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Analysis of Environmental Contamination by Metals Using Wood Mouse *Apodemus sylvaticus* Hair as a Biomonitor: An Appraisal

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Abstract: This study assessed environmental metal and metalloids (TE) levels using hair of *Apodemus sylvaticus* as a non-lethal biomonitor. TE decreased as follows: Zn > Al > Fe > Cu > Pb > Cr > Ni > Mn > Cd > Se > As > Hg; TE widely distributed in soils as Zn, Al, Fe, and Cu, are more abundant than those of ecotoxicological interest, such as Cd, Se, As and Hg. Cd, Pb, Cu, and Cr concentrations are highly variable, while Zn, Fe, and Mn are less variable. TE in hair are below the threshold levels in soil and decrease the same way in both sexes. Concentrations in soil and hair are significantly related, and their level can be modulated both by homeostatic control of essential metals and absorbance from the soil by keratin. Slight differences in Ni and Cr can be related to the differing behaviour of males and females during reproduction. A scarce tendency toward mercury bioaccumulation has been observed in both sex and age classes; from an ecological point of view, these data suggest that the species is a primary consumer, feeding more on the leaves and seeds than on small invertebrates.

Keywords: mammal hairs; trace elements; environmental pollution; biomonitor; wood mouse; *Apodemus sylvaticus*

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1. Introduction

Anthropogenic activities cause a large diffusion of heavy metals, which can accumulate in living organisms and circulate in trophic chains [1]; trace elements can be directly uptaken by organisms through ingestion of contaminated prey items, external exposure, or inhalation. Metals occur in different chemical forms in the environment and are often over the natural background; thus, organisms are exposed to their concentrations [2].

Metals can have differing effects on animals since they can accumulate in different organs; moreover, their toxicity may depend on concentration. Potentially toxic elements (PTE) have been a matter of environmental concern in recent decades; high levels may have acute or chronic toxic effects on animals [3,4]. On the other hand, metals such as Zn, Cu, and Fe are involved in several vital processes; they are essential for mammals but, at high levels, can induce pathological alteration and oxidative damage and interfere with cellular activity [1,2]. Environmental exposure to non-essential metals is known to be associated with a wide range of toxic effects in mammals [5]; metals such as Cd, Pb, Hg, and Ni lead to harmful effects with respect to excretion, reproduction, and other functions in mammals [5]. Again, at high levels, their uptake or accumulation in organs can lead to severe pathological changes, and chronic overloading and/or exposure can initiate oxidative damage and interfere with important cellular events, promoting synergic physiological processes [6]. Trace elements, on the other hand, play an important role in vertebrate physiology and an insufficient amount can result in deficiency diseases [7]. Chromium and zinc play a role in the control of oxidative stress and the proper functioning of lipid and glucose metabolism. Iron has a strong binding affinity for haemoglobin and myoglobin and a primary function in delivering oxygen to organs and tissues. Nickel plays an important role in regulating hormone metabolism, while in mammals, the integrity of cell membranes depends on glutathione peroxidase, an anti-oxidative system that in turn depends on selenium. In general, the effects of oligoelements such as arsenic, chromium, copper, iron, manganese, nickel, selenium, and zinc depend on trace element intake, ranging from deficiency to toxicity [7–10].

Thus, exposure to PTE may have opposite effects depending on whether the metal is essential or non-essential, its concentration, and the sex, age, and reproductive status of the animals [1,10,11].

Wild small mammals are used as bioindicators of terrestrial ecosystem disturbance in ecotoxicology [2,12]. Studies based on euthanized small rodents, including the wood mouse *Apodemus sylvaticus*, have focused on the pollution status of industrial areas or mines [13,14], the transfer of metals in trophic chains [12,15,16], their effect on physiological parameters (immunocompetence [17]), and the influence on behaviour, including food selection [18]. In these cases, TM concentrations were obtained from internal tissues and organs such as blood and plasma [5,19], kidney [19–21], liver [14,16,22,23], spleen [5,24], and bones [21,25,26]. On average, the frequency of metals analyzed in wood mice is around 4, clearly depending on primary factors such as the source of pollution, research funding, facility, and toxicological significance. Of 23 papers published on the contamination level in wood mice, Cd was analyzed in 19, Pb in 16, Cu and Zn in 15, As in 5, and Ni and Cr in only three; typically, the papers present data on Cd, Cu, Zn, and Pb.

The wood mouse *Apodemus sylvaticus* has long been used as a biomonitor of pollution in field studies [5,14,19,20,27,28] since it is an omnivorous, abundant, and ubiquitous rodent species [29]. As in other rodent species, hair stalks form hair follicles that undergo successive growth cycles; hair remains in contact with the bloodstream and has a strong affinity for various metals, reflecting an individual's exposure to these elements [27,30].

Hair is considered a good probe of environmental contamination, being a suitable method to monitor the transfer of PTE from the environment to organisms [31,32]; recently, the use of hair or other appendices as feathers in birds for biomonitoring has increased because it minimizes stress on small mammals [20,33,34].

This study aims to assess the contamination of the wood mouse population *Apodemus sylvaticus* living in the nearby of Pavia (NW-Italy); we adopted the hair of wood mice to detect the concentration of 12 trace elements, namely, aluminium, arsenic, cadmium, chromium, copper, iron, mercury, nickel, manganese, lead, selenium, and zinc.

2. Materials and Methods

2.1. Study Area, Trapping Techniques, and Soil Sampling

The study area coincided with the Nature Reserve "Bosco Negri", a wooded area located on the outskirts of Pavia (NW Italy). The protected area covers 34 ha on the right bank of the River Ticino; forested areas consist of deciduous riparian woods with oak Quercus robur and elms Ulmus spp. The areas surrounding the study area are highly urbanized and consist of suburban, industrial, and commercial areas and a dense road network.

The wood mouse *Apodemus sylvaticus* is a small (ca. 20 g) rodent; it is a widespread species, abundant in wooded areas and relatively common in agricultural environments; its strong adaptability allows it to survive in suburban environments. During spring 2023, wood mice were sampled by means of a line of 36 traps (Sherman live-traps folding model, 8 × 9 × 24 cm) baited with sunflower seeds; traps were spaced at 20 m from each other, and each trapping point was pre-baited for one night. Trapping session lasted 3 days and included two daily trap checks at dawn and dusk. Each captured rodent was individually marked by fur clipping, and sex, body weight, and reproductive condition were recorded. Before the release, nearly 0.5 g of wad hair, the lower and most dense layer of fur, was removed from the back and stored in a small plastic bag. We collected hair from 54 individuals (males = 22; females = 32).

Twenty soil samples were opportunistically collected in the low Po plain in order to improve comparison with metal concentration in hair. Soils from study areas were defined as loamy, mixed, and mesic following the USDA classification, while the land use is defined as "arable land". At each sampling point, 20 g of soil was collected and stored in airtight plastic containers. After transfer to the laboratory, they were left to dry at room temperature, homogenized, sieved (70 mesh), and stored in PET containers (residual humidity < 0.5%) until the microwave digestion.

2.2. Reagents and Apparatus

Certified multi-standard solution Merck VI for ICP-MS, Trace-SELECT®, ultrapure HNO_3 (65% *w*/*w*), and H_2O_2 (30% *w*/*w*) were purchased from Merck (Milan, Italy), and ultrapure water was purchased from Carlo Erba Reagents (Cornaredo, Milan, Italy). A microwave oven (Mars 5, CEM s.r.l., Cologno al Serio, Italy) equipped with 8 PFA polytetrafluoroethylene modified vessels (PTFE-PFA, 10 mL, Xpress) and 3 TFM polytetrafluoroethylene modified vessels (PTFE-TFM, 100 mL, EasyPrep) was used for trace elements extraction from wad hair and soil samples. A single quadrupole inductively coupled plasma mass spectrometer (SQ-ICP-MS, iCAP RQ Thermo Fisher Scientific), equipped with a quartz cyclonic chamber cooled at 3 °C, a MicroMist nebulizer 179 (400 µL/min), a quartz torch, a Ni sampler, skimmer cones, and a QCell pressurized with helium (3V, KED mode), was used for trace elements analysis in hair and Hg determination in soil. Inductively coupled plasma optical emission spectroscopy (ICP-OES iCAP 7400, Thermo Fisher Scientific) equipped with a concentric nebulizer, a cyclonic spray chamber, and a ceramic duo-torch, was used for trace elements analysis in soil samples.

2.3. Analytical Procedure

Before digestion, each hair sample was washed to remove all organic and inorganic residues on their surface. The washing system consisted of a vacuum flask, 0.45 µm filter, and a washing column. A moderate flow rate and stirring were maintained to achieve effective cleaning. Approximately 200 mL of tap water, about 50 mL of 1M acetone, and around 50 mL of ultrapure water were used sequentially. These solvents were selected to remove the majority of water-soluble fractions and residual organic compounds. At the end of the washing phase, the sample was transferred onto filter paper and then placed on a watch glass for drying in an oven at 60 °C for 24 h. Then, each sample was accurately weighed and introduced into the PFA vessel of the microwave oven with 0.2 mL of HNO_3 and 0.5 mL of H_2O_2. Digestion was performed at 800 W and 200 °C for 15 min to guarantee the complete dissolution of the samples. After cooling, each extract was diluted to 2.5 mL with ultrapure water in calibrated polypropylene tubes, filtered (0.22 µm, nylon filter), and analyzed by SQ-ICP-MS. In parallel, blanks, consisting of reagents only, were submitted to the same digestion procedure. Soil samples (150 mg) were placed into the microwave vessels, and 5 mL HNO_3, 2 mL of H_2O_2, and 1 mL of HF were added. Then, vessels were irradiated at 800 W for 15 min at 200 °C after a temperature ramp of 20 min. The solutions obtained from digestion were gently evaporated to a small volume (0.5–1 mL). After cooling, the samples were dilute to 25 mL with ultrapure water, filtered (0.22 µm, nylon filter, Whatman®, purchased from Merck KGaA, Darmstadt, Germany) and analyzed for the trace elements content by ICP-OES. A total of twelve trace elements—aluminium (Al), arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), iron (Fe), lead (Pb), manganese (Mn), mercury (Hg), nickel (Ni), selenium (Se), and zinc (Zn)—were determined via external calibration curves. Results are reported as µg/g dry weight.

2.4. Statistical Methods

Differences in metal concentrations were analyzed using parametric and non-parametric univariate tests. Analyses were carried out via SPSS 20.0.

3. Results

3.1. Heavy Metal Levels in the Wood Mouse: Effect of Sex, Age, and Weight

We analyzed the concentration of 12 metals (Al, As, Cd, Cr, Cu, Fe, Hg, Ni, Mn, Pb, Se, and Zn) in the hair of 54 individuals (Table 1); the average concentration of trace elements accumulated in hair of wood mice decreased as follows: Zn > Al > Fe > Cu > Pb > Cr > Ni > Mn > Cd > Se > As > Hg.

Table 1. Mean concentrations (µg/g), standard error, variation coefficient (sd/mean), minimum and maximum of metals concentration in wood mouse hair (n = 54).

	Mean	Se	VC	Min–Max	n
Zn	128	3	0.2	96–189	54
Al	86	11	0.9	19–419	54
Fe	60	4	0.5	28–168	54
Cu	18	3	1.2	8–119	54
Pb	6	2	1.2	0.7–34	54
Cr	4.3	0.5	0.9	0.6–17	54
Ni	3.7	0.4	0.8	0.8–12	54
Mn	1.9	0.1	0.4	0.8–3,6	54
Cd	0.3	0.1	2.9	0.03–5.1	54
Se	0.13	0.01	0.3	<LOD–0.21	54
As	0.09	0.01	0.5	0.04–0.23	54
Hg	0.07	0.01	0.5	0.03–0.20	54

The concentration values of several metals, such as Cd, Pb, Cu, Cr, and Al, are highly variable, as shown by a coefficient of variation greater than or close to 1. In contrast, ubiquitous metals, such as Zn, Fe, Se, and Mn, have a lower variability with a coefficient of variation less than or close to 0.5.

Males and females showed different patterns of variability in concentrations (Table 2). In males, high variability is observed for Al, Cu, Cd, and Pb, with values close to or above 100% of the within-group variability. In females, the within-group variability of concentrations seems to be higher than in males; the coefficient of variation is above or close to 1 for Al, Pb, Cr, Ni, and Cd. Only Pb and Cd concentrations are highly variable in both sexes, while Zn, Mn, and Se shows low variability of concentration both in males than females.

Table 2. Mean concentrations (µg/g), standard error, coefficient of variation (sd/mean), minimum and maximum of metals concentration in male and female wood mice. ns*= not significant.

	MALES					FEMALES						
	Mean	**Se**	**VC**	**Min–Max**	**n**	**Mean**	**Se**	**VC**	**Min–Max**	**n**	**t**	***p***
Zn	121	15	0.1	96–148	22	133	5	0.2	98–189	32	–2.09	*0.041*
Al	80	14	0.8	19–262	22	89	17	1.0	24–419	32	–0.30	*ns*
Fe	59	4	0.3	39–95	22	61	6	0.6	28–168	32	0.55	*ns*
Cu	24	7	1.3	8–119	22	14	1	0.5	8–35	32	1.09	*ns**
Pb	5	2	1.5	1–25	22	6	2	1.7	0.7–34	32	–0.01	*ns*
Cr	5.1	0.5	0.5	2.5–10	22	3.8	0.8	1.2	0.6–17	32	3.17	*0.003*
Ni	4.5	0.7	0.7	1.3–12	22	3.1	0.5	0.9	0.8–11	32	2.19	*0.003*
Mn	2.0	0.2	0.4	1.1–3.0	22	1.9	0.1	0.4	0.8–3.6	32	0.72	*ns*
Cd	0.15	0.02	0.7	0.04–0.4	22	0.4	0.2	2.8	0.03–5	32	–1.02	*ns*
Se	0.13	0.01	0.2	0.08–0.17	22	0.14	0.01	0.4	<IDL–0.21	32	–0.71	*ns*
As	0.10	0.01	0.5	0.04–0.23	22	0.08	0.01	0.4	0.04–0.14	32	1.61	*ns*
Hg	0.07	0.01	0.4	0.04–0.15	22	0.07	0.01	0.6	0.03–0.20	32	0.35	*ns*

The average concentration of trace elements decreases in the same way as for both sexes combined (Table 2).

Although the within-group variability is high, especially for females, we observed statistically significant differences only for Zn, whose concentration is significantly higher in females, and for Cr and Ni, which have significantly higher concentrations in males (Figure 1).

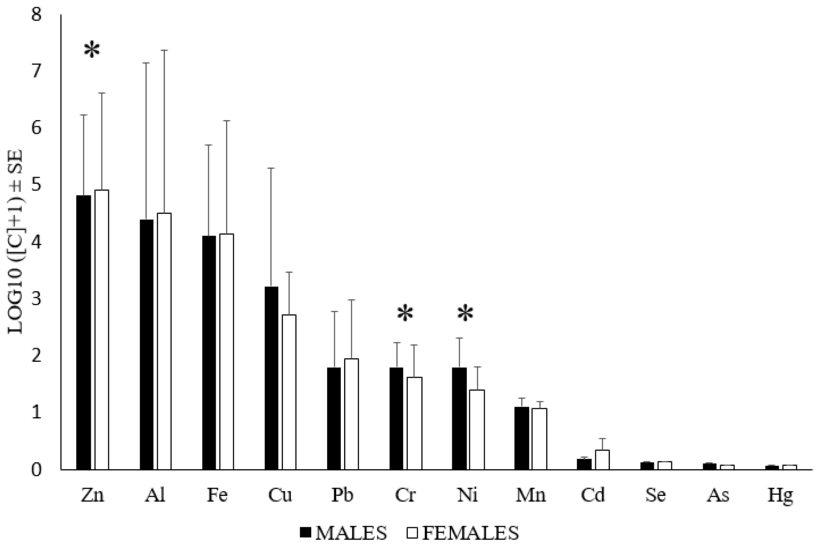

Figure 1. Trace elements mean male and female concentrations (µg/g ± SE). Differences were tested by *t*-test carried out on log normalized data (n = 54); an asterisk (*) indicates a significant difference at $p < 0.05$.

A pattern of substantial uniformity in contamination was ultimately observed between adults and subadults (Table 3): concentration levels are very similar, and slight differences, though not significant, were observed only for Al, Fe, and Cu; as a general comparison, we can observe that the Zn, Cu, and Cd level are higher in adults; Al, Fe, and Cr in subadults; while the concentration of other metals is nearly equal.

Table 3. Mean concentrations (µg/g), standard error, coefficient of variation (sd/mean), minimum and maximum of metals in adult and subadult wood mice.

	ADULTS				SUBADULTS			
	Mean	**Se**	**VC**	**n**	**Mean**	**Se**	**VC**	**n**
Zn	130	5	0.2	34	127	3	0.1	16
Al	69	10	0.9	34	74	9	0.5	16
Fe	53	3	0.4	34	74	11	0.6	16
Cu	20	4	1.3	34	14	2	0.6	16
Pb	6	2	1.7	34	6	2	1.6	16
Cr	4.0	0.5	0.7	34	5	1	1.0	16
Ni	3.6	0.5	0.8	34	4.1	0.9	0.9	16
Mn	1.8	0.1	3.0	34	2	0.2	0.4	16
Cd	0.4	0.2	5.1	34	0.12	0.03	0.8	16
Se	0.14	0.01	0.2	34	0.12	0.02	0.5	16
As	0.09	0.01	0.2	34	0.08	0.01	0.4	16
Hg	0.06	0.01	0.1	34	0.08	0.01	0.6	16

The reproductive condition seems to affect contamination level in females; Al, As, and Hg concentration in the hair of females with imperforate vagina, a condition of no reproductive activity, were significantly higher than in pregnant/lactating females (Al = 103.3–47.0, $z = -2.1$, $p = 0.037$; As = 0.087−0.59, $z = -2.62$, $p = 0.008$; Hg = 0.077–0.044, $z = -2.27$, $p = 0.023$).

Adult weight is positively and significantly correlated with Cr, Mn, Fe, Ni, Cd, and Hg concentrations (Figure 2). In contrast, in juveniles, we observed significant but inverse correlations with Cu and Pb concentrations (Figure 3). Weight is also positively correlated with Ni in males ($\delta = 0.47$, $n = 22$, $p = 0.028$) and negatively correlated with Al and Pb in females ($\delta = -0.60$, $p < 0.001$; $\delta = -0.44$, $p < 0.018$; $n = 28$). In pregnant/lactating females, there is a strong and positive correlation with Se concentration ($\delta = 0.99$, $n = 6$, $p < 0.0001$).

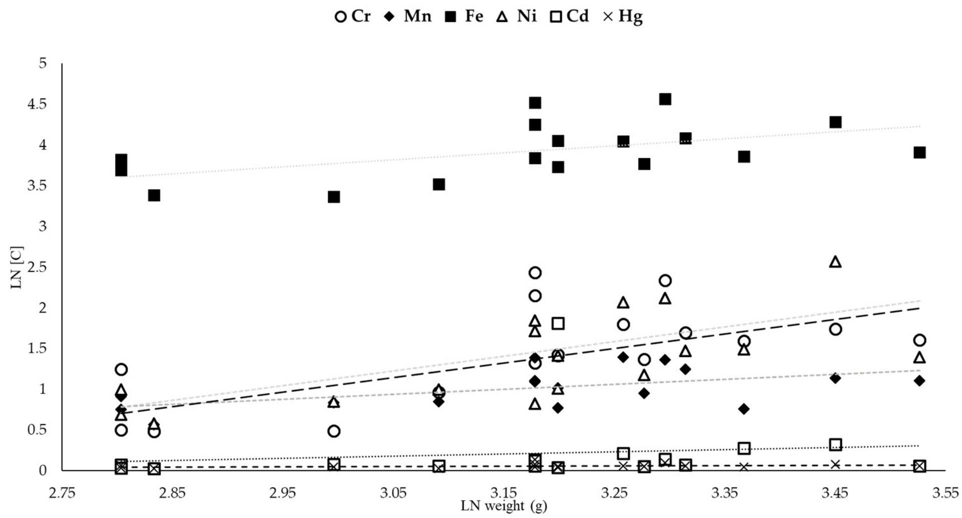

Figure 2. Correlation between adult weight and Cr, Mn, Fe, Ni, Cd, and Hg concentration in hair (Spearman δ.) Only significant correlations were included. All data (n = 34) are log-transformed to allow a better comparison.

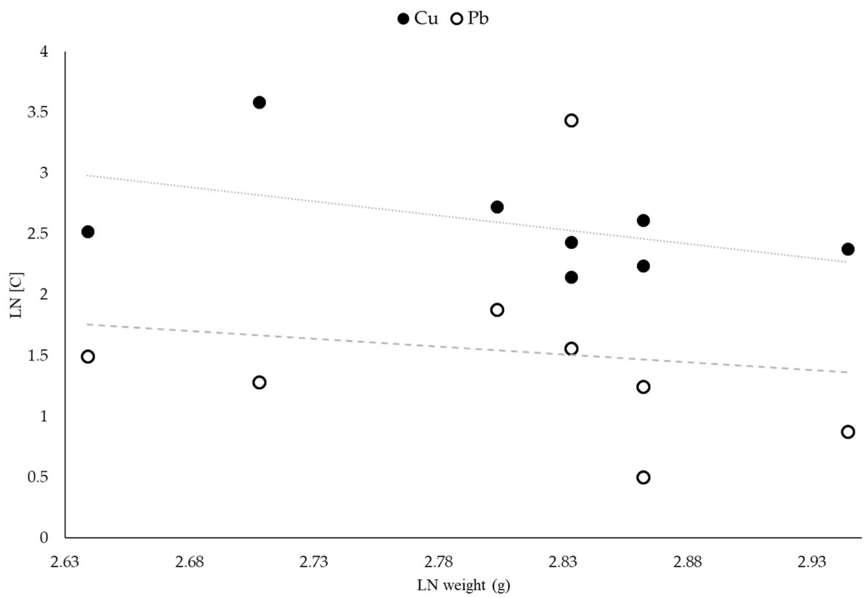

Figure 3. Correlation between subadult weight and Cu, and Pb concentration in hair (Spearman δ). Only significant correlations were included. All data (n = 14) are log-transformed to allow for a better comparison.

3.2. Heavy Metal Levels in Soil

In Table 4, we show the metal concentration in the soil around the study area; although this comparison is purely indicative, as the concentration of metals in alluvial soils is highly variable, our data seem to largely agree with those from agricultural monitoring by the Environmental Agency of NW Italy and geological studies carried out in the Po basin (Table 4); the average concentration value are indeed very close to our for Zn, Pb, Cr, Cd, As, and Hg and only slightly different for Ni and Se. Consequently, we are confident that our data on metal concentration in soil are representative of the general composition of planitial soil and can serve as a knowledge source for the distribution of metals in the soils of the Italian Po Plain.

Table 4. Average concentration of trace elements in 20 soil samples (μg/g ± standard error, min-max; n = 20) collected in the nearby of study area and comparison with data from other sites of Northern Plain.

	Zn	Al	Fe	Cu	Pb	Cr	Ni	Mn	Cd	Se	As	Hg
Mean	98	/	2581	26	29	97	59	66	0.38	6.8	14	0.37
se	7	/	97	1	2	7	5	3	0.04	0.8	1	0.06
min	46	/	1530	13	14	46	21	35	<IDL	<IDL	4	0.06
max	211	/	4069	60	91	211	156	113	1.2	17	35	1.97
Po plain [1]	74	/	/	28	20	93	70	/	/	/	9	/
Po plain [2]	93	/	/	47	21	134	91	/	0.28	/	3	0.50
Po plain [3]	111	/	/	66	34	162	130	/	0.54	0.91	28	0.08

[1]—[35]; [2]—[36]; [3]—[37].

3.3. Heavy Metal Levels in Hair: Comparison with Other Studies

We observed a significant and positive correlation between element concentrations in the soil and in the hair of wood mice (Figure 4), suggesting a direct effect of fossorial life on the level of contamination. The concentrations of most of the metals analyzed in the fur of our wild mice are consistent with those found by other authors, at least for the relative importance of concentration value, especially for Hg [38,39], As [40,41], Cd [20,27,42], Mn, Cr [42], and Zn [27,41]; while the concentrations of Pb and Cu are in agreement with the data collected by ref. [27] and ref. [41] for Cu and by refs. [20,41] for Pb, but not with those collected by ref. [42] for the two metals and by ref. [27] for Pb. Metal analyses in the hair of *Apodemus sylvaticus* for Al, Fe, and Se are not available to the best of our knowledge.

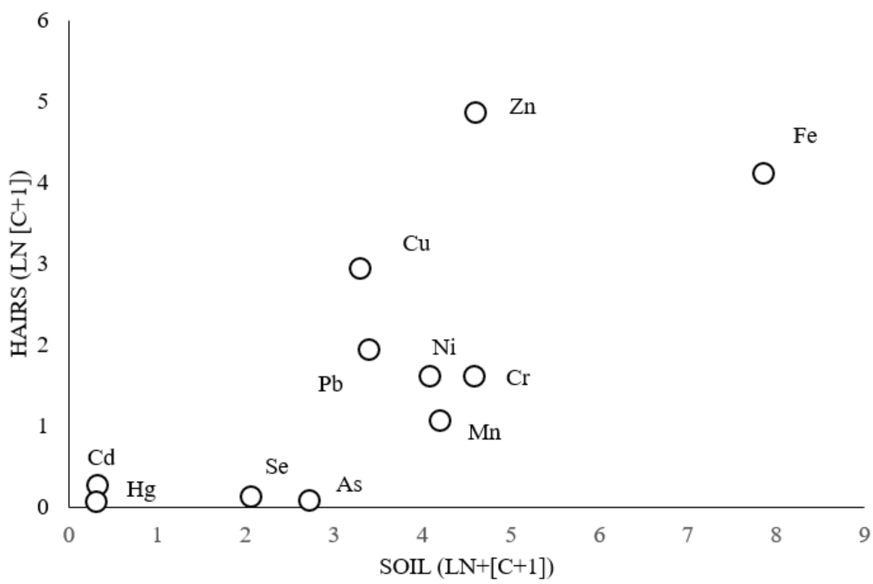

Figure 4. Correlation between concentration of metals in soil and hair (Spearman r_s = 0.79, p = 0.003).

A comparison of the concentration level of nine metals analyzed in other similar studies (Figure 5) in the fur of wood mice shows that it decreases in approximately the same way for seven metals, with the following sequence: Zn, Cu, Pb, Mn, Cr, Cd, and Hg. On the other hand, there are large differences between the median concentrations of each metal, ranging from lows of 0.3 (Zn) to highs of more than 10 (As). Taking into account the fact that these studies were carried out in different European areas, the comparison of the data obtained from the analysis can be considered reliable at least in documenting the relative importance of certain metals in the hair.

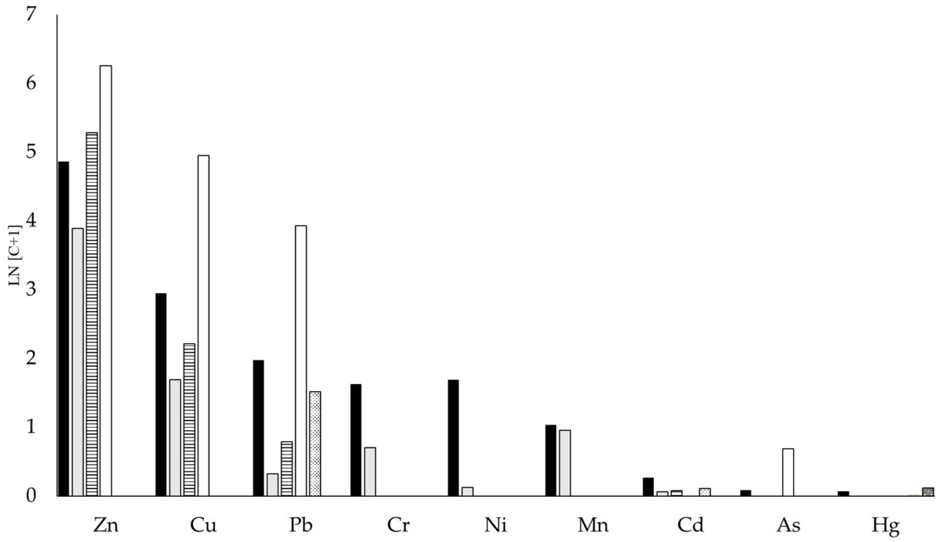

Figure 5. Comparison of metal concentration in wood mice hair. Data are log-transformed to allow for comparison. Black bar [present study]; grey bar [42]; stripes bar [27], white bar [41], black dot bar [20], white dot bar [39].

4. Discussion

4.1. Metal Level in Wood Mouse Hair: Sex and Age Differences and Similarities

The data from this study are comparable with the results of a few other papers on metal levels in hair. We have observed that ubiquitous metals, such as Zn, Al, Fe, and Cu, are more abundant than those of ecotoxicological interest, such as Cd, Se, As, and Hg (Table 1), and that the concentrations of Cd, Pb, Cu, and Cr are more variable than those of Zn, Fe, and Mn. Concentration variability is higher in females than in males (Table 2), and no significant age-related differences were observed (Table 3). Differences in accumulation between male and female mammals have been highlighted in the literature but, as reported by ref. [5], data on the effect of biotic factors such as age and sex on metal bioaccumulation in the wood mouse are scarce, and those available do not show clear patterns as they vary significantly between populations [5,27,43]. Several authors have reported slight differences in the variation of metal concentrations in male and female wood mice [27,44], and no differences were observed by ref. [39]. Our data confirmed slight sex differences for Zn, but only Cr and Ni are markedly and significantly higher in males than in females (Table 2 and Figure 1). The social system of the wood mouse is polygynous, and the home ranges of males overlapped those of several females [45,46]; males increase the home range at the beginning of the reproductive season, and the home range size can exceed 1 hectare depending on habitat [29]. It is likely that some males moving or dispersing around our study area, a woodland surrounded by a densely urbanized area with small galvanic and electroplating industries, have come into contact with Cr and Ni, metals widely used in this production chain.

More interestingly, we observed large differences between females in different reproductive status, with pregnant/lactating females having lower levels of Al, Hg, and As than non-reproductive females. The low level of some toxic metals in lactating females may reflect an increased metabolic activity during pregnancy, and fat tissue transformation during lactation activates the release of some metals in blood and from there into the hair [47]. A decrease in some metals in lactating females has been observed by refs. [47,48]; maternal transfer during lactation and a higher capacity for demethylation [49] is the probable cause for reproductive females having lower levels of Hg in their bodies. On the other hand, milk is the secretory product of proteins, amino acids, and fats synthesized and secreted by mammary epithelial cells in all mammals [48]. Its production imposes a significant energy cost on females and releases many compounds stored in reserve tissues into the bloodstream; this may explain why lactating female wild mice have significantly lower concentrations of potentially toxic metals than non-reproductive females. Also, the strong correlation between the weight of reproductive females and Se level in hair may also be related to the energetical effort in lactation. High Se levels in lactating females may improve immunocompetence and have a bactericidal effect on mammary epithelial cells; its high level in lactating female wood mice, and the corresponding stable and physiological concentration in imperforate females, could be the effect of an enhanced protective activity [50,51].

Another interesting observation is the very low concentration of Hg in every statistical group. Its concentration ranges from 0.06 to 0.08 μg/g in adults and subadults and around 0.07 μg/g in both sexes (Table 3); these values are considerably lower than in soil (Table 4), and since Hg can be assumed through food, this may suggest a low tendency to bioaccumulate in the wood mouse tissues. From an ecological point of view, the low level of Hg also suggested that the wood mouse should be considered as a primary consumer, more than a generalist feeder that included animals in its diet. The diet of the wood mouse in our study area was analyzed by ref. [52]; the annual diet of the wood mouse species is dominated by seeds that account for nearly 77% of the relative proportion, while insects are usually less than 2%. These data confirm the generalistic feeding habits of the wood mouse in our study area and, since leaves and seeds accumulate lower levels of some metals as Hg, they probably explain the low level of Hg in hair, thus confirming the statement by ref. [38] about the role of the wood mouse as primary consumer in the oak–elm forest [53].

4.2. Relation Between TE Concentration in Soil and Wood Mouse Weight

Weight and the concentration of some metals are significantly and positively related in our sample (Figures 2 and 3); the relationship between weight and contamination has already been reported by ref. [5]. We interpreted these data as a response to the growth of the individuals; however, hair were moulted periodically, and the metals level increased as the body size of the rodent increased, which is ultimately reflected in the concentration levels of some metals (Cr, Fe, Mn, Ni, Cd, Hg) in the hair. Moreover, we also observed a negative correlation between Pb concentration and subadult weight (Figure 3). The harmful effect of Pb is very well known since it can affect several physiological functions in homeotherm vertebrates [19,32,33,41]; the negative relationship with weight (a growth factor of each individual) and the high variability of Pb concentration in the hair of males and females may indicate a negative effect of Pb on wood mice and an active metabolic activity leading to its excretion.

Contrary to [5], we did not observe significant differences in metal levels between adults and subadults; several authors explained a higher metal concentration in juveniles and its decrease with age with the more significant energy requirements of juveniles, implying a high feeding uptake and incorporation during the growth. Discordant results may be attributed mainly to inter-population variation caused by differences in exposure and uptake of elements or to different classification criteria of "young" mice. According to [54], juveniles can be defined s small individuals weighing less than 16.5 g, with a grey or greyish fur and absence of reproductive activity (imperforate vagina or abdominal testes); these individuals are in full growth phase and can probably dilute the metal load during growth. The young individuals in our study areas were subadults weighing more than 16.5 g; at that time, males dispersed while females were more philopatric and stayed close to adults. Since females remain in the same microhabitat as adults, and males disperse in the uniform microhabitat of a mesic forest, they assimilate similar metal loads from their habitat through food or contact with soil.

A significant correlation between metal levels in soil and hair showed that the wood mouse is partially influenced by soil chemistry; essential metals, such as Fe and Zn, are the most abundant metals we found in soil and show a higher concentration in wood mouse hair (Table 4; Figure 4). Significant correlations between metal in soils and hair are in agreement with the results of ref. [42], who found a positive relationship between Cd, Ni, Pb, and Zn in soil and hair, and the results of ref. [20], who did not find a significant relation between concentrations but observed that levels of Cd and Pb tend to increase with increasing concentration in soil. Our data do not establish a causal relationship between metals in soil and hair as the correlation is significant but weak; however, it may indicate that the fossorial behavior of the wood mouse plays a partial role in hair contamination. Hg, Pb, Cd, and As do indeed have a high affinity for sulfhydryl groups of keratin, a protein forming mammal fur; sulfhydryl groups can interact with metals through metal thiolate coordination and incorporate metal into hair [55]. Metal incorporation can be important in metal removal from the body; blood route and metal binding from keratin are the most important ways in which several non-essential metals are removed from the body and stored in the hair. Still, ref. [41] suggested that Pb adhesion from soil to hair may be an integrative accumulation pathway, rather than ingestion followed by metabolism and storage. The low level of TE in hair with respect to soils may be an effect of homeostatic physiological control, which tends to maintain and lower the level of essential metals in the body; in addition, the possibility that hair layers may adsorb some proportion of metals from soil cannot be ruled out as the wood mouse is a fossorial species living underground for much of its life.

4.3. Hair as a Biomonitor of TE Contamination

The lethal approach in biomonitoring research has increasingly ethical implications, and some studies have assessed the reliability of conservative methods using the hair of wild individuals subsequently released into the wild (i.e., the conservative approach). However, there are surprisingly few conservative studies on synanthropic species, such as the wood mouse *Apodemus sylvaticus*; to the best of our knowledge, only seven studies have adopted a conservative approach to analyzing contamination levels in the wood mouse, a number lower than those on species less associated with the human, such as seals and sea lions (i.e., [56–58]). Some papers consider the reliability of hair as an index of metal contamination; ref. [38] found that mean Hg concentrations in organs decreased from the hair to the brain, confirming that the hair is a suitable index of the maximum level of contamination by Hg in the wood mouse. The same pattern was observed in otters [59] and the red fox *Vulpes vulpes* [60,61], with hair having higher Hg concentrations than soft tissues. Ref. [27] suggested that hair from wood mice can be used to monitor exposure to non-essential metals (Cd and Pb), but to a minor extent for regulated metals such as Cu or Zn. Significant correlations between hair and internal tissues for Cd, Ni, Pb, and Zn were found by ref. [42], demonstrating that hair proved to be a suitable and non-invasive tool for monitoring metal responses in small mammals; similarly, ref. [20] suggested that hair should be used with caution to predict Cd concentrations but can be considered as an accurate non-invasive estimator of internal Pb concentrations. The implications of these studies for data collection protocols are still limited, and further studies are needed. Intra-populational variability in metal concentration may be higher than the inter-populational variability, thus affecting the detection of differences in the bioaccumulation in the population [62]; moreover, essential metals can be regulated by the organism, resulting in a weakened pattern of contamination by metals [20,27].

It could be argued that unlike in birds, rodents shed their hair continuously, and that this should be taken into account when interpreting the results since it is an additional source of variability. However, a large proportion of adult mice show a patchy moulting over the whole body with no seasonality [63]; since the process of hair replacement is constant and widespread, it is unlikely to significantly influence the result of our analysis. In conclusion, we accept the results of some experimental studies that currently recognize hair as a biomonitor of the contamination status of organisms and the environment [20,42,64].

5. Conclusions

Our results are generally consistent with those of several other authors (Figure 5) and support the reliability of hair, at least, for comparisons between different sites. In addition to providing data of general interest for an understudied species, this study adopts a conservative approach to biomonitoring contamination [20]. Hair may be an interesting alternative to lethal methods, and there are good reasons to enhance its use [65]: first, for ethical reasons, since the sacrifice of wild animals for research is no longer acceptable; second, because the wood mouse is essentially a primary consumer and thus an organism capable of intercepting metals that are eventually transferred from the soil to the green plant; third, the hairs are continuously connected to the bloodstream and are a biomonitor of the current contamination; last but not least, the reason for using hair is that there is no organ or tissue that can be considered as the ultimate indicator of a "true level" of contamination; it therefore seems prudent and habitat-friendly to use hair as an initial probe, i.e., as a first, simple, and quick level of environmental contamination analysis. This approach should be used with caution, taking into account a possible systemic error for some essential metals, and should only be followed by more in-depth analyses involving the euthanasia of wild animals if necessary.

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