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Original article

Influence of age on the bioaccumulation of heavy metals in *Apodemus sylvaticus* at Merja Zerga lagoon, Morocco

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ABSTRACT

The influence of age and sex on the bioaccumulation of heavy metals in Apodemus sylvaticus was studied in Merja Zerga lagoon in northern Morocco. Five trace metal elements (Zn, Pb, Cr, Cu and Fe) were quantitatively analyzed by Varian AA 240 atomic absorption spectroscopy with graphite furnace in three organs (Liver, Kidney and Heart) from animals of different age and sex. The maximum metal level of the analyzed samples was recorded in adults and was limited to 46.62 µg/g for Pb and 35.1 µg/g for Cu, while it reached 22.69 µg/g, 7.59 µg/g and 6.78 µg/g for Cr, Zn and Fe, respectively. Highly significant differences were found for bioaccumulation of heavy metals according to animal ages and no significant differences were observed between the two sexes among the studied animals. Our results revealed also the existence of a strong correlation ($r > 0.65$) between the majority of biometric parameters and the trace element concentrations. In general, we found that age is a critical factor in estimating the level of heavy metal pollution. Other characteristics such as habitat, feeding habits and anti-predator behavior of the species need to be studied.

1. Introduction

Since the 20th century, the knowledge concerning the environment contamination by metallic trace elements has unceasingly evolved. Although these elements are naturally present in the earth's crust, most environmental contaminations result from numerous human activities (Aziz et al., 2013). Metal trace elements are one of the main polluting groups, for they pose a serious problem for life in general (Nasrabadi et al., 2010) and a real threat to the environment since they cannot be biodegraded and therefore persist in it for long periods of time (Okuku and Peter, 2012). The impact of these substances is very complex as long as their toxic actions can be direct or indirect on individuals, populations or ecosystems, and that synergistic phenomena among metal trace elements are possible and could triggers complex effects (Haider et al., 2004).

A field study has shown that living organisms require varying amounts of essential metals; however, some excessive levels can be harmful to the body. Therefore, when these organisms are exposed to metals either actively or passively, all their development stages got hindered (Serbaji et al., 2012).

Because of their wide distribution, micromammals are suitable for studying the effects of pollution as bioindicators (Chardi, 2007; Wijnhoven et al., 2008; Beernaert et al., 2008; Fritsch et al., 2010; Tête et al., 2014; Tifarouine et al., 2018). The wood mouse, *Apodemus sylvaticus* (Linnaeus 1758), is an example of these species widely distributed in North West Africa and Western Europe (Lalis et al., 2016). They accumulate larger quantities of heavy metals (Sawicka-Kapusta et al., 1995; Martiniaková et al., 2012), but this does not necessarily mean that these species are the most exposed to the toxic effects of pollution. The sensitivity of the

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species is highly variable and many mechanisms exist to deal with toxins.

In this perspective, this study aims to better understand the transfer and impacts of metallic pollutants in the ecosystems, by characterizing the response mechanisms of certain biological models, especially rodents, to this type of pollution. The approach adopted is based on the analysis of data on the influence of individual characteristic (age) and the effects of trace metals.

2. Materials and methods

2.1. Biological model

The Wood Mouse is a rodent generally found in forest areas in northern Morocco, but it can also be found in cultivated land, its food is 80% composed of plants (Khidas et al., 2002). This rodent, which lives in shallow burrows, is characterized by long ears and a tail covered with hair on all its length, it presents, as most of its congeners, hind limbs that are more developed than the anterior ones. It resembles to the gray mouse (*Mus musculus*), which can be distinguished by its larger size, its large eyes (those of nocturnal rodents), its back is gray-brown to red and its belly is white.

2.2. Study area

The study is carried out in the Ain Felfel forest near the lagoon of Merja Zerga located in northern Morocco (near the seaside village of Moulay Bousselham, between the cities Kenitra and Larache). This lagoon is a biological reserve and also the RAMSAR site (1980) the best known in Morocc. Her environment is mainly of tectonic origin and occupies a depression of about 5000 ha located at the northern limit of the Gharb plain and near the southwestern margin of the Rif mountain range. It is surrounded by low sand dunes, one of which, in the form of a shoreline, separates the lagoon from the ocean and is interrupted by a gully that provides permanent communication with the Atlantic Ocean. The climate in this region is Mediterranean with dominance of oceanic influences (Benhoussa, 2000; Bazaïri et al., 2003).

Thirty-seven specimens of *Apodemus sylvaticus* were captured (22 males and 15 females) in the Ain Felfel forest, dominated by wild olive trees, cork oak and pine trees, the preferred habitats of this species. About fifty Sherman traps are deposited in the afternoon, and are recovered early the following morning. The different baited traps (bread, olive oil, peanut butter, dates) are placed in the vegetation, at least 5 m apart (Fig. 1).

2.3. Morphometric parameters and chemical analyses

The collected population samples consisted of twenty-one juveniles and sixteen adults that were clearly separable by body weight (W), the length of which included the head and body (TL), tail (T), hind paw (PL) and ear (E) (Fig. 2).

Captured specimens are sacrificed with chloroform, sexed, weighed and measured (head and body length, tail length, hind leg length, ear length). Kidney, liver, and heart samples were maintained at −18 °C until assayed (the absorption of metals is mainly distributed at the level of the liver and the kidney, where finally concentrates almost half of the total load of the organism). Thereafter, three doses of about 0.5 g of organ samples are hot-mineralized at 120 °C for 4 h in the presence of 3 ml of suprapur nitric acid (65% Merck), the mineralization solution was diluted to the final volume of 25 ml with bidistilled water as described by Auger (1989). The concentrations of Pb, Cu, and Cr in the various samples were analyzed by atomic absorption spectroscopy with graphite furnace (Varian AA 240 120 GTA Z) (Chiffoleau and Truquet, 1994). The background correction is made by a Zeeman effect, while the Zn and Fe dose is carried out by flame atomic absorption spectroscopy (VARIAN AA40 FS), in the accredited laboratory of toxicology at the National Institute of Hygiene (Rabat, Morocco). To reduce the chemical interference and volatility of Cr,

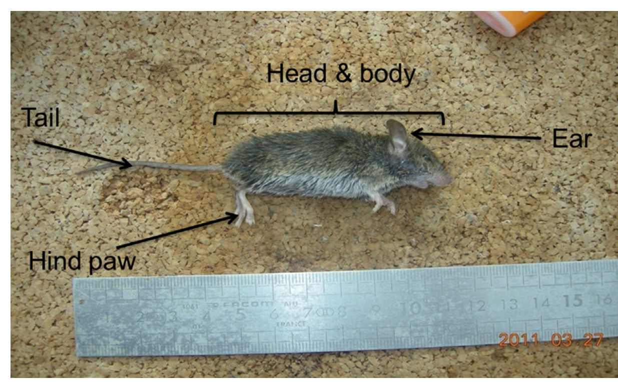

Fig. 2. Morphometric measurements taken on rodents.

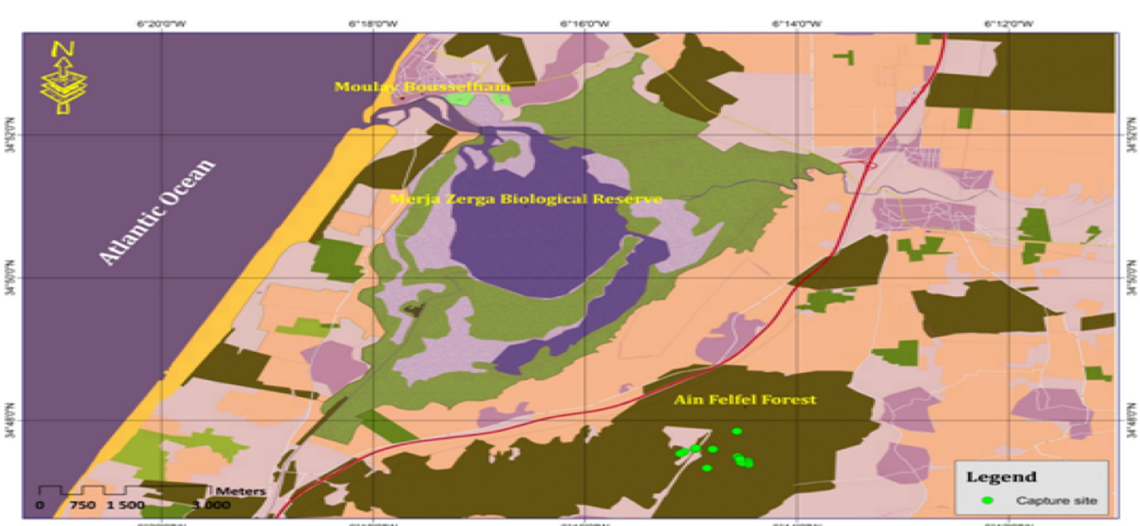

Fig. 1. Geographical location of the sampling points.

Cu and Pb in an oven, a matrix modifier was used (mixture of PdCl2 and MgNO3). The calibration curve was generated by the "MSA (standard addition method)". The validity of this method is verified by internal control using standard samples (National Research Council of Canada: DORM-2) and by external control using inter-calibration exercises (IAEA-MESL-2014-01-TE) which uses blood doping samples. After the linearity of the curve, the accuracy was verified by three successive readings for each sample and the average of these measurements would be taken into consideration if the RSD (relative standard deviation) was less than 10%.

2.4. Statistical analyses

The data concerning the morphometric parameters, the metallic trace element quantities as well the animal sexes (male or female) and ages (adults or juveniles) were a matrix structure which was subjected to the next analysis:

- A multivariate analysis of variance to assess the effect of sex and age on metal elements bioaccumulation.
- A comparison of means to detect possible morphological variations between Moroccan specimens and those of other countries.
- Pearson (r) correlation coefficients were calculated to establish the relationship between trace elements and individual size. Significant differences were accepted at $p < 0.05$.

All statistical analyzes were performed using the "R 2.7.1" software (R Development Core Team, 2006) with the following additional packages: ggfortify, lfda, ggplot2, devtools, FactoMineR, cluster and ade4.

3. Results

3.1. Morphometrics parameters

Morphometry is an important criterion to identify the species. The body measurements of the various captured individuals reveal certain homogeneity with the limit set by other authors (Ben Salem et al., 2012; Çolak et al., 2004; Stoetzel et al., 2010). The average weight of *Apodemus* is 17.16 ± 3.31 g. The body measurements show that head and body length vary between 74.0 and 101.0 mm (mean = 90.08 ± 7.00 mm). The length of the tail is between 57.0 and 116.00 mm with an average of 80.78 ± 22.70 mm. The hind paw has an average of 20.45 ± 1.91 mm.

An Ascending Hierarchical Classification was made from the morphometric data of averages of *Apodemus sylvaticus* from Morocco (Stoetzel et al., 2010, 2012; the present study), Tunisia (Stitou et al., 2001; Saïdi, 2006; Ben Salem et al., 2012), France (Rampaud, 1988), Turkey (Çolak et al., 2004; Krystufek and Vohralik, 2009), Germany (Düsseldorf, 2007) and the Middle East (Filippucci et al., 1989; Abu Baker and Amr, 2008). This procedure made it possible to distinguish between three groups (Fig. 3). Group 1 includes species from Morocco, France and Germany with a dissimilarity distance of about 0.10. The second group includes those from Tunisia and Turkey with a difference of 0.45, and the last group comprises the one from Middle East.

3.2. Bioaccumulation of metals by age and sex

The integration of the results in a Geographic Information System (GIS) allowed the realization of several thematic maps relating to the spatial distributions of the contents with metallic trace elements (Fig. 4).

Analysis of the assay of the different HMs in the liver of adult *Apodemus sylvaticus* shows that, at the level of the liver, the highest concentrations are those of Pb and Cu with maximum values of about 46.62 μg/g and 33 μg/g respectively. The accumulation of Cr, Zn and Fe in the same organ alternately reaches maximum concentrations of 10.87 μg/g; 7.59 μg/g and 6.56 μg/g. At the level of the kidney, the concentrations of the HMs follow the same hierarchy with maximums which are limited to 39.75 μg/g for Pb and 29.17 μg/g for Cu. As for Cr, Zn and Fe, they do not exceed 12.53 μg/g; 7.31 μg/g and 6.78 μg/g respectively. Finally, the recorded concentrations of the HMs in the heart show that the latter constitutes the organ that accumulates less compared to the kidney and the liver.

In juveniles of *Apodemus sylvaticus*, the overall rate of HM accumulation is generally lower than that seen in adults. In this age group, the liver remains the organ that concentrates the largest quantities of heavy metals, in particular Pb and Cu whose maximum concentrations reach 29.18 μg/g and 14.83 μg/g respectively. While chromium, zinc and iron concentrations do not successively exceed 6.57 μg/g; 5.89 μg/g; 5.17 μg/g. Moreover, the dosages of these HMs in the kidneys and the heart of these same (juvenile) individuals give much lower concentrations than those observed in the liver.

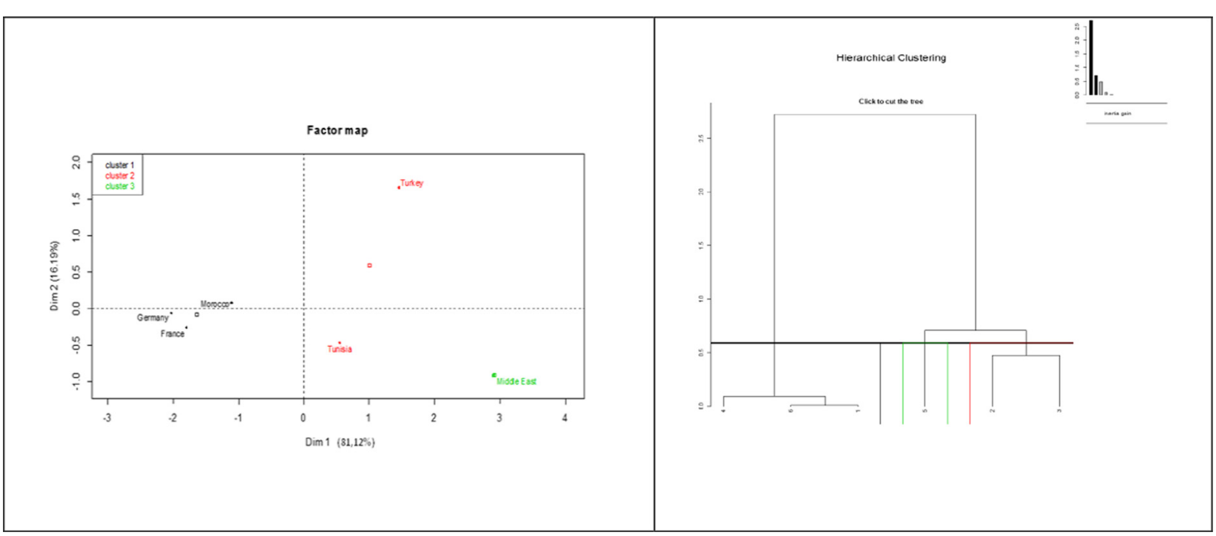

Fig. 3. Principal component analysis applied to *Apodemus sylvaticus* averages from Morocco, France, Germany, Turkey, Tunisia and the Middle East.

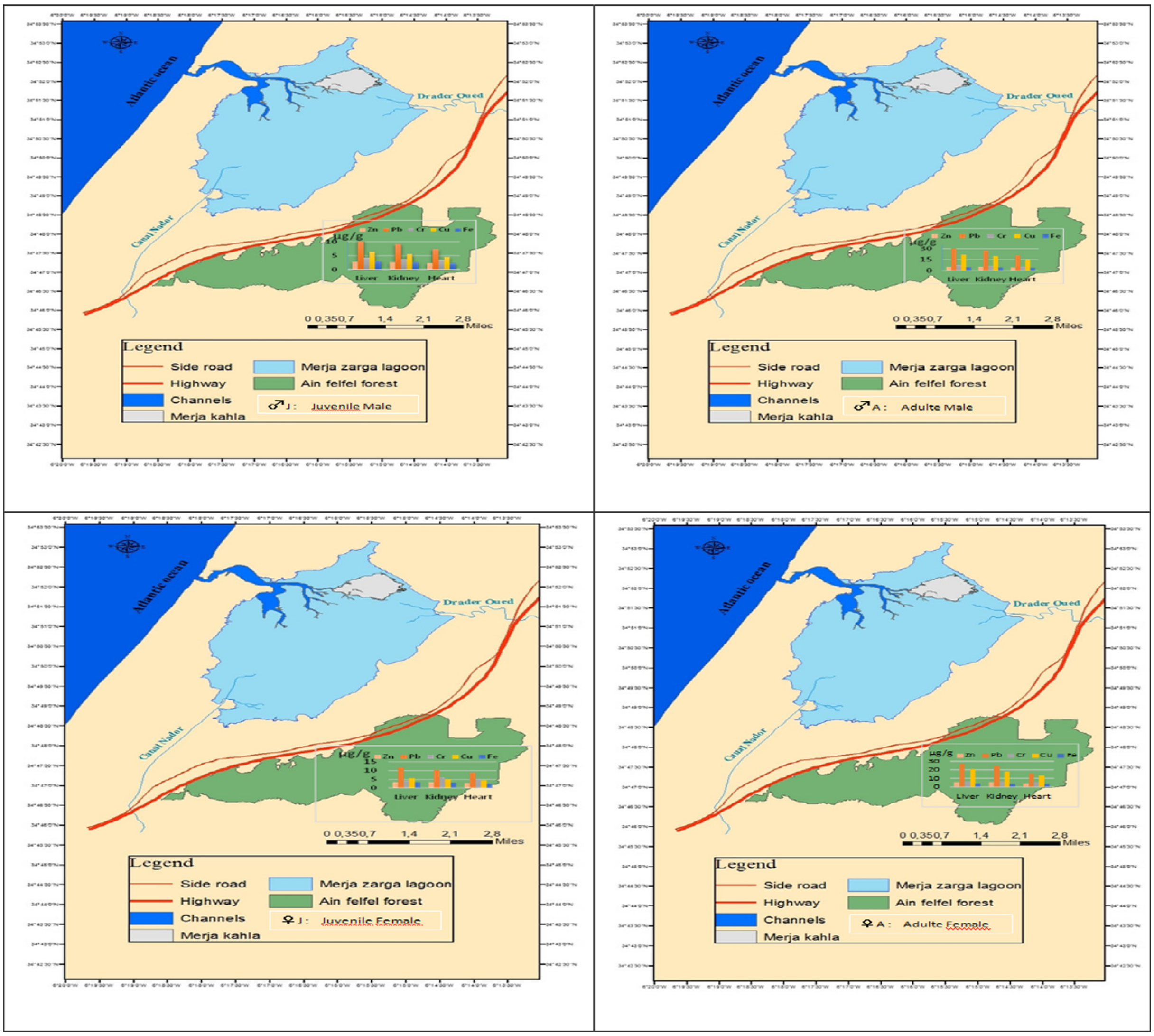

Fig. 4. Map based on the concentrations of the trace elements (Zn, Pb, Cr, Cu and Fe) in the organs (Liver, Kidney and Heart) of the different captured individuals (MA, MJ, FA and FJ) and their geographical distributions (map by BENMOKHTAR Salma).

In all the individuals in our sample (male, female, adult and juvenile) the liver remains the organ that concentrates the largest amount of HMs followed by the kidney and heart. The contents of the metallic trace elements in the different organs, have respected the same hierarchy, as the concentrations of Pb and Cu are the highest followed by that of Cr, Zn and Fe.

The application of an ANOVA analysis to the matrix of all of our data showed statistically significant differences in the concentrations of the HMs according to the age of the individuals (Table1). However, no significant difference, were detected between the two sexes. This would seem to be due to the high proportion of juveniles in our sample (21 juveniles and 16 adults). The same analyzes were repeated on adult individuals only, but no sex-dependent variation was detected for the concentrations of these metals.

3.3. Correlations between parameters (morphometric and metals)

The correlation matrix gives estimates of the magnitude of the links between the different morphometric variables and the rate of bioaccumulation of metals in the organs considered in this study

Table1
Two-factor ANOVA results to determine the effects of Sex and Age in the organ of Apodemus sylvaticus (Liver, Kidney & Heart) and interactions on metal tissue concentrations (Zn, Pb, Cr, Fe, and Cu) ns no significant differences: $^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

Source	Zn	Pb	Cr	Cu	Fe
Sex (F_{Lead})	0.2412 **ns**	0.2715 **ns**	0.1508 **ns**	0.7934 **ns**	0.2675 **ns**
Age (F_{Lead})	36.7736***	36.2853***	42.3619***	67.2870**	14.8237 ***
as.factor(sex):as.factor(age)	0.1720 **ns**	0.4598 **ns**	0.0039 **ns**	0.0006 **ns**	1.0388 **ns**
Sex (F_{Kidney})	0.6711 **ns**	0.1689 **ns**	1.4129 **ns**	1.2059 **ns**	0.0562 **ns**
Age (F_{Kidney})	32.6485***	36.5374***	2.3005 **ns**	85.0764***	10.3070**
as.factor(sex):as.factor(age)	0.3073 **ns**	0.4356 **ns**	0.5554 **ns**	0.1208 **ns**	1.8110 **ns**
Sex (F_{Heart})	0.3250 **ns**	0.7368 **ns**	1.1903 **ns**	1.1186 **ns**	0.7168 **ns**
Age (F_{Heart})	29.2530***	25.7746***	2.3890 **ns**	92.1182***	14.1362***
as.factor(sex):as.factor(age)	0.6951 **ns**	2.3744 **ns**	0.6487 **ns**	0.3101 **ns**	0.8231 **ns**

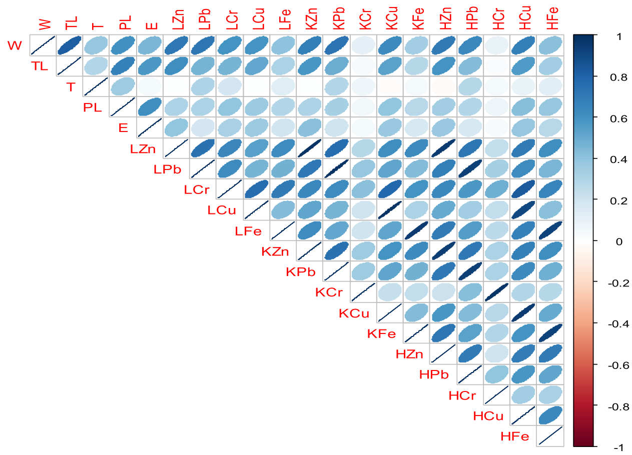

Fig. 5. Correlations between the different variables: Morphometric & heavy metals in the organs (Liver: LZn, LPb, LCr, LCu, LFe; Kidney: KZn, KPb, KCr, KCu, KFe; Heart: HZn, HPb, HCr, HCu, HFe).

(Liver, kidney and heart). These links are translated by coefficients of correlations which vary between −1 and 1. It is estimated that 2 variables are strongly correlated if their coefficient has a value R > 0.7 whereas if 0.5 < R < 0.7 the correlation between the parameters is considered moderate.

The analysis of this matrix (Fig. 5) shows that the weight of the individuals (W) is strongly correlated with TL (R = 0.82), KPb (R = 0.73), LPb (R = 0.72). LZn (R = 0.71), and moderately correlated with KZn (R = 0.68) and HCu (R = 0.68). On the other hand, TL has a strong correlation with Pp (R = 0.87), and an average with HCu (R = 0.68) and LZn (R = 0.61). Other correlations could be detected between the different metals, which reflects their implications for the main processes that take place at the level of the organs (LZn-LPb (0.75); LZn-KPb (0.76); LZn-HPb (0.73); LZn-HCu (0.70); LPb-KZn (0.72); LCr-LCu (0.76); LCr-LFe (0.71); LCr-KCu (0.78); LCr-HCu (0.83); LFe-HZn (0.70)).

To confirm the different results obtained, and in order to identify a typology of all individuals according to their morphological characteristics and the accumulation rates of the HMs in their organs, we performed a principal component analysis (PCA) on the matrix of all of our data. The projection of the coordinates of the different variables on the F1 × F2 plane of the first two principal components, which account for 64.30% of the total variance, shows that the F1 axis is strongly correlated with the biometric variables (W, TL, T, PL, E) and the concentrations of Cu, Zn, Pb and Fe in the various organs. While only the concentrations of Cr in the kidney and the heart show a strong correlation with the F2 axis (Fig. 6).

The organization of the different individuals in the same factorial plane F1 × F2 makes it possible to distinguish between 4 groups (cluster) which are distributed along the axis F1

Group 1: positioned towards the positive values of the F1 axis and consisting of adult male and female individuals who are characterized by a large size (w and TL) and having high concentrations of trace metal elements (Cu, Zn, Pb, Fe) in the three organs.
Group 2, located on the opposite side of the first group, contain juvenile individuals of both sexes with small sizes and low levels of accumulation of trace metal elements.
Group 3 (intermediate position between groups 2 and 4) includes the MA7, MA9, MA3, FA3, MA6, FA2, MJ8, MJ13 and FJ15 individuals with average levels of HM.

The FJ1 specimen, which is a subadult, individualises towards the positive values of the F2 axis due to its high Cr content in the heart (17.63 µg/g) and the kidneys (22.69 µg/g).

4. Discussions

The obtained results indicate that the Moroccan populations of *Apodemus sylvaticus* have almost the same morphological characteristics as those of France, Germany, Turkey, Tunisia and the Middle East. It also emerges that the tail (T) of individuals increases with the rate of increase of their posterior paw (PL) and that the individuals of low weight, their hind legs tend to have a significant length allowing these individuals to jump. According to the fossil record, the species *Apodemus sylvaticus* colonized Morocco and

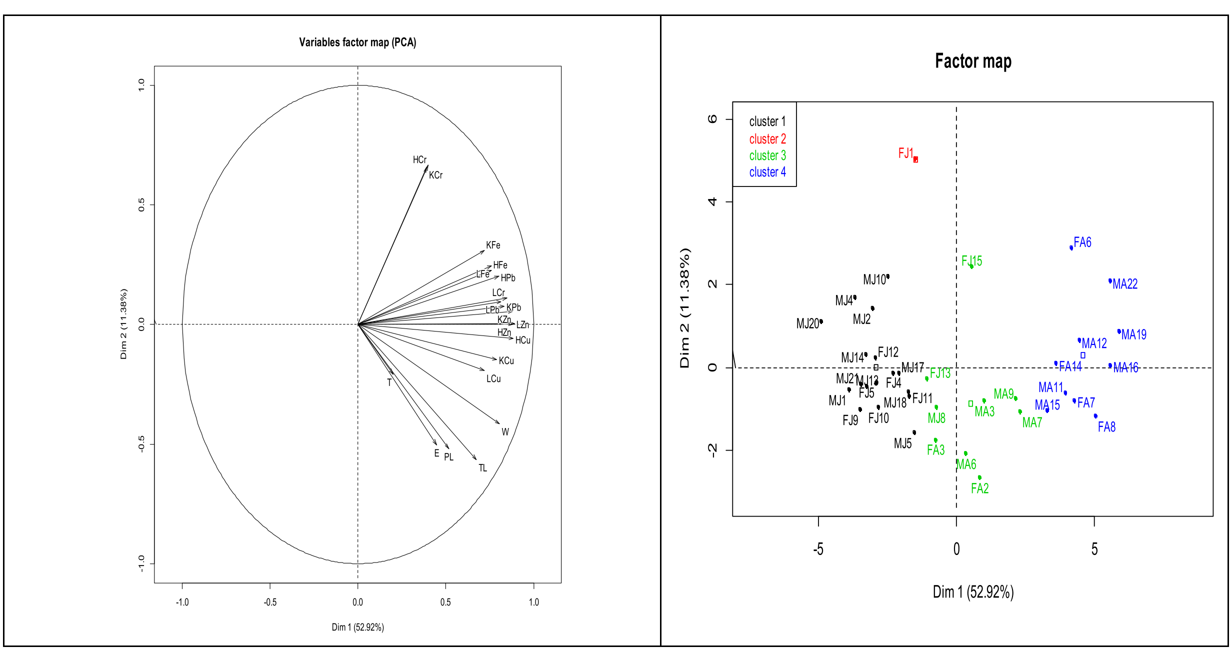

Fig. 6. Principal component analysis applied to the concentration of trace elements of captured *Apodemus sylvaticus*.

Algeria during the Holocene (Stoetzel, 2013), after its migration from Europe by the Strait of Gibraltar (Lalis et al., 2016) whereas it has been present in Europe since the beginning of the Pleistocene (Cuenca-Bescos et al., 2010). Several hypotheses have been put forward to explain the arrival of the wood mouse in Africa; they assume that this species is native to South-West Europe and that their introduction is probably anthropogenic (Libois et al., 2001). This confirms the great similarity observed between our Moroccan specimens and those of other European countries. This percentage of similarity between all these populations can reach 95%. According to Tchernov (1979); a high genetic affinity between Middle Eastern and Balkan populations has been demonstrated, suggesting that the genus *Apodemus* has probably invaded the Middle East from southern Europe. A mitochondrial and microsatellite analysis performed by Lalis et al. (2016); revealed a low variability in the North African lineage (Morocco, Algeria and Tunisia) suggesting that North African wood mice form a single population. It should also be noted that the differences between populations are probably due to the fact that these geographically separated populations live in distinctly different ecological conditions. The studies of Bernardo (1994) and Klaassen (1995) demonstrated that life history traits and metabolic rates vary across populations, apparently as an adaptive response to different environments.

As previously described by Martiniaková et al. (2012), Tête et al. (2014) and Tifarouine et al. (2018); rodents have been widely used as bioindicators of metal contamination. Indeed, they can provide information on the characteristics, quantities and types of pollutants present in the environment. The results we obtained showed highly significant differences in the bioaccumulation of HMs by age of individuals in the population. Adults accumulated approximately twice as much Pb and Cu in liver, kidney and heart tissues compared to juveniles. These results also showed that hepatic lead concentration reached a maximum of 46.62 μg/g in adults and 25.07 μg/g for juveniles, which explains their strong ability to regulate HMs homeostatically in their soft tissues. In addition, the accumulation of lead in different organs follows the same hierarchy Kidney > Liver > Heart. Unlike lead, copper accumulation peaked in the heart ($F = 94.02$, $p < 0.0001$), which may be related to the need for myocardial function. Indeed Angelova et al. (2004), have shown that essential metals are necessary for the proper functioning of living organisms. However, they can also produce toxic effects when their consumption reaches high concentrations. In this respect, our results confirm significant differences in the accumulation of Zn, Fe and Cr. Hepatic Cr concentrations were higher than those of Zn and Fe. However, no difference in Cr was observed in renal and cardiac tissues, whereas the hierarchy of concentrations in these tissues was [Zn] > [Fe]. Nevertheless, high levels of metals in the body could be caused by poor kidney excretion.

On the basis of the results obtained, the correlations observed between the HMs of the *Apodemus* indicate significant values of the Zn-Pb couples ($R > 0.70$); Cr-Cu ($R > 0.75$); Fe-Zn ($R > 0.70$). In addition to their toxic levels, the interaction between metals has a very detrimental effect, since these mixtures can increase the toxic effects by synergy (Açikel and Alp, 2009; Desaunay, 2011). Our data are consistent with reported relationships for Fe-Zn, Cr-Cu, and Zn-Pb (Goix, 2012). Indeed, there are also interactions between metals and other chemicals such as pesticides and major ions (sodium, potassium, calcium and magnesium). The results also indicate very important correlations, in particular between the body variables and the concentrations of HMs, W-Pb ($R > 0.70$), W-Zn ($R > 0.65$) and TL-Cu ($R > 0.65$), it can be said that the accumulation of HMs is influenced by the size of individuals, the duration of exposure to pollutants and the age of the population (Scheirs et al., 2006). The large variation in the concentration of metals in the same population could be justified mainly by biotic and abiotic parameters.

In a global way, we can say that our research shows an increased accumulation of metallic trace elements in the organs of the *Apodemus* species studied; this fact can be explained by the study area which is essentially agricultural. Merja Zerga is located on two large agricultural perimeters: Gharb and Loukkos. The Gharb plain is one of the most important vegetable production areas in Morocco (ORMVAG, 2010). At the same time, Loukkos covers a large agricultural area nationally, mainly in the production of strawberry (80%), rice, peanuts (20%) and sugar crops (15%). In general, these crops consume a lot of pesticides. According to the ORMVAG (2007), 12 pesticide residues were recorded at the Gharb perimeter in the water and soil resources, which may lead to a subsequent accumulation of specific elements, notably Cu and Zn in the soil. Near the capture locality, there is heavy and dense road traffic considered as a source of risk elements, in this case Pb (Blagojevic et al., 2012), particularly in summer, when climatic conditions favor the dispersion of pollutants (absence of wind and rain). According to the bibliography, the abrasive and mechanical wear of braking systems mainly contributes to emissions of HM by vehicles. Nevertheless, although road traffic emissions are generally recorded, geographical differences exist because of the additives and lubricants used, which vary from one country to another (Sternbeck et al., 2002; Lin et al., 2005). In addition, runoff water from the road consists mainly of Cu, Zn, and Pb (Coggins et al., 2006). There is also a possibility of contamination from the dust produced, by the industrial zones in Kenitra, Larache, and Tangier, and transported in the air. This hypothesis should be confirmed by other studies highlighting the possibility of transporting long-range toxic elements. Among the most important sources of environmental contamination is the coal industry as its dust contains zinc, copper, lead and cadmium, and this contamination can increase the content of elements in mammal tissues that populate these areas. We have also hypothesized that anthropogenic input of HMs into the environment result from industrial sources, agricultural applications and urban activities (Onder et al., 2007).

5. Conclusion

The present study analyzes both the bioaccumulation of metals in the organs of *Apodemus sylvaticus* of Merja Zerga and the geographical variation in body size of this species in other countries. We found that the effectiveness of the process of bioaccumulation depends only on the individual age, while sex has no effect. Our results showed that adults, despite the low rate of their metabolism, accumulated about twice the HMs in their tissues compared to juveniles. It asserts also, that the transfer of pollutants from the environment to *Apodemus* is due to contamination by direct ingestion of soil particles and/or transfer through the food web (Gonzalez et al., 1999). We suspect that exposure time, metal dispersion, and population structure may be important factors in the bioaccumulation of HMs in this species.

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