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Heavy Metals in Sediments and Greater Flamingo Tissues from a Protected Saline Wetland in Central Spain

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Abstract: Aquatic ecosystems often act as sinks for agricultural, industrial, and urban wastes. Among potential pollutants, heavy metals can modify major biogeochemical cycles by affecting microorganisms and other biota. This study assessed the distribution and concentration of heavy metals (Cd, Hg, Cu, Pb, and Zn) in Pétrola Lake, a heavily impacted area in central Spain where the greater flamingo *Phoenicopterus roseus* breeds. This study was designed to determine the concentration and identify the potential sources of heavy metals in Pétrola Lake protected area, including sediments, agricultural soils, and tissues of the greater flamingo. A six-step sequential extraction was performed to fractionate Cu, Pb, and Zn from lake sediments and agricultural soil samples to gain insight into different levels of their bioavailability. Our results showed that Pb and Cd accumulated in lake sediments and agricultural soils, respectively, most likely derived from anthropogenic sources. Multivariate analysis revealed differences between these (Pb and Cd) and the remaining studied elements (Cu, Hg, and Zn), whose concentrations were all below the pollution threshold. Lead pollution in sediments was apparently dominated by organic matter binding, with fractions up to 34.6% in lake sediments. Cadmium slightly accumulated in agricultural soils, possibly associated with the use of fertilizers, but still below the pollution thresholds. In the flamingo samples, low bioaccumulation was observed for all the studied elements. Our study suggests that human activities have an impact on heavy metal accumulation in sediments and soils, despite being below the pollution levels.

Keywords: heavy metals; geochemical fractionation; lake sediments; *Phoenicopterus roseus*; saline wetland

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1. Introduction

Heavy metals are naturally present in waterbodies in dissolved, colloidal, and particulate phases. Some of them, for example copper (Cu) and zinc (Zn), are essential for living organisms when occurring at trace levels. Overexposure to heavy metals, however, negatively affects human health and the environment [1]. These harmful effects include the formation of metal complexes with proteins, inactivating enzymatic systems, which compromises the course of biogeochemical cycles [2–4]. In aquatic ecosystems, heavy metal pollution may severely affect all organisms along the trophic levels, from microorganisms to higher organisms, including birds [5–11].

Human activities from ancient times to the present have multiplied environmental concentrations of Cu, lead (Pb), and Zn in all regions of the world [12,13]. Importantly, biosphere pollution by heavy metals has increased drastically since the beginning of the industrial revolution [14–16]. In contrast to organic pollutants, for example, heavy metals are nondegradable. Their long persistence, tendency to bioaccumulate, and their toxicity

have made them a target of intensive research over the last few decades [17–24]. Based on these features, heavy metals cause toxic effects at locations distant from the pollution source. In aquatic environments, they are poorly soluble in water and, therefore, are ultimately adsorbed into the sediments. For this reason, heavy metal concentrations in water are sometimes below detection limits and many studies have focused on sediment analyses [22,23,25–28]. Heavy metals in sediments may bioaccumulate to benthic organisms, thus entering the food chain [29,30]. Recent studies have also highlighted the positive correlation between the concentration of heavy metals and antibiotic resistance genes in aquatic environments [31–33]. The main anthropogenic sources of heavy metals in waters are urban and industrial effluents, agricultural fertilizers, and mining [34,35]. Moreover, heavy metals can enter waterbodies after atmospheric transport and subsequent deposition [36]. Fertilizers not only provide macronutrients and micronutrients (e.g., Cu and Zn) but are also often contaminated with other heavy metals, such as cadmium (Cd) and Pb [37,38]. The European Union has listed Cd and Pb, together with mercury (Hg), as priority substances in the field of water policy in Directive 2008/105/CE [39]. However, there is no unified European legislation regarding sediment quality criteria [40].

A better understanding of heavy metal bioavailability requires considering chemical interactions [41]. Mineral components and organic matter in sediments and soils are the main reservoir of heavy metals in aquatic environments and farming areas [27]. Heavy metals in sediments are most often associated with clays, iron and manganese (Fe–Mn) oxides, and organic matter due to ion exchange, complexation, and chemical adsorption [42]. Many studies have linked pollution sources with total concentrations of heavy metals in sediments and soils around the world [25,43–51]. Although nondegradable, heavy metals show different levels of mobility in soils and sediments, mainly depending on their chemical speciation. This, in turn, is highly dependent on several factors, including pH, redox potential, organic matter content, and ion exchange processes [28]. In contaminated anoxic sediments, for instance, the reoxidation of metal sulfide species can lead to significant releases of soluble species into the aquatic phase and therefore increase the bioavailability of the respective metal [52]. Despite the different chemical forms in which heavy metals can occur in sediments, the total concentration is the most commonly used parameter in assessment studies [53]. Such complexity prompted numerous studies to focus on the mobility in different geochemical phases in sediments by applying a sequential extraction before measurement [47,54–57]. This procedure releases heavy metals into different fractions, including “exchangeable”, “bound to carbonates”, “occluded in Fe–Mn oxides”, “organic fractions”, and the “residual fraction” [58,59]. In general, heavy metals derived from anthropogenic sources (i.e., Cd, Cu) are present in the more easily extractable fractions, contrary to those derived from lithogenic sources and which are mainly associated with the residual fraction [56,60]. Thus, sequential extraction approaches are valuable to complement source identification studies.

Among aquatic ecosystems, saline lakes are highly vulnerable to heavy metal pollution. These ecosystems usually function as sinks for agricultural, industrial, and urban wastes, acting to some extent as barriers against pollution [61]. Moreover, they are mainly located in closed hydrological systems in arid and semiarid regions. These basins are often closed drainage areas with no outlet other than evaporation. This type of environment, combined with the low precipitation and high evaporation rates typical of arid climates, accumulates and biomagnifies pollutants compared to freshwater systems [62]. Moreover, salinity plays an important role in the bioavailability of labile organic matter and, thus, in the release of chemical species [63]. Importantly, saline lakes are characterized by high primary productivity mainly related to microorganisms adapted to extreme conditions. This yields a wide range of unique biogeochemical features [64–67]. These environments not only host a rich microbial diversity, but also a high diversity of larger organisms (e.g., birds), which can play an important role in nutrient dynamics [68,69].

In this study, we compare heavy metal patterns in sediments, soils, and greater flamingo (*Phoenicopterus roseus*) tissues from the Pétrola Lake protected area (Central

Spain). The lake is a heavily modified endorheic waterbody that can reach hypersaline conditions during the driest periods. Moreover, it is home to a significant colony of the greater flamingo, which has nested there in recent years [70]. The inputs of pollutants from agricultural sources and urban wastewater to the waterbody affect the water quality of the ecosystem [71,72]. We hypothesized that heavy metal concentrations in sediments and soils of the study area may exceed the threshold levels for lacustrine environments, which in turn may lead to bioaccumulation of these metals in the food web (i.e., flamingos). Despite its importance, few studies have addressed heavy metal pollution in wetlands from these two angles simultaneously. This study was designed to determine the concentration and identify the potential sources of heavy metals in the Pétrola Lake protected area, including both soil/sediments and greater flamingo tissues.

2. Materials and Methods

2.1. Study Area

Pétrola Lake is one of the most representative saline wetlands in the Castilla–La Mancha region, in the High Segura river basin (SE Spain, Figure 1). The lake is an important flamingo feeding and breeding site, with up to 2600 breeding pairs and 2365 hatched chicks recorded in 2017 [73]. However, it is classified as a heavily modified waterbody because of the pollutant inputs from agricultural return flows (inorganic synthetic fertilizers), urban wastewater inflows, and accidental spills. These include significant loads of nitrogen compounds (i.e., ammonium and nitrate) [74] and endocrine-disrupting chemicals (i.e., biocides, PAHs, body care products, and abused drugs) [72]. The lake is shallow (<2 m) and occupies the terminal discharge zone of an endorheic basin in a semiarid area. Various small streams discharge into the lake in a radial pattern. The catchment area of the Pétrola lake–aquifer system extends over 43 km^2, where agricultural cultivation and livestock are the main activities. Farming encompasses approximately 75% of the total area, with the remaining area being occupied by semi-natural land cover types [71]. In addition, urban wastewaters are discharged directly into the lake without proper treatment. The basin is mainly composed of Mesozoic sequences, whereas the main aquifer is formed of Lower Cretaceous sediments [75].

2.2. Sample Collection

Between September 2014 and January 2016, a total of 41 sediment and soil samples were collected from 14 control points located in the Pétrola endorheic basin. These control points were positioned in Pétrola Lake as well as in streams and springs near the wastewater discharge areas (Figure 1C). The sampling points were distributed in four areas according to the main anthropogenic pressures and their location. These are lake bottom organic-rich sediment samples (sites 2635, 2643, 2648, 2649, 2650, 2651, 2652), soil samples from surrounding areas of the lake affected by wastewater inflows from Pétrola village (site 2575, a wastewater pond, and 2641, agricultural soil affected by wastewaters), soil samples from dryland farming area (sites 2554, 2640, 2642), and soil samples from the irrigation area (sites 2571, 2602).

Samples were taken from the upper part of the sediment/soil, with depths ranging from 5 cm to 30 cm, using acrylic coring tubes (5 cm inner diameter). Coring tubes were capped at top and bottom with silicone rubber stoppers, cooled, and transported to the laboratory. Once there, cores were sliced into 5 cm thick sections using ceramic knives and then frozen at −20 °C. Subsequently, these slices were freeze-dried for 48 h in a lyophylizer (Telstar-Cryodos-80, Telstar, Spain) at the Institute for Regional Development (University of Castilla–La Mancha). Dry sediment samples were homogenized in a porcelain mortar, sieved through a 1 mm steel sieve, and stored until further analysis.

A strong hailstorm in August 2015 killed approximately 300 flamingos in the lake area. Freshly deceased carcasses were transported to the closest wildlife rehabilitation center (Albacete) by competent authorities. There, flamingo samples (liver, fat, and breast muscle) were obtained from 12 individuals by the environmental agents of the Junta

de Comunidades de Castilla–La Mancha and provided for our research. Samples were transported to the Institute for Regional Development immediately after collection, where they were lyophilized.

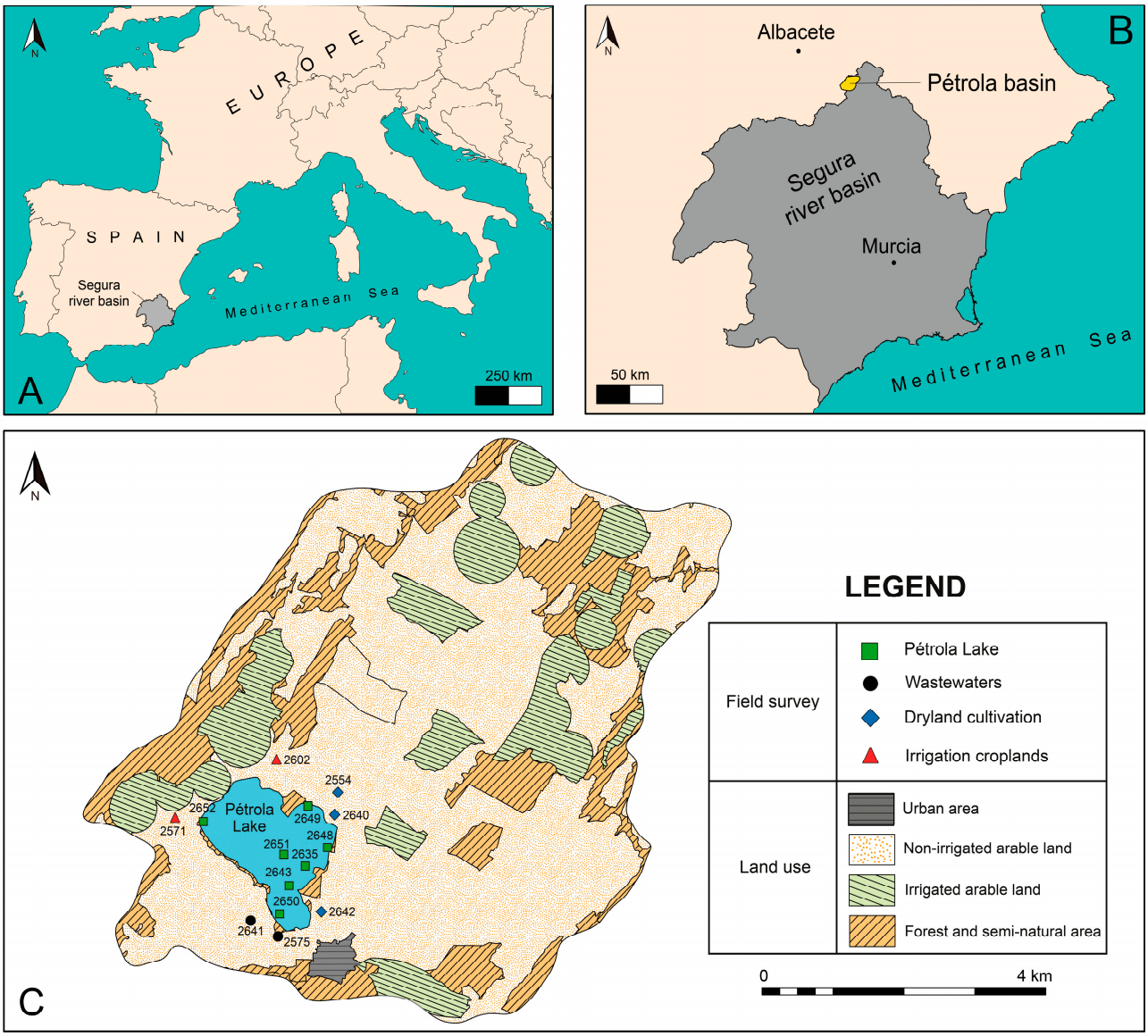

Figure 1. (**A**) Location of the Segura river basin in Europe. (**B**) Location of Pétrola basin in Segura river basin. (**C**) Land use map of Pétrola basin and location of control points from the studied areas: Pétrola Lake (green squares), affected by wastewaters (black circles), dryland cultivation (blue rhombus), and irrigated croplands (red triangles).

2.3. Sequential Extraction of Metals from Dried Sediment and Soil Samples

A six-step sequential extraction procedure, based on the approach proposed by [58], was applied to fractionate the heavy metals from sediment and soil samples into six fractions: exchangeable (F1), bound to carbonates (F2), bound to Mn oxides (reducible) (F3), bound to organic matter (F4), bound to Fe oxides (reducible) (F5), and residual (F6). Extractions were performed using dried samples at the Institute for Regional Development (University of Castilla–La Mancha). First, approximately 1 g of the dried samples from cores BA–BN (n = 14) were weighed and sealed in 50 mL polyethylene (PE) vials. The subsequent extraction used different extraction media (see following paragraph). After each successive step, samples were centrifuged at 7000 rpm for 15 min to separate the extract (supernatant) from the residual sediment. Extracts were then filtered through a

0.45 µm nylon filter (Millipore®, Burlington, MA, USA), transferred into 15 mL PE vials, and acidified by adding 0.5 mL of ultrapure HNO_3 (69%, TraceSELECT® Fluka, Hong Kong, China). Then, the residual pellet was washed with 10 mL Millipore water and centrifuged at 7000 rpm for 5 min, the supernatant was discarded, and the pellet used for the next extraction. All chemicals used for the extraction (Sigma-Aldrich, St. Louis, MO, USA) were of analytical grade (p.a.) unless otherwise stated.

The exchangeable fraction (F1) was extracted using 40 mL of 1 M KNO_3 (pH = 7) for 14 h under constant shaking (adapted from [76]). The fraction "bound to carbonates" (F2) was extracted by adding 35 mL of 1 M sodium acetate solution to the residue from step 1 and adjusting to pH 5.0 with glacial CH_3COOH, followed by continuous agitating for 5 h on an orbital shaker at 300 rpm (adapted from [77]). The residue was treated with 35 mL of 0.1 M $NH_2OH{\cdot}HCl$ in 0.1 M HNO_3 (pH = 2) for 2 h [77] to obtain the fraction "bound to Mn oxides" (F3). The fraction "bound to organic matter" (F4) was obtained from the residue from the previous step by adding 35 mL of 0.1 M $Na_4P_2O_7$ solution (pH = 10) and leaving it for 36 h [77]. Then, the residue was used to extract the fraction "bound to Fe oxides" (F5) by adding 35 mL of 0.04 M $NH_2OH{\cdot}HCl$ in 25% (v/v) CH_3COOH at 96 °C with occasional agitation for 5 h [59]. Finally, the residual fraction (F6) was extracted from the residue from F5 with 30 mL of ultrapure 4 M HNO_3 at 85 °C with occasional agitation for 12 h [78]. All the extracts were shipped to the lab of the Institute of Inorganic Chemistry at the University of Vienna for element analysis.

In addition to this sequential extraction, a pseudo-total digestion was carried out in the laboratory at the University of Vienna. This digestion should yield a 70–90% recovery of total metal concentration in the sample, depending on the species and bonding of the metal with the silica matrix [79]. Approximately 0.5 g of the homogenized dry sample were digested. Acid leaching was conducted in glass tubes equipped with air-coolers in a heating block for 120 min at 130 °C after adding 9 mL of 34% HNO_3 (TraceSELECT® Fluka) and 1 mL H_2O_2 30% (TraceSELECT® Fluka). Digested samples were transferred into 20 mL flasks and brought to volume with Millipore water. Afterwards, samples were filtered through 0.2 µM PTFE syringe filters (VWR). For quality assurance and to determine the recovery rates for this digestion method, reference samples comprising 0.3 g dry weight (dw) of certified reference material (marine sediment PACS-2, Natural Research Council Canada) were digested as described above for sediments.

2.4. Chemical Analysis

The organic matter content (OM%) in dried sediment and soil samples (n = 41) was determined as loss of ignition by combustion of dried samples for 2 h at 550 °C, as described by [80]. In leachates of sediments and soils, Cu, Pb, and Zn were detected using an inductively coupled plasma–optical emission spectrometer (ICP–OES, Optima 5300DV, Perkin Elmer, Waltham, MA, USA) and by total reflection X-Ray fluorescence spectrometry (TXRF, S2 PicoFox TXRF, Bruker Nano GmbH, Berlin, Germany). Mercury was measured in the samples immediately after digestion by cold vapor atomic absorption spectrometry (CV-AAS, FIMS 400, Perkin Elmer Inc., Waltham, MA, USA). *To* detect Cd, a graphite furnace atomic absorption spectrometer was used (GF-AAS, PinAAcle 900Z, Perkin Elmer Inc.). For TXRF analysis, platinum (Pt) was used as internal standard. The analysis of lyophilized tissue samples (n = 28) involved homogenizing them with a mortar and pestle, and approximately 0.2 g was digested in 9 mL 34% HNO_3 (TraceSELECT® Fluka) and 1 mL of 30% H_2O_2 using a microwave MARS XPRESS system (CEM Corporation, Matthews, NC, USA). The digested samples were transferred into 15 mL volumetric flasks and brought to volume using Millipore water. They were filtered through 0.2 µm PTFE pre-syringe filters (VWR). Trace element concentrations in the samples were determined using TXRF and, when necessary, GF-AAS. For TXRF, LOD were determined by preparing analytical blanks without insertion of a sample. The limits of detection (all given in mg/kg dw) were 0.005 for Cd; 0.5 and 0.25 for Cu in ICP-OES and TXRF, respectively; 0.008 for Hg; 1.0 and 0.2 for Pb in ICP-OES and TXRF, respectively; and 1.0 and 0.35 for Zn in ICP-OES and TXRF,

respectively. Recovery rates ranged from 96 to 112%, demonstrating the suitability of the methods used.

2.5. Statistical Analysis

Data were analyzed using the open-source software R version 4.1.0 [81]. Variables were checked for normality using the *bestNormalize* package [82] and transformed to logarithmic to reduce heteroscedasticity and obtain normally distributed residuals. Variables, however, were not standardized (i.e., mean centering and scaling) because comparisons of rescaled coefficients across datasets are problematic [83]. Once the transformations were performed, correlations were calculated using Pearson's correlation coefficients. One-way ANOVA was used to test differences of heavy metal concentrations among depths and control points, followed by the Tukey's post hoc test (homogeneous variances) or by the Games–Howell post hoc test (heterogeneous variances). To assess differences between background soil values and measured values, one-sample two-tailed *t*-tests were used. In addition, principal component analysis (PCA) was performed for dimension reduction. This analysis was followed by K-means clustering. This non-hierarchical clustering method aims to partition the studied variables into *k* groups such that the sum of squares from points to the assigned cluster centers is minimized. Results of statistical tests were considered to be significant at the confidence level 95% ($p < 0.05$).

3. Results and Discussion

3.1. Heavy Metals in Sediments

Mean values are shown in Table 1, whereas detailed concentrations are provided in the Supplementary Information (Table S1). Cadmium was above the LOD (0.005 mg/kg dw) in 99% of the samples. The highest mean concentration was recorded in soil samples from the irrigation area (0.142 ± 0.053 mg/kg dw), which is significantly lower than the background soil value for Cd (0.265 mg/kg dw) described by [84]. In lake samples, the highest Cd concentrations were recorded at sediment depths 5–10 cm (0.063 ± 0.060 mg/kg dw) and 10–15 cm (0.066 ± 0.046 mg/kg dw). Compared with other lake studies, our results were slightly but significantly lower than those reported in Lake Acigöl (0.185 mg/kg dw) [85], considered as unpolluted by Cd, and about ten times lower than those reported in Yangzonghai Lake (0.68 mg/kg dw) [86], where Cd pollution in surface sediments is mainly attributed to high inputs of Cd-bearing pesticides and industrial wastewaters. The Cd concentrations in the lake samples did not differ significantly with depth ($F_{(5,85)} = 1.36$). Nevertheless, comparison of sediments from different areas at 5 cm revealed significant differences ($F_{(3,36)} = 14.45$). Tukey's post hoc tests separated the samples into two homogeneous subsets consisting of (i) surface lake samples and (ii) wastewater ponds, dryland cultivation, and irrigation croplands. "Surface lake sample" concentrations of Cd at 5 cm were significantly lower (0.035 ± 0.032 mg/kg dw) compared to the other group. The sequence of decreasing Cd concentration within this group was irrigation > dryland > wastewaters.

Table 1. Mean (±SD) concentrations of heavy metals (mg/kg dry weight) and organic matter content (OC, %), and comparison with other locations and sediment quality guidelines. ERM: effects range median. ISQG: interim.

Sediments Zone	Sediment Layer (cm)	Cd	Hg	Cu	Pb	Zn	OC	Reference
Pétrola Lake (n = 28)	0–5	0.035 ± 0.032	0.014 ± 0.034	4.28 ± 4.83	16.2 ± 25.4	18.7 ± 17.9	9.5 ± 6.1	This study
Pétrola Lake (n = 17)	5–10	0.063 ± 0.060	0.031 ± 0.058	6.92 ± 6.55	34.0 ± 27.7	29.0 ± 25.6	10.8 ± 5.6	This study
Pétrola Lake (n = 17)	10–15	0.066 ± 0.046	0.038 ± 0.039	8.37 ± 4.41	51.6 ± 48.7	37.4 ± 35.5	14.6 ± 5.6	This study
Pétrola Lake (n = 28)	>15	0.047 ± 0.020	0.029 ± 0.044	5.77 ± 3.12	32.4 ± 22.8	24.8 ± 9.73	10.6 ± 4.8	This study
Wastewaters (n = 4)	0–5	0.053 ± 0.042	0.030 ± 0.043	6.39 ± 4.79	32.3 ± 31.6	27.5 ± 23.3	11.4 ± 5.6	This study

Table 1. *Cont.*

Sediments Zone	Sediment Layer (cm)	Cd	Hg	Cu	Pb	Zn	OC	Reference
Dryland (n = 4)	0–5	0.099 ± 0.039	0.016 ± 0.012	7.30 ± 5.23	12.2 ± 6.27	26.3 ± 15.6	10.5 ± 5.0	This study
Irrigation (n = 4)	0–5	0.142 ± 0.053	0.022 ± 0.010	9.01 ± 4.51	14.0 ± 4.08	30.2 ± 13.9	8.2 ± 1.9	This study
Lake Acigöl	0–5	0.185	na	17.4	8.05	52.9	26.0	[85]
Yangzonghai Lake	0–10	0.68	0.13	na	38.7	114.3	na	[86]
Maharlu Saline Lake	0–5	4.4	na	13.8	29.0	37.0	0.3	[87]
Caspian Sea	0–5	na	na	21.8	15.9	73.2	3.24	[88]
Persian Gulf	0–10	0.8	na	32.1	48.3	62.5	na	[26]
Moknine Continental Sebkha	0–20	na	na	10.3	7.66	52.4	1.5	[89]
Marine Quality Guidelines NOAA (ERM)	-	9.6	0.71	270	218	410	na	[90]
Canadian Quality Guidelines in freshwater (ISQG)	-	0.6	0.17	35.7	35	123	na	[91]
Background soil	-	0.265	0.13	29.6	12.3	56.5	na	[84]
Castilla-La Mancha agricultural soils	0–2	na	na	17.38	25.77	43.48	na	[92]

Mercury was above the LOD (0.008 mg/kg dw) in 74.5% of the sediment samples (Table S2). The highest values were measured in subsurface lake sediments and in surface soils/sediments affected by wastewaters (≈0.030 mg/kg dw; Table 1). Measured Hg concentrations in this study were significantly lower than mean background soil values reported in soils from UK (0.13 mg/kg dw) [84], in soils from other European countries (<1 mg/kg dw) [93], or the values specified in the Spanish national directive RD1310/1990 (< 1 mg/kg dw) [94]. The highest Hg concentration was found in core AL (0.251 mg/kg dw) between 5 and 10 cm depth. Nevertheless, these values are far below the effects range median (ERM) quality guideline value from NOAA (0.71 mg/kg dw) [90]. No statistically significant differences were detected between depths in lake sediment samples ($F_{(5,85)}$ = 0.85) or among land use areas ($F_{(3,36)}$ = 1.03). A Hg contamination in sediments and soil can therefore be excluded.

Copper was well detectable in all sediment and soil samples (Table S2). Although maximum mean values were recorded in the irrigation croplands area (9.01 ± 4.51 mg/kg dw; Table 1), no statistically significant differences in Cu concentrations were found at 5 cm depth ($F_{(3,36)}$ = 2.63). Concerning lake samples, the highest Cu concentrations were recorded in the subsurface sediment layer 10–15 cm (8.37 ± 4.41 mg/kg dw) (Table 1), again showing no significant differences from samples at other depths ($F_{(5,85)}$ = 1.30). Compared to other lacustrine areas, such as Lake Acigöl (17.4 mg/kg dw) [85], Maharlu Saline Lake (13.8 mg/kg dw) [87], or the Caspian Sea (21.8 mg/kg dw) [88], the values we measured in Pétrola Lake sediments were even lower. In both dryland and irrigation agricultural soils, mean values were also lower than those in areas unpolluted by agricultural soils from in the Castilla–La Mancha region (17.4 mg/kg dw) [92]. Detailed results regarding speciation are included in the Supplementary Information (Table S2). In all studied locations, Cu was mainly accumulated in the residual fraction (F6), ranging from 56.5% of the total Cu (lake sediments) to 69.2% of the total Cu (irrigation cropland soils) (Figure 2). An important fraction of Cu (16.1–21.7%) was bound to Mn oxides (F3 fraction). In lake samples, the fraction bound to carbonates (F2) was significantly higher (19.1%) than in that same fraction in the remaining areas (5.5–8.6%), whereas the fraction bound to organic matter (F4) was more abundant in agricultural soils (5.6% and 3.5%, in irrigation and dryland, respectively).

Lead concentrations were above the LOD in all samples (Table S2). As shown in Table 1, mean Pb concentrations for all sediment locations were slightly higher than the background soil value (12.3 mg/kg dw) [84]. A maximum value of 202.4 mg/kg dw was measured in core AN (control point 2652, lake) in the sediment layer 10–15 cm, below the ERM quality guideline value of NOAA (270 mg/kg dw) [90]. Pb concentrations differed significantly among lake sediment depths ($F_{(5,85)}$ = 2.83). The highest mean Pb concentration was found

in the 10–15 cm lake sediment layer (51.6 ± 48.7 mg/kg dw) (Figure 3). In the top layer (0–5 cm), the mean Pb concentration was similar to values observed in the Caspian Sea (15.9 mg/kg dw) [88], both below the threshold effect range low (ERL) values reported in the Marine Quality Guidelines NOAA (46.7 mg/kg dw) [90] and the Canadian sediment quality guidelines (ISQG) (30.2 mg/kg dw) [91]. However, the remaining mean values of the profile were close to the threshold value or, as noted above in layer 10–15 cm, above it (51.6 mg/kg dw). Concerning surface soils and sediments (5 cm depth), we determined differences between the background soil value (12.3 mg/kg dw) [84] and mean values per sediment location. Soils affected by wastewaters (32.3 mg/kg dw; Table 1) had significantly higher values of Pb ($t_{(3)}$ = 5.37), unlike lake sediments (16.2 mg/kg dw; $t_{(27)}$ = 0.81), dryland cultivation sediments (12.2 mg/kg dw; $t_{(3)}$ = 0.04), or irrigation cropland sediments (14.0 mg/kg dw; $t_{(3)}$ = 0.82). In the samples analyzed by sequential extraction, surface samples showed that Pb was mostly bound to the residual fraction, ranging from 37.4% (lake) to 67.4% (irrigation croplands) (Figure 2). In samples from the lake and from those affected by wastewaters, however, the fraction of Pb bound to organic matter (F4; 34.6% and 28.1%, respectively) and to Fe oxides (F5; 11.9% and 8.7%) was significantly higher than in agricultural soils (10% and 6.2% for dryland and irrigation croplands, respectively).

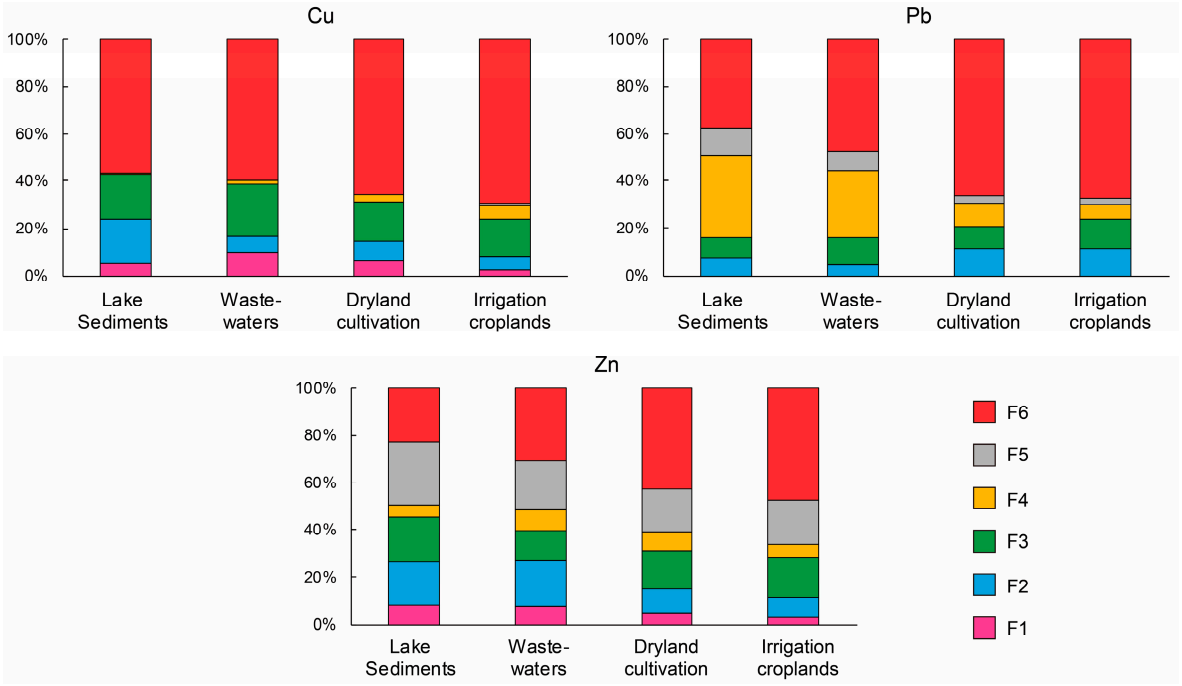

Figure 2. Distribution of Cu, Pb, and Zn in the different fractions for the studied sediment samples (n = 14). F1: exchangeable fraction. F2: bound to carbonates. F3: bound to Mn oxides. F4: bound to organic matter. F5: bound to Fe oxides. F6: residual fraction.

Zinc was detectable in all studied samples (Table S2), but no significant differences in concentration were found either among areas ($F_{(3,36)}$ = 2.43) or among lake sediment depths ($F_{(5,85)}$ = 1.12). Table 1 and Figure 3 show the maximum mean value in lake sediments at 10–15 cm depth (37.4 mg/kg dw). The maximum value (161.5 mg/kg dw) was also measured at this depth in core AF (control point 2648, lake sediments). This still is far below the ERM quality guideline value of NOAA (410 mg/kg dw) [90]. Surface sediments from Pétrola Lake also showed a concentration (18.7 mg/kg dw) lower than that reported in other unpolluted lacustrine sediments (e.g., Maharlu Saline Lake, 37.0 mg/kg dw) [87]. Moreover, all mean Zn concentrations were lower than the general background soil value (56.5 mg/kg dw) [84]. The sequential extraction indicated that Zn was predominantly present in the residual fraction (F6) in wastewater pond sediments (30.5%), dryland (42.9%), and irrigation cropland soils (47.2%). In lake sediments, however, the most Zn-rich fraction was that bound to Fe oxides (26.8%). Finally, lake sediments and wastewater sediments

showed a significant contribution of the fraction bound to carbonates (18.1% and 19.4%, respectively) (Figure 2).

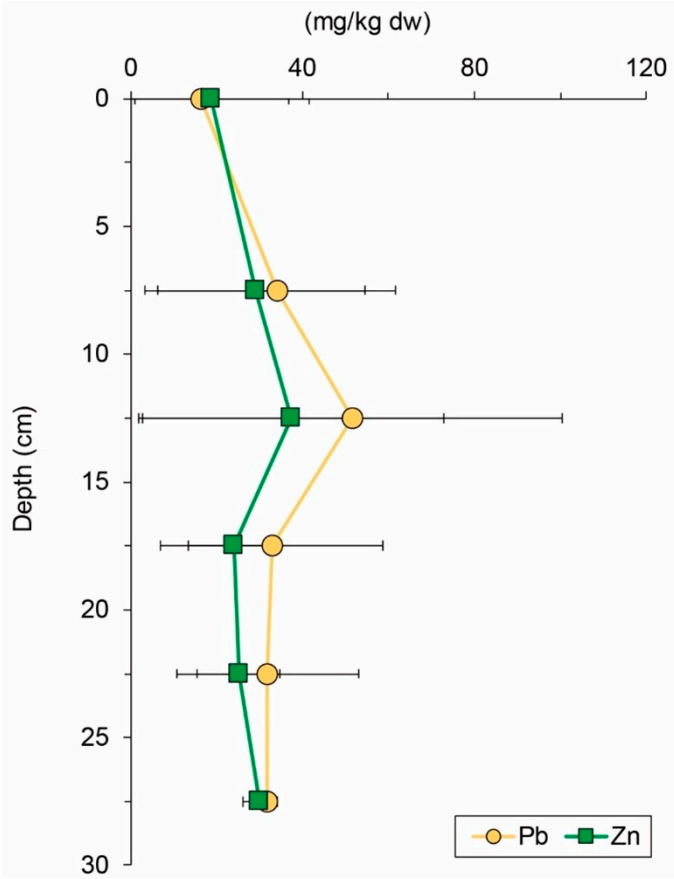

Figure 3. Depth profiles of Pb (circles) and Zn (squares). Mean values (±SD) in sediments from Pétrola Lake (n = 90).

Overall, these results show a certain Pb enrichment in subsurface (below 5 cm) sediments, with a maximum in the 10–15 cm layer from Pétrola Lake (Figure 3). These values were close to or above the threshold levels in marine and lacustrine guidelines (NOAA and ISQG). In addition, soils affected by wastewaters also had values slightly exceeding the background levels. Even though other elements (i.e., Zn) in lake sediments followed the same depth pattern as Pb, the measured concentrations remained below the quality guideline values and/or were similar to comparable lacustrine systems [86–88]. Sequential extraction provided important information about the mobility of Cu, Pb, and Zn. Zinc was the element showing the smallest accumulation in the residual fraction (i.e., higher mobility), whereas Pb was mainly bound to organic matter (Figure 2).

3.2. Levels of Heavy Metals in Flamingo Tissues

In flamingo samples, mean measured values are shown in Table 2, with full data provided in the Supplementary Information (Table S3). Data on heavy metals in flamingos are limited, frequently involving non-destructive biomonitoring tools (e.g., feathers) [10]. Although the measured concentrations were not alarming, our results showed that the liver was the primary organ for heavy metal accumulation. In contrast to our soil/sediment results, Pb was the element that accumulated the least in flamingo tissues. As discussed below, major differences were generally observed between the liver and muscle or fat. Our results suggest that the low availability of heavy metals in the study area translates into low bioaccumulation in flamingo tissues. Nonetheless, the risk of accumulation of such

pollutants for the flamingos inhabiting the lake cannot be ruled out, given the constant input of pollutants into the Pétrola lake–aquifer system [71,72].

The highest Cd values were measured in liver (mean = 0.304 mg/kg dw) and were within the range of previous studies on liver tissues of flamingos from the Ebro Delta (0.55 mg/kg dw) [95], but significantly lower than those reported by [96] in the protected area of Migliarino San Rossore Massaciuccoli (MSRM) Park (1.30 mg/kg dw). The Cd values in muscle (mean = 0.040 mg/kg dw) were higher than those measured in fat (mean = 0.007 mg/kg dw) and were well comparable to those in flamingos from the Camargue region (0.040 mg/kg dw) [97].

Highest Hg values were recorded in liver samples (mean = 0.534 mg/kg dw) as opposed to muscle and fat tissue, as has been described for birds before [8]. Although these levels appear quite high, they are lower than those reported in liver tissues of flamingos from MSRM Park (3.83 mg/kg dw) [96]. The bird muscle tissues from Pétrola Lake were also significantly lower (0.088 mg/kg dw) than those in MSRM Park (0.59 mg/kg dw) [96] or Carmargue (2 mg/kg dw) [97]. The comparably low Hg concentrations in flamingos reflect the low levels detected in sediments and point to a very low mercury burden in the system.

Table 2. Mean (±SD) concentrations of heavy metals in collected in *Phoenicopterus roseus* samples (mg/kg dry weight), and comparison with other locations. BLD: below the limit of detection; na: not available.

Tissue	Location	Cd	Hg	Cu	Pb	Zn	Reference
Liver	Pétrola Lake	0.304 ± 0.549	0.534 ± 0.461	34.6 ± 28.5	0.160 ± 0.115	382 ± 257	This study
Muscle	Pétrola Lake	0.040 ± 0.089	0.088 ± 0.06	24.0 ± 19.7	0.025 ± 0.014	56.2 ± 23.7	This study
Fat	Pétrola Lake	0.007 ± 0.005	BLD	1.87 ± 0.69	0.075 ± 0.044	14.0 ± 5.1	This study
Liver	Doñana	na	na	na	0.885	203.4	[98]
Liver	Doñana	0.01 ± 0.02	na	79.9 ± 13.3	0.06 ± 1.5	192 ± 80	[99]
Liver	Ebro Delta	0.019 ± 0.011	na	na	0.138 ± 0.071	na	[100]
Liver	Ebro Delta	0.55 ± 0.12	na	56 ± 11	149 ± 58	403 ± 45	[95]
Liver	Carmargue	0.9	4	100	2.0	600	[97]
Liver	MSRM Park	1.30 ± 0.31	3.83 ± 2.95	na	313 ± 48.1	na	[96]
Muscle	Doñana	na	na	14.3 ± 3.24	0.05 ± 0.03	53.7 ± 66.4	[99]
Muscle	Carmargue	0.04	2	60	0.09	100	[97]
Muscle	MSRM Park	0.15 ± 0.03	0.59 ± 0.47	na	3.36 ± 0.75	na	[96]

The concentrations of Cu were similar in both liver (mean = 34.6 mg/kg dw) and muscle (mean = 24.0 mg/kg dw) tissues (Table 2). This is surprising and stands in contrast to many other publications on copper in anatid birds [8,99], where copper usually occurs in significantly higher concentrations in liver versus muscle tissue. Cu in muscle samples was well comparable with values obtained by [99] (14.3 mg/kg dw), whereas those authors reported mean levels of 79.9 ± 13.3 mg/kg in liver. The uptake and excretion of copper, as an essential trace element, is usually highly regulated metabolically [101], and the low liver values here correlate well with the low concentrations in sediments. This points to a copper depletion in the system rather than a contamination with this element.

We also recorded the highest Pb concentrations in flamingo tissues in liver (mean = 0.160 mg/kg dw), similar to the values reported by [100] in birds from the Ebro Delta (0.138 mg/kg dw). In contrast, muscle and fat samples showed much lower mean Pb values (0.025 and 0.075 mg/kg dw, respectively), also well comparable to the values found by [99] in Doñana National Park (0.05 mg/kg dw). Lead in birds has been frequently investigated because gunshot pellets made from Pb continue to be distributed widely everywhere, and hunters still use them on waterfowl [102,103]. This can lead to lead poisoning through ingestion [8], potentially explaining the higher values in liver tissues compared to muscle and fat. The highest Pb concentrations in sediments were recorded at 10–15 cm depth. This may also reflect the accumulation of gunshot pellets from hunting. Such activity was prohibited by law in Pétrola Lake in 1993. It is, however, still ongoing in

other wetlands within the Pétrola–Corral–Rubio–La Higuera Saline Complex. Although this has become progressively restricted in recent decades, flamingos can create craters in soft sediments by treading with their feet when feeding [104]. This exposes the pellets buried in the sediment, which can then be ingested [105]. Pb concentrations are of particular concern because this element may negatively affect chick body composition as well as microbial diversity and composition [10].

The highest mean value of Zn in flamingo tissues occurred in liver (382 mg/kg dw), considerably higher than the mean values from muscle and fat (56.2 and 14.0 mg/kg dw, respectively) (Table 2). Liver values were within the range of those reported by [98] in Doñana National Park (203.4 mg/kg dw) or [95] in the Ebro Delta (403 mg/kg dw). Regarding Zn in muscle samples, our data are close to those reported by [99] in birds from Doñana National Park (53.7 mg/kg dw). Zinc is an essential element required for feather formation and is toxic only at very high levels [106]. Recent studies link it beneficially to gut homeostasis in birds [10]. Nonetheless, Zn concentrations in tissues are weak indicators of exposure in birds [107]; long-term exposure is required for bioaccumulation in flamingos [108]. In sediments, Zn is more bioavailable than Pb [109]. Based on our results, the values from sediments (Table 1) and flamingo tissues (Table 2) are within the range reported in other studies, suggesting no contamination by Zn in Pétrola Lake.

3.3. Interaction between Heavy Metals and Organic Matter

The mobility and solubility of heavy metals in the environment depend on their complexation with organic matter. We found similar patterns for Pb and Zn in sediment cores (Figure 3). Moreover, studied metals and organic matter content tended to accumulate in a similar way within the sediment layers (Table 1). Correlation among heavy metal concentrations may reveal the source, transport, and fate of those elements [44,110]. Table S4 shows the results of the Pearson correlation analysis. All elements showed a positive and significant correlation with the organic matter content (OM%) at $p < 0.05$. Pb showed a positive correlation (0.40) with organic matter. Sequential extraction experiments also showed the greater influence of organic matter in controlling Pb fate. This phenomenon may be explained by lead adsorption processes by organic colloids, as suggested by previous authors in similar environments [48]. The highest Pb concentrations occurred in lake sediments and wastewater-affected sediments/soils. Possible explanations for the accumulation in Pétrola Lake include the use of Pb in gasoline in Spain until the late 1990s or the use of gunshot pellets in hunting. Further information on sedimentation rates and Pb isotopes would be necessary to determine the origin of this element in the lake. In wastewater-affected soils and ponds (sites 2641 and 2575), concentrations may also derive from industrial wastes.

Regarding the remaining elements, particularly noticeable were the positive correlations Hg–Zn (0.79), Cu–Hg (0.65), and Cu–Zn (0.54). Cu, Hg, and Zn seem to share a common source and/or behavior. Their concentrations were close to background values (Table 1). These findings suggest that this group of metals is derived from natural sources, such as mineral weathering [111]. Silicate minerals are abundant in the study area, potentially constituting a source. In fact, the amount of Cu, Pb, and Zn in the residual fraction (F6; Figure 2) is higher in samples from irrigation and dryland sandy siliciclastic soils. These three elements (Cu, Hg, and Zn) behave differently with respect to Hg and Cd, based on the PCA (Figure 4). PCA of the transformed variables (log) showed that the first two components explained 81% of the variance. Subsequent K-means clustering analysis revealed two main groups of explanatory variables. Cluster A includes Cu, Hg, and Zn, whereas cluster B contains Cd and Pb. Cluster A elements have the highest loading in component 1, showing that these elements accumulated mainly in flamingo tissues, particularly in the liver. In contrast, cluster B elements showed the highest loading for component 2, highlighting the accumulation in lake sediments and agricultural soils for Pb and Cd, respectively. Therefore, the influence of anthropogenic origins for the latter should not be neglected. For instance, agricultural sources involve the use of inorganic synthetic fertiliz-

ers with considerable concentrations of trace elements (i.e., Cd) [37]. This interpretation is supported by the significant accumulation of Cd in dryland and irrigation agricultural soils. In lake sediments, heavy metals can form strongly dissolved complexes with organic matter and inorganic ligands under high ionic strength, conditions that are characteristic of Pétrola Lake, with dissolved organic carbon concentrations up to 646 mg/L [75]. This may help to explain the differences in Cd concentrations between irrigation/dryland soils and lake sediments. Accordingly, transport of metals may start in the agricultural and wastewater-affected soils, where the highest Cd concentrations were found, and may end in the lake, where metals can be accumulated and immobilized in organic-rich sediments. However, Pb accumulation in subsurface lake sediments may be linked to other anthropogenic sources as discussed above (e.g., fuels, hunting). Organic-rich sediments seem to be the main sink for metals, which can probably become remobilized under changing redox conditions.

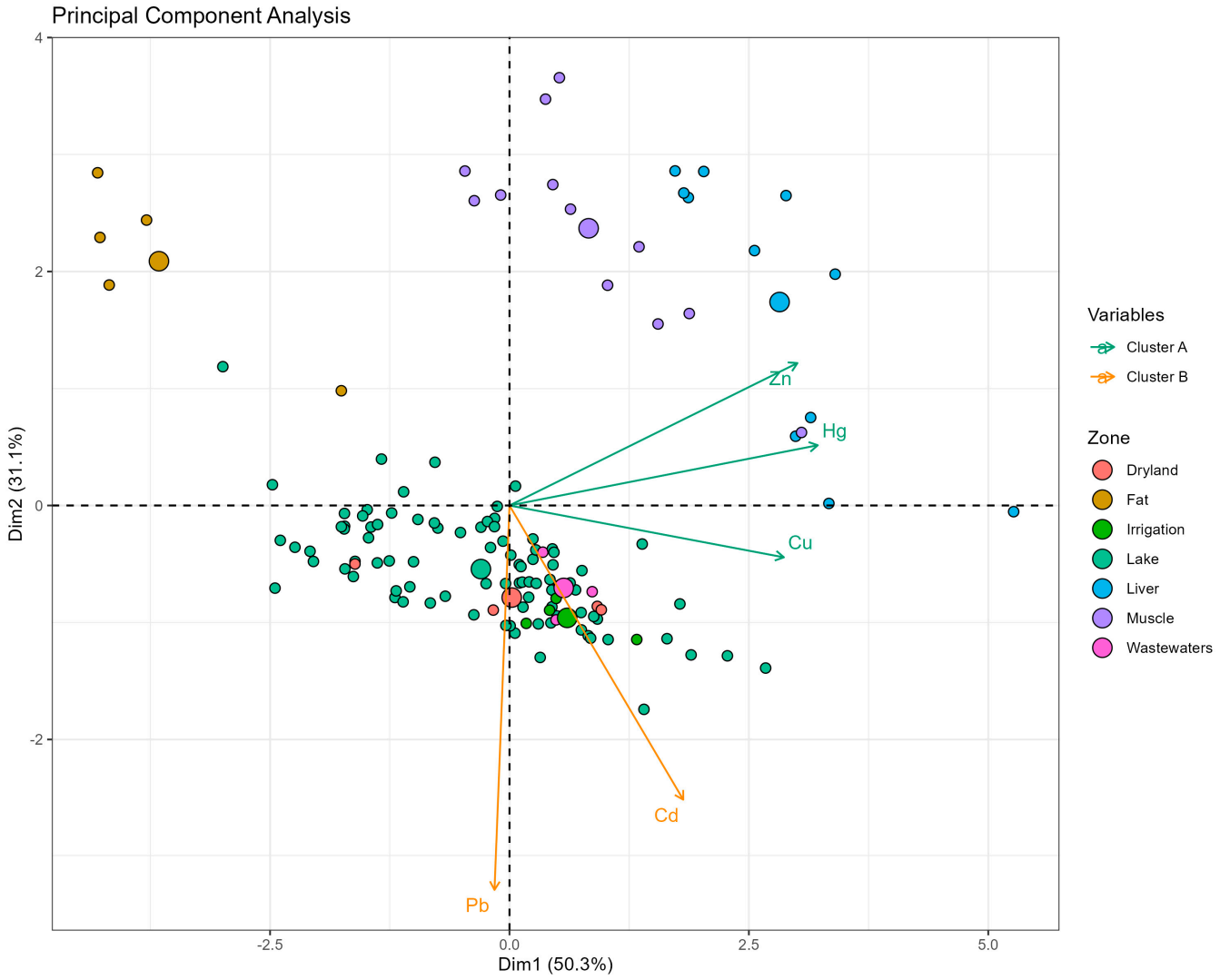

Figure 4. PCA analysis of the log-transformed variables depicting the two main principal components (PC) of parameter loading with the two defined clusters labeled in green and orange (clusters A and B, respectively). The two PCA axes explain 81.4% of total variability in the dataset.

4. Conclusions

The present study assessed heavy metal pollution and source identification in the Pétrola Lake protected area. Our findings provide a better understanding of heavy metal accumulation not only in soils and lacustrine sediments affected by anthropogenic activities,

but also in greater flamingo tissues. Our results highlight a certain degree of Pb and Cd accumulation in subsurface lake sediments and agricultural soils, respectively, and low bioaccumulation of the studied elements in flamingo tissues. Both the above elements are most likely derived from anthropogenic sources. Lead pollution in sediments is apparently dominated by organic matter binding and may be derived from human sources such as gasoline and gunshot pellets. Conversely, the highest Cd concentrations were found in agricultural soils (both irrigation and dryland), probably derived from the use of fertilizers, but still below the quality guideline levels to consider them as polluted. The remaining elements (Cu, Hg, and Zn) may have originated from a common source linked to siliciclastic weathering and carbonate mineral dissolution in the soil profile. The evidence from this study suggests that human activities have an impact on the accumulation of certain heavy metals such as Pb or Cd, regardless of whether these are below the pollution thresholds. Considerably more work will need to be carried out to assess the pollution risk of other elements that are commonly used in consumer and agricultural products, such as Cr or As. Further research is also required to understand how heavy metals bioaccumulate and adversely affect the health of natural bird populations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app12125769/s1, Table S1: Complete results of the chemical analyses in sediment and soil samples; Table S2: Cu, Pb, and Zn concentrations from fractions F1–F6 during sequential extraction; Table S3: Complete results of the chemical analyses in flamingo tissue samples; Table S4: Pearson correlation analysis among total concentrations of heavy metals and organic matter content.

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