaters [19]	0.1	1	2	7	1
Ecotoxicological threshold for sediments [20]	0.6-3.5	35-91.3	35.7-197	123-315	37.9-90

Highlighted values mean that HMs concentration surpassed ecotoxicological threshold levels of water or sediments.

Heavy metal content and distribution in plant organs

Total HMs concentration for both plant species, distribution, and concentration levels in each organ of *V. anagallis-aquatica* and *C. maritima* collected in the Ave River estuary and the Douro River estuary are shown in Table 2 and Fig. 2. Fig. 2 shows normalized distribution of HMs in plant organs and in the corresponding water and sediments for comparative analysis. All HMs were found in the two plant species, except for Cd in *C. maritima* from the Ave River estuary (Table 2 and Fig. 2). Plant metal concentrations were typically higher in the Douro River estuary compared to the Ave River estuary, except for Zn (Table 2, Fig. 2). Interestingly, though Cd was found in both water and sediments in the Ave River estuary, this element was not found in *C. maritima* collected at sampling point SA2 (Fig. 2). In the Ave estuary, all elements showed higher values in sediments than in the roots, except at SA1, where for Pb and Zn the higher values were found in roots (Fig. 2). In the Douro estuary, all elements also showed higher values in sediments than in roots, except for Cd (Fig. 2). HMs plant organ distributions in *V. anagallis-aquatica* decreased according to the order root>leaves>stem in the Ave River estuary, while in the Douro River estuary the order was leaves>root>stem. For *C. maritima*, plant organ element distribution was also different between estuaries. In the Douro estuary, the distribution pattern was root>leaves>stem, but in the Ave River estuary the highest value for most of the elements was found in leaves, followed by the root or stem, depending on the element (Fig. 2). Regarding element distribution, for both specimens collected in the Ave estuary, the following order was found in all organs: Zn>Cu>Cr>Pb>Cd, though Cd was not determined in *C. maritima*. In the Douro River estuary, element order was Zn>Cu>Pb>Cr>Cd. In contrast to the Ave estuary, Cd was found in both species collected in the Douro River estuary and at similar concentrations of Cr (Table 2, Fig. 2). The higher values of Pb, Cu and Zn were found in leaves, followed by root (and stem for Zn), whereas the highest value of Cr was found in the root, followed by the stem.

Significant differences were found for Cd and Pb between species collected in the Ave and the Douro estuaries (0.792 *vs.* 0.108 and 3.87 *vs.* 0.295 mg/kg, respectively, Table S1), with higher values found in the Douro River estuary. Significant differences were found between Zn concentrations in the two species, with *V. anagallis-aquatica* showing higher values than *C. maritima*, but independently of the estuary (median of 163 *vs.* 48.9 mg/kg, respectively, Table S2). Although *V. anagallis-aquatica* also showed a higher metal concentration of Cd, Cr, Cu and Pb, results were not significantly different (Table S2).

In the Ave River estuary, in *V. anagallis-aquatica*, the BAF decreased in the order Pb>Cu>Cr>Zn>Cd, whereas in the Douro River estuary the trend was Cd>Zn>Cu>Cr>Pb (Fig. 2, Table 2). However, no significant differences were found between plant organs and metal levels (Table S3).

Table 2. Determination of HMs in *V. anagallis-aquatica* and *C. maritima* (mg/kg dry weight).

V. anagallis-aquatica		**Cd**	**Pb**	**Cu**	**Zn**	**Cr**	**Total**
Ave	Root	0.26	1.87	18.3	377	4.33	**401.8**
River	Stem	0.22	0.27	8.14	215	0.83	224.5
Estuary	Leaves	0.28	0.44	20.0	323	1.19	344.9
(**SA1**)	**TOTAL**	0.76	2.58	46.4	915	6.34	
	BAF	0.03	**7.13**	**1.30**	0.23	0.29	
	TF stem	0.82	0.14	0.45	0.57	0.19	
	TF leaves	**1.06**	0.24	**1.09**	0.86	0.28	
Douro	Root	0.84	7.36	18.5	85.2	3.73	115.6
River	Stem	0.47	0.57	8.60	64.4	0.43	74.5
Estuary	Leaves	0.74	2.18	19.4	112	1.49	**135.8**
(**SD1**)	TOTAL	2.06	10.1	46.5	261	5.65	
	BAF	**8.26**	0.02	0.31	**1.94**	0.13	
	TF stem	0.56	0.08	0.46	0.76	0.11	
	TF leaves	0.89	0.30	**1.05**	**1.31**	0.40	
C. maritima		**Cd**	**Pb**	**Cu**	**Zn**	**Cr**	
Ave	Root	-	0.152	2.83	29.2	0.69	32.9
River	Stem	-	0.096	2.71	39.3	0.72	42.8
Estuary	Leaves	-	0.319	3.53	53. 7	0.49	**58.0**
(**SA2**)	TOTAL	-	0.567	9.07	122	1.90	
	BAF	-	0.06	**1.11**	**1.50**	0.18	
	TF stem	-	0.63	0.96	**1.35**	**1.05**	
	TF leaves	-	**2.10**	**1.25**	**1.84**	0.72	
Douro	Root	1.13	5.55	17.4	120	2.73	**146.8**
River	Stem	0.64	1.40	6.50	44.1	0.51	53.2
Estuary	Leaves	0.90	10.4	26.0	100	1.66	139.0
(**SD1**)	TOTAL	2.67	17.3	49.9	264	4.90	
	BAF	**9.48**	0.03	0.22	0.62	0.10	
	TF stem	0.56	0.25	0.37	0.37	0.19	
	TF leaves	0.79	**1.87**	**1.49**	0.83	0.61	

Highlighted values mean values higher than 1.

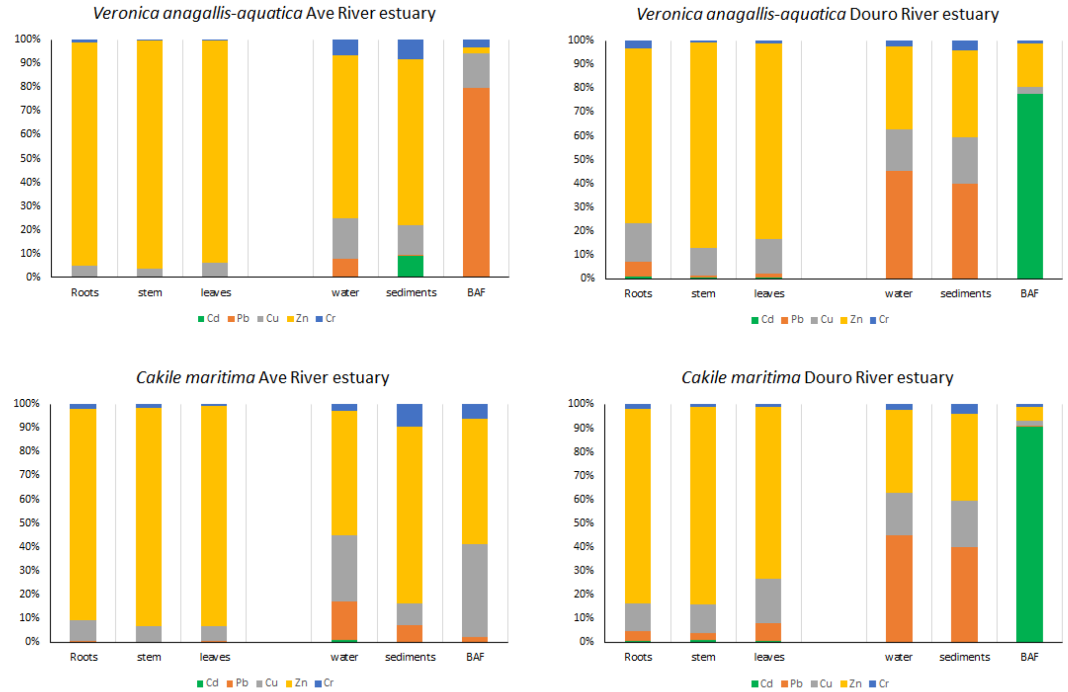

Figure 2. Normalized distribution of HMs values in plant organs, surface water, sediments and bioaccumulation factor (BAF), for each plant species collected in both the Ave River estuary and the Douro River estuary.

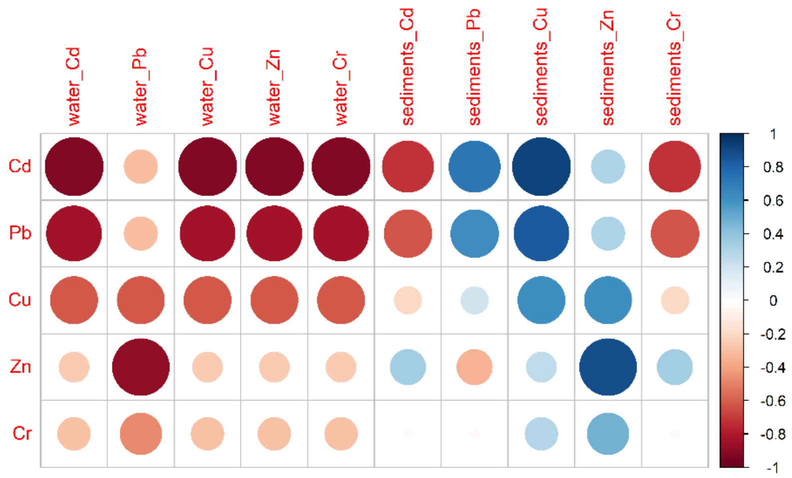

Figure 3. Correlation plot between plant metal concentrations (*y* axis) and surface water and sediment metal concentrations (*x* axis). The blue color represents a positive correlation, while red represents an inverse correlation.

Logistic regression models showed that concentrations of Pb in plants were significantly associated with the estuary. Additionally, specimens collected in the Douro River estuary had, on average, 25 times higher risk of showing high Pb concentration (> 0.98 mg/kg) independently of the species (Fig. 3). No significant associations were observed for the other metals (Table S4).

Discussion

Both rivers receive large amounts of effluents and wastewaters from urban, industrial and agricultural activities, resulting in the occurrence of diverse pollutants at high concentrations [4, 5, 21]. Though high values were found for most HMs levels in both surface water and sediments, HMs levels are within or lower than other reported in estuarine systems around the world [22]. Nevertheless, attention should be given as most of the HMs levels were higher than safety ecotoxicological threshold recommended levels. Thus, harmful effects are expected to occur and should be investigated in local non-target species. For instance, several tissue damages were reported in fish collected in tributaries of the Douro River estuary [23]. In fact, these five HMs are among the priority metals of higher health public concern, as they may cause several damages, including carcinogenesis, in both animals and humans [1,2]. A deeper discussion considering spatial-temporal occurrence of various trace elements in both estuaries can be found elsewhere [4,5].

In the Douro River estuary, the concentration of HMs in surface water was lower than in the sediments. This may be attributed to the adsorption or combination of HMs with organic matter from suspended solids that then settle into the bottom. The same was verified in the Ave River estuary, though, at SA2, high values were found for most elements (Cd, Pb and Cu) in surface water, which may indicate punctual discharges. Further, this sampling point is in the natural reserve of the Ave River estuary, which is highly impacted by local agricultural practices, in which soil lixiviates drain into this sampling point [5].
Element order followed the same descending order for plant species collected in the same estuary but were different between estuaries. This may be attributed to the different capability for metal accumulation between the two species, but also to the different environmental conditions of the two estuaries, as HMs availability on both surface waters and sediments. For most of the HMs, higher values were found in the roots rather than in the aerial parts. Higher content of metals in roots has also been reported in macrophyte species living in saline soils, attributed to the possible adaptation of the mechanisms of salt excretion for trace element elimination, as a response to the stress caused by these contaminants [16, 24, 25]. Nevertheless, the pattern of element distribution among plant organs was different among species and estuaries. The higher values of Pb and Cu were found in leaves, followed by roots, whereas the highest value of Cr was found in the root, followed by the stem. In the Ave estuary, all elements showed higher values in sediments than in the roots, except at SA1, where for Pb and Zn the higher values were found in roots. In the Douro estuary, all elements also showed higher values in sediments than in roots, except for Cd (Fig. 2). This is in accordance with other studies that showed the immobilization of metals in belowground plant tissues for Cd, Cu and Zn, in tidal marshes, caused by their lower sorption capacity to soil components [26, 27]. Nevertheless, in the Ave estuary, high levels of Pb, Cu and Zn were found in leaves. This is in accordance with the high concentration of the HMs found in surface water at this sampling point. In fact, at this sampling point, sediment HMs levels were lower than threshold safety concentrations. Nevertheless, results showed that levels of metals found in plants were not positively correlated with metal levels found in surface water, but positively correlated with concentrations found for metals in sediments of the respective estuarine system, except for Cr and Cd (Fig. 3).
In both species and estuaries, Zn was the element found at the highest concentration in all organs, while Cd was the least accumulated element (Fig. 2). High Zn content has been reported in several aquatic and terrestrial plant species, possibly due to the high mobility of the Zn cation in soil and water [28].

Heavy metal bioaccumulation and translocation factor
The BAF pattern in both species was different among estuaries. BAF > 1 for Pb and Cu were found in *V. anagallis-aquatica* in the Ave estuary, but TF < 1 was determined for Pb, showing that this species can contribute to the immobilization of this element. In the Douro estuary, BAF > 1 and TF < 1 were found for Cd; all other elements were medium to low accumulated. This shows that *V. anagallis-aquatica* may be a suitable species for phytostabilization of Cd in the Douro estuary. TF > 1 was found for Cu in both estuaries and TF > 1 for Zn in the Douro estuary. Cu and Zn are essential elements required for growth and maintenance mechanisms. Regarding *C. maritima*, the BAF pattern was also different among estuaries. In the Ave River estuary (SA2), the order was Zn>Cu>Cr>Pb, with values higher than 1 for Zn and Cu; in the Douro River estuary (SD1), the order was the same than that found in *V. anagallis-aquatica* in the same estuary: Cd>Zn>Cu>Cr>Pb. Similarly to *V. anagallis-aquatica*, *C. maritima* collected in the Ave River estuary showed BAF > 1 for Zn and Cu, showing accumulation of these elements, but low accumulation of Pb. BAF > 1 was found for Cd in specimens collected in the Douro estuary, while other elements showed medium to low accumulation. Cd was not accumulated in *C. maritima* from the Ave estuary, indicating that this element may not be in an available form, but, in *C. maritima* collected in the Douro estuary, Cd showed the highest BAF (> 8) and TF < 1, demonstrating that this species is able to accumulate this element. These differences may be the result of metals being in different forms in the sediments of the two estuaries and the different HMs content found in sediments collected in both estuaries. Almeida *et al.* studied metal content and the influence of *Juncus maritimus* in sediments of the Douro estuary [26]. Authors reported that *J. maritimus* was able to change sediment metal composition, namely for Cd, influencing metal mobility, availability, and toxicity. It is also important to stress the high accumulation of Pb in *V. anagallis-aquatica* from the Ave estuary. Pb is a nonessential toxic element to plants and, therefore, it was not expected to be accumulated in plants.

Aquatic macrophytes as bioindicators
Aquatic macrophytes are widely distributed in wetland ecosystems. They have various characteristics that favor metal accumulation, as they are predominant organisms, sessile and therefore constantly exposed to contaminants as metals. Additionally, rooted species can absorb metals from their roots and rhizome system and through their leaves they can trap particulate matter, sorb metals and accumulate them. Various studies have been reporting the capability of aquatic macrophytes to accumulate large quantities of metals and tolerate highly contaminated ecosystems. However, different aquatic species accumulate variable amounts of diverse HMs as a function of biological characteristics (age, life cycle) and environmental conditions, including metal availability (free ions or humic complexes), season, temperature, and physicochemical characteristics as pH, salinity and nutrients. Ahmed *et al.* described a hydroponic experiment using *V. anagallis-aquatica* and *Epilobium laxum*. The effect of different fertilizers and plant growth regulators was evaluated

on growth, biomass, free proline, phenolics, and chlorophyll contents, and their role in Cd phytoaccumulation was investigated; *V. anagallis-aquatica* demonstrated a hyperaccumulator potential for Cd, with a BAF of about 8, specially in roots, as shown in our study in specimens collected in the Douro estuary [29]. Nevertheless, specimens collected in the Ave estuary did not show high BAF for Cd, suggesting that environmental conditions are a key factor for the capability of absorption of metals by plants. Another study conducted by Khalid *et al.* showed that *V. anagallis-aquatica* accumulated high HMs quantities, according to metal uptake percent as follows: Ni>Cu>Zn>Mn>Pb>Fe, mainly in the roots [28]. The role of 29 plant species, including *V. anagallis-aquatica*, in metal cycling in tidal wetlands was also investigated [27]. This species was the third out of 29 plants with higher metal accumulation, showing a high accumulation potential towards As, Cr, Pb, Cd, Fe, Cu, Mn, and Zn. Kroflic *et al.* also studied the influence of extensive agriculture on the concentrations of As, Cr, Cu, Cd, Se, Pb and Zn in sediments and in *V. anagallis-aquatica* [30]. Most of the elements studied, except for Zn and Cu, were accumulated mainly in root tissues [30]. The highest concentration of the selected elements was found in roots; leaves and stems showed at least 7 times lower concentrations than roots, except for Cu and Zn, as was also found in this study.

Comparison between morphological organs of the collected plants showed that, except for *C. maritima* collected in the Ave River estuary, roots consistently presented higher metal concentrations than either the stems or leaves. Also, stems accumulate less metals than the leaves. In fact, in *C. maritima* specimens collected in the Ave River estuary, leaves showed the higher concentration for some elements, Pb, Zn and Cu. These results suggest that roots and leaves of these species are good accumulators of the selected elements. The differences between the same species collected in different estuaries can be related to different uptake mechanisms, influenced by environmental conditions and by HMs concentrations that are different among estuaries. In fact, the elements found at the highest concentrations in both water and sediments in the Ave River estuary are Zn and Cu, followed by Pb, Cr and the lowest one, Cd. The pattern in the Douro River estuary was different, with the highest concentration found for Pb. The other elements followed the same trend observed in the Ave River estuary: Zn>Cu>Cr>Cd. The Cd-phytoextraction ability of *C. maritima* was evaluated comparatively to that of *Brassica juncea*. *C. maritima* showed to be more tolerant to Cd than *B. juncea*, being able to maintain growth and photosynthesis despite high levels of Cd accumulation in the shoots [15]. However, accumulation in aboveground plant tissues may increase toxicity. In fact, high accumulation of these elements in the leaves might transfer to the food chain by direct consumption of leaves or metal-contaminated litter [27]. This also supports the fact that, aquatic plants influence sediment composition and metal availability, acting as HMs scavengers in sediments [26, 31].

Conclusions and Future Perspectives

Positive correlations were found between the levels of metals in plants and in sediments, except for Cd and Cr. This suggests that HMs in sediments of both estuaries occur in bioavailable forms and are being taken up and accumulated in macrophytes. These results support that metal concentration in aquatic macrophytes can indicate contamination of local wetlands.

Both species can be used as bioindicators of metal contamination in both estuaries. *C. maritima* is an annual plant that completes its life cycle in a short period, while *V. anagallis-aquatica* is a perennial hemicryptophyte. Thus, *V. anagallis-aquatica* can be a more suitable plant species as bioindicator of HMs contamination and for phytoremediation. Nevertheless, accumulation of elements was dependent on the species. *V. anagallis-aquatica* showed to accumulate Pb and Cu and *C. maritima* Cu and Zn in the Ave estuary, while in the Douro estuary both species showed to be good accumulators of Cd.

Knowledge about the differences in trace metal accumulation between plant species is crucial to evaluate the effect of plant growth on toxic metal cycling within HMs-contaminated zones and to develop appropriate management options. Additionally, this knowledge is valuable in the risk assessment of metal toxicity in restoration projects in contaminated sites.

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Author Contributions

CR and CC conceptualized the work, implemented the methodology, performed formal analysis, and drafted the manuscript. CR, CC and AAA provided the resources, reviewed and edited the manuscript. CR and AAA acquired the funding. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no competing interests.

References

1. Briffa, J.; Sinagra, E.; Blundell, R. Heavy metal pollution in the environment and their toxicological effects on humans. *Heliyon* **2020,** 6, (9), e04691, doi:10.1016/j.heliyon.2020.e04691.
2. Yang, F.; Yun, Y.; Li, G.; Sang, N. Heavy metals in soil from gangue stacking areas increases children health risk and causes developmental neurotoxicity in zebrafish larvae. *Sci Total Environ* **2021,** 794, 148629, doi:10.1016/j.scitotenv.2021.148629.
3. Bai, L.; Liu, X. L.; Hu, J.; Li, J.; Wang, Z. L.; Han, G.; Li, S. L.; Liu, C. Q. Heavy Metal Accumulation in Common Aquatic Plants in Rivers and Lakes in the Taihu Basin. *Int J Environ Res Public Health* **2018,** 15, (12), doi:10.3390/ijerph15122857.
4. Ribeiro, C.; Couto, C.; Ribeiro, A. R.; Maia, A. S.; Santos, M.; Tiritan, M. E.; Pinto, E.; Almeida, A. A. Distribution and environmental assessment of trace elements contamination of water, sediments and flora from Douro River estuary, Portugal. *Sci Total Environ* **2018,** 639, 1381-1393, doi: 10.1016/j.scitotenv.2018.05.234.
5. Couto, C.; Ribeiro, C.; Ribeiro, A. R.; Maia, A.; Santos, M.; Tiritan, M. E.; Pinto, E.; Almeida, A. A. Spatiotemporal Distribution and Sources of Trace Elements in Ave River (Portugal) Lower Basin: Estuarine Water, Sediments and Indigenous Flora. *Int J Environ Res* **2019,** 13, (2), 303-318, doi: 10.1007/s41742-019-00174-z.
6. Stoichev, T.; Coelho, J. P.; De Diego, A.; Valenzuela, M. G. L.; Pereira, M. E.; de Chanvalon, A. T.; Amouroux, D. Multiple regression analysis to assess the contamination with metals and metalloids in surface sediments (Aveiro Lagoon, Portugal). *Mar Pollut Bull* **2020,** 159, 111470, doi: 10.1016/j.marpolbul.2020.111470.
7. Truchet, D. M.; Buzzi, N. S.; Negrin, V. L.; Botté, S. E.; Marcovecchio, J. E. First long-term assessment of metals and associated ecological risk in subtidal sediments of a human-impacted SW Atlantic estuary. *Mar Polluti Bull* **2022,** 174, 113235, doi: 10.1016/j.marpolbul.2021.113235.
8. Mishra, A. K.; Farooq, S. H. Trace metal accumulation in seagrass and saltmarsh ecosystems of India: comparative assessment and bioindicator potential. *Mar Pollut Bull* **2022,** 174, 113251, doi: 10.1016/j.marpolbul.2021.113251.
9. Bonanno, G.; Vymazal, J.; Cirelli, G. L. Translocation, accumulation and bioindication of trace elements in wetland plants. *Sci Total Environ* **2018,** 631-632, 252-261, doi: 10.1016/j.scitotenv.2018.03.039.
10. EU-Directive, Directive of the European Parliament and of the Council 2000/60/EC, establishing a framework for community action in the field of water policy. *Off J Europ Comm* **2000,** L 327, 1-72.
11. Wani RA; Ganai BA; Shah MA; B, U. Heavy Metal Uptake Potential of Aquatic Plants through Phytoremediation Technique - A Review. *J Bioremediat Biodegrad* **2017,** 8, (4), 404, doi: 10.4172/2155-6199.1000404.
12. Jenačković, D. D.; Zlatković, I. D.; Lakušić, D. V.; Ranđelović, V. N. Macrophytes as bioindicators of the physicochemical characteristics of wetlands in lowland and mountain regions of the central Balkan Peninsula. *Aquat Bot* **2016,** 134, 1-9, doi: 10.1016/j.aquabot.2016.06.003.
13. Pasricha, S.; Mathur, V.; Garg, A.; Lenka, S.; Verma, K.; Agarwal, S. Molecular mechanisms underlying heavy metal uptake, translocation and tolerance in hyperaccumulators-an analysis: Heavy metal tolerance in hyperaccumulators. *Environ Challenges* **2021,** 4, 100197, doi: 10.1016/j.envc.2021.100197.
14. Sharma, P.; Ngo, H. H.; Khanal, S.; Larroche, C.; Kim, S.-H.; Pandey, A. Efficiency of transporter genes and proteins in hyperaccumulator plants for metals tolerance in wastewater treatment: Sustainable technique for metal detoxification. *Environ Technol Innov* **2021,** 23, 101725, doi: 10.1016/j.eti.2021.101725.
15. Taamalli, M.; Ghabriche, R.; Amari, T.; Mnasri, M.; Zolla, L.; Lutts, S.; Abdely, C.; Ghnaya, T. Comparative study of Cd tolerance and accumulation potential between *Cakile maritima* L. (halophyte) and *Brassica juncea* L. *Ecol Eng* **2014,** 71, 623-627, doi: 10.1016/j.ecoleng.2014.08.013.
16. Pachura, P.; Ociepa-Kubicka, A.; Skowron-Grabowska, B. Assessment of the availability of heavy metals to plants based on the translocation index and the bioaccumulation factor. *Desalin Water Treat* **2016,** 57, (3), 1469-1477, doi: 10.1080/19443994.2015.1017330.

17. Usman, A. R.; Alkredaa, R. S.; Al-Wabel, M. I. Heavy metal contamination in sediments and mangroves from the coast of Red Sea: *Avicennia marina* as potential metal bioaccumulator. *Ecotoxicol Environ Saf* **2013,** 97, 263-70, doi: 10.1016/j.ecoenv.2013.08.009.
18. Yoon, J.; Cao, X.; Zhou, Q.; Ma, L. Q. Accumulation of Pb, Cu, and Zn in native plants growing on a contaminated Florida site. *Sci Total Environ* **2006,** 368, (2-3), 456-64, doi: 10.1016/j.scitotenv.2006.01.016.
19. CCME, Canadian Sediment Quality Guidelines for the Protection of Aquatic Life. In Council, C., Ed. 2015; Vol. http://st-ts.ccme.ca/en/index.html, accessed 02-10-2017.
20. CCME, Water Quality Guidelines for the Protection of Aquatic Life. http://stts.ccme.ca/en/index.html **2019**.
21. Ribeiro, C.; Tiritan, M. E.; Rocha, E.; Rocha, M. J. Seasonal and Spatial Distribution of Several Endocrine-Disrupting Compounds in the Douro River Estuary, Portugal. *Arch Environ Contam Toxicol* **2009,** 56, (1), 1-11.
22. Wang, Z.; Lin, K.; Liu, X. Distribution and pollution risk assessment of heavy metals in the surface sediment of the intertidal zones of the Yellow River Estuary, China. *Mar Pollut Bull* **2022,** 174, 113286.
23. Santos, R.M.B.; Monteiro, S.M.V.; Cortes, R.M.V.; Pacheco, F.A.L.; Fernandes, L.F.S. Seasonal Differences in Water Pollution and Liver Histopathology of Iberian Barbel (*Luciobarbus bocagei*) and Douro Nase (*Pseudochondrostoma duriense*) in an Agricultural Watershed. *Water* **2022,** 14, 444.
24. Cambrollé, J.; Redondo-Gómez, S.; Mateos-Naranjo, E.; Figueroa, M. E. Comparison of the role of two *Spartina* species in terms of phytostabilization and bioaccumulation of metals in the estuarine sediment. *Mar Pollut Bull* **2008,** 56, (12), 2037-42, doi: 10.1016/j.marpolbul.2008.08.008.
25. Arbelet-Bonnin, D.; Ben-Hamed-Louati, I.; Laurenti, P.; Abdelly, C.; Ben-Hamed, K.; Bouteau, F. Chapter Two - *Cakile maritima*, a promising model for halophyte studies and a putative cash crop for saline agriculture. In *Advances in Agronomy*, Sparks, D. L., Ed. Academic Press: 2019; Vol. 155, pp 45-78.
26. Almeida, C. M. R.; Mucha, A. P.; Vasconcelos, M. T. S. D. Influence of the sea rush *Juncus maritimus* on metal concentration and speciation in estuarine sediment colonized by the plant. *Environ Sci Technol* **2004,** 38 11, 3112-8.
27. Teuchies, J.; Jacobs, S.; Oosterlee, L.; Bervoets, L.; Meire, P. Role of plants in metal cycling in a tidal wetland: implications for phytoremidiation. *Sci Total Environ* **2013,** 445-446, 146-54, doi: 10.1016/j.scitotenv.2012.11.088.
28. Khalid, K. M.; Ganjo, D. G. A. Native aquatic plants for phytoremediation of metals in outdoor experiments: implications of metal accumulation mechanisms, Soran City-Erbil, Iraq. *Int J Phytoremediation* **2021,** 23, (4), 374-386, doi: 10.1080/15226514.2020.1815645.
29. Ahmad, A.; Hadi, F.; Ali, N.; Jan, A. U. Enhanced phytoremediation of cadmium polluted water through two aquatic plants *Veronica anagallis-aquatica* and *Epilobium laxum*. *Environ Sci Pollut Res Int* **2016,** 23, (17), 17715-29, doi: 10.1007/s11356-016-6960-2.
30. Kroflič, A.; Germ, M.; Golob, A.; Stibilj, V. Does extensive agriculture influence the concentration of trace elements in the aquatic plant V*eronica anagallis-aquatica*? *Ecotoxicol Environ Saf* **2018,** 150, 123-128, doi: 10.1016/j.ecoenv.2017.10.055.
31. Almeida, C. M. R.; Mucha, A. P.; Vasconcelos, M. T. S. D. Comparison of the role of the sea club-rush *Scirpus maritimus* and the sea rush *Juncus maritimus* in terms of concentration, speciation and bioaccumulation of metals in the estuarine sediment. *Environ Pollut* **2006,** 142, (1), 151-159, doi: 10.1016/j.envpol.2005.09.002.

Supplementary material

The aquatic macrophytes as bioindicators of heavy metal contamination in estuarine ecosystems

Cláudia Ribeiro [1,2], Agostinho A. Almeida [3], and Cristina Couto [1,3]

Table S1. Median and interquartile ranges for the concentration of metals found in plants, grouped by estuary.

	Ave River estuary (*n*=6)	**Douro River estuary (*n*=6)**	**Total (*n*=12)**	***p* value***
Plant Cd				0.004
Median	0.108	0.792	0.376	
Q1, Q3	0.000, 0.251	0.664, 0.883	0.162, 0.768	
Plant Cr				0.631
Median	0.775	1.575	1.009	
Q1, Q3	0.695, 1.099	0.758, 2.463	0.644, 1.930	
Plant Cu				0.150
Median	5.834	17.972	13.020	
Q1, Q3	3.005, 15.739	10.811, 19.140	5.736, 18.718	
Plant Pb				0.010
Median	0.295	3.871	0.981	
Q1, Q3	0.182, 0.410	1.594, 6.907	0.307, 3.028	
Plant Zn				0.873
Median	134.228	92.645	92.645	
Q1, Q3	42.916, 295.933	69.580, 108.705	51.273, 143.801	

*Mann-Whitney test

Table S2. Median and interquartile ranges for the concentration of metals found in plants, grouped by species.

	***Veronica anagallis-aquatica* (*n*=6)**	***Cakile maritima* (*n*=6)**	**Total (*n*=12)**	***p* value***
Plant Cd				0.747
Median	0.376	0.319	0.376	
Q1, Q3	0.266, 0.676	0.000, 0.832	0.162, 0.768	
Plant Cr				0.337
Median	1.338	0.704	1.009	
Q1, Q3	0.919, 3.170	0.558, 1.428	0.644, 1.930	
Plant Cu				0.109
Median	18.389	5.000	13.020	
Q1, Q3	11.020, 19.140	3.005, 14.696	5.736, 18.718	
Plant Pb				0.631
Median	1.217	0.859	0.981	
Q1, Q3	0.471, 2.105	0.194, 4.518	0.307, 3.028	
Plant Zn				0.037
Median	163.176	48.875	92.645	
Q1, Q3	91.769, 295.933	40.518, 88.509	51.273, 143.801	

*Mann-Whitney test

Table S3. Median and interquartile ranges for the concentration of metals found in plants, grouped by structure.

	Root (*n*=4)	**Stem (*n*=4)**	**Leaves (*n*=4)**	**Total (*n*=12)**	***p* value***
Cd					0.690
Median	0.551	0.345	0.511	0.376	
Q1, Q3	0.197, 0.913	0.162, 0.515	0.208, 0.782	0.162, 0.768	
Cr					0.098
Median	3.231	0.618	1.338	1.009	
Q1, Q3	2.219, 3.880	0.493, 0.748	1.015, 1.531	0.644, 1.930	
Cu					0.146
Median	17.855	7.305	19.663	13.020	
Q1, Q3	13.786, 18.331	5.532, 8.255	15.395, 21.481	5.736, 18.718	
Pb					0.309
Median	3.713	0.418	1.312	0.981	
Q1, Q3	1.439, 6.008	0.227, 0.773	0.410, 4.230	0.307, 3.028	
Zn					0.584
Median	102.655	54.231	105.844	92.645	
Q1, Q3	71.165, 184.433	42.893, 101.983	88.509, 164.421	51.273, 143.801	

*Kruskal-Wallis test

Table S4. Logistic regression models. Estimates are presented in odds ratios and respective 95% confidence intervals (CI).

	High Pb			High Cu			High Zn			High Cr		
Predictors	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*
Location (Douro)	25.00	1.74 – 1058.40	**0.038**	5.00	0.41 – 131.10	0.239	1.00	0.08 – 12.21	1.000	5.00	0.41 – 131.10	0.239
Plant (*Cakile maritima*)	1.00	0.03 – 30.40	1.000	0.20	0.01 – 2.43	0.239	0.25	0.02 – 2.52	0.258	0.20	0.01 – 2.43	0.239