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Tissues and hair residues and histopathology in wild rats (*Rattus rattus* L.) and Algerian mice (*Mus spretus* Lataste) from an abandoned mine area (Southeast Portugal)

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The bioaccumulation of As and Cd and signs of renal histopathological injury proved the value of Algerian mice as a bioindicator species in the risk assessment of contaminated sites.

Abstract

Data gathered in this study suggested the exposure of rats and Algerian mice, living in an abandoned mining area, to a mixture of heavy metals. Although similar histopathological features were recorded in the liver and spleen of both species, the Algerian mouse has proved to be the strongest bioaccumulator species. Hair was considered to be a good biological material to monitor environmental contamination of Cr in rats. Significant positive associations were found between the levels of this element in hair/kidney ($r = 0.826$, $n = 9$, $p < 0.01$) and hair/liver ($r = 0.697$, $n = 9$, $p = 0.037$). Although no association was found between the levels of As recorded in the hair and in the organs, the levels of this element recorded in the hair, of both species, were significantly higher in animals captured in the mining area, which met the data from the organs analysed. Nevertheless, more studies will be needed to reduce uncertainty about cause–effect relationships.

Keywords: Rodents; Heavy metals; Exposure assessment; Liver; Spleen; Kidney; Hair; Histopathological changes

1. Introduction

Although metals are naturally occurring elements, their concentration in environmental compartments is significantly increased by anthropogenic activities, such as mining. Metallic and non-metallic ore extraction activities are responsible for serious impacts on the environment, which most of the times persist for several decades, after mineral exploitation has ceased (Helms, 1993; Pereira et al., 1995, 1999a; Lopes et al., 1999).

Terrestrial vertebrates have been reported to be exposed to heavy metals in contaminated areas and to bioaccumulate them in different tissues (Hyvärinen and Nygrén, 1993; Burger et al., 1994, 2000; Gochfeld et al., 1996; Erry et al., 1999; Pereira et al., 1999b; Kålås et al., 2000; Mertens et al., 2001). Therefore, animals have long served to evaluate inorganic compound exposures, especially for Cd, Hg and Pb (Talmage and Walton, 1991). Data collected from animals, which are exposed to contaminants in their natural habitat, are very important for human and environmental health risk

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assessments. Such data provide information about environmental and food chain contamination with chemicals or chemical mixtures, potential wildlife and human exposures, and about hazard effects on animals themselves (NRC, 1991).

Chronic exposures to heavy metals have been associated with various effects on blood biochemistry (WHO, 1998; Silva et al., 1999; Liu et al., 2000), enzymes and transport proteins activity (Foulkes, 1996; Vogiatzis and Loumbourdis, 1998; Wright et al., 1998; Liu et al., 2000; Lopes et al., 2002), and on cells structure and function (Markovich and James, 1999). Most of these effects are yielded by hepatotoxic and nefrotoxic mechanisms causing severe histopathological alterations (Silva et al., 1999; Jeong et al., 2000; Liu et al., 2000). Therefore, tissue lesions are an important assessment endpoint for the effects induced by chronic exposure to heavy metals in natural conditions, a fact that is corroborated by Talmage and Walton (1991), Stehr et al. (1997) and Silva et al. (1999). Even when cellular responses are not toxic manifestations, such as degeneration or necrosis, but adaptive ones, they may be important indicators of exposure that should be assessed (Haschek and Rousseaux, 1998).

By obvious reasons, field conditions are always difficult to mimic in lab experiments, especially when wild organisms are exposed to complex mixtures of contaminants where synergistic and/or antagonistic effects are to be expected. Additionally, available models for exposure and bioaccumulation prediction can introduce considerable uncertainty into screening-level ecological risk assessment (Torres and Johnson, 2001). Thus, in order to overcome these constraints, wild rats (*Rattus rattus* L.) and Algerian mice (*Mus spretus* Lataste) were the species chosen in this study to assess an integrated exposure to a complex mixture of heavy metals, and subsequent toxic effects, in the vicinity of a derelict pyrite mine. These species satisfied the criteria to be considered good indicators, namely: (i) they have a large geographic distribution and can be found in both contaminated and non-contaminated areas; (ii) they may cohabit with humans and eat their food, which makes them potential indicators of human exposure; (iii) they are a component of some terrestrial ecosystems and occupy a middle position in many food chains; (iv) they contact with soil during their entire life cycle, being exposed to heavy metals mainly by ingestion of contaminated food or soil and through dermal absorption; (v) they have small home ranges, typically less than 90 m (MacDonald and Barret, 1993), which makes them appropriate site-specific indicators of contamination; and finally, (vi) their populations are usually large enough to support harvesting without a major adverse effect at the population level (NRC, 1991). Regarding the Algerian mouse, it has been widely used as a bioindicator species in some environmental studies performed in Portugal and Spain (Ruíz-Laguna et al., 2001; Nunes et al., 2001a, 2001b; Lopes et al., 2002).

The present study is integrated in the risk assessment process planned for the abandoned St. Domingos mine (Pereira et al., 2004). The specific objectives of this work were: (i) to assess the exposure of rats and Algerian mice to metals and arsenic present in the vicinity of the mine; (ii) to identify adverse effects, namely histopathological and weight changes, in the liver, kidney and spleen of rats and mice, and to compare them with exposure levels; (iii) to make some insights about the bioavailability of metals, to wild mammals, on the mining area; (iv) to discuss the suitability of histopathological features as an endpoint in wildlife and human health risk assessment in metal-contaminated scenarios, and (v) to evaluate the suitability of hair as a monitoring tool for assessing wild mammals exposure. For the matter, the constraints imposed by the European Union legislation in force (EC, 1986), regarding the protection of animals used for experimental and other scientific purposes, were considered in this study.

2. Material and methods

2.1. Study area

The St. Domingos mine is a cupric pyrite mine, located in Southeast Alentejo (Portugal), where tons of mining tails where left behind, exposed to atmospheric conditions (Fig. 1). The exploitation finished in 1965 without any attempt to take reclamation measures in order to prevent the aerial transportation of the finest particles and the secondary pyrite oxidation, with subsequent production of acidic conditions, favourable for heavy metals mobilization (Helms, 1993; Gerke et al., 1998). Acid mine drainage (AMD) has been responsible for serious threats to ecosystems since high concentrations of toxic metals were found in the aquatic compartment of the St. Domingos mine area (Pereira et al., 1995, 2000; Lopes et al., 1999). The impacts of AMD on aquatic organisms and communities have already been assessed with laboratory and in situ bioassays, and field studies (Canteiro, 1994; Lopes et al., 1999; Pereira et al., 1999a, 2000; Castro et al., 2003). Few researches have been performed in the terrestrial compartment of the mine area. Total metal concentrations, pH, organic matter, and conductivity, in surface soils, were assessed at different distances from the mine (Pereira et al., in press) (Table 1). Higher concentrations of As, Cu, Fe, Pb, and Zn were recorded in the mining area when compared to the north part of the mine (Fig. 1). As, Cd, Cr were above toxic levels in both areas, according to the soil quality criteria recommend by MEE and DEPA (1995). Cu and Pb overcame these criteria only in the mining area. However, the low pH values recorded by Pereira et al.

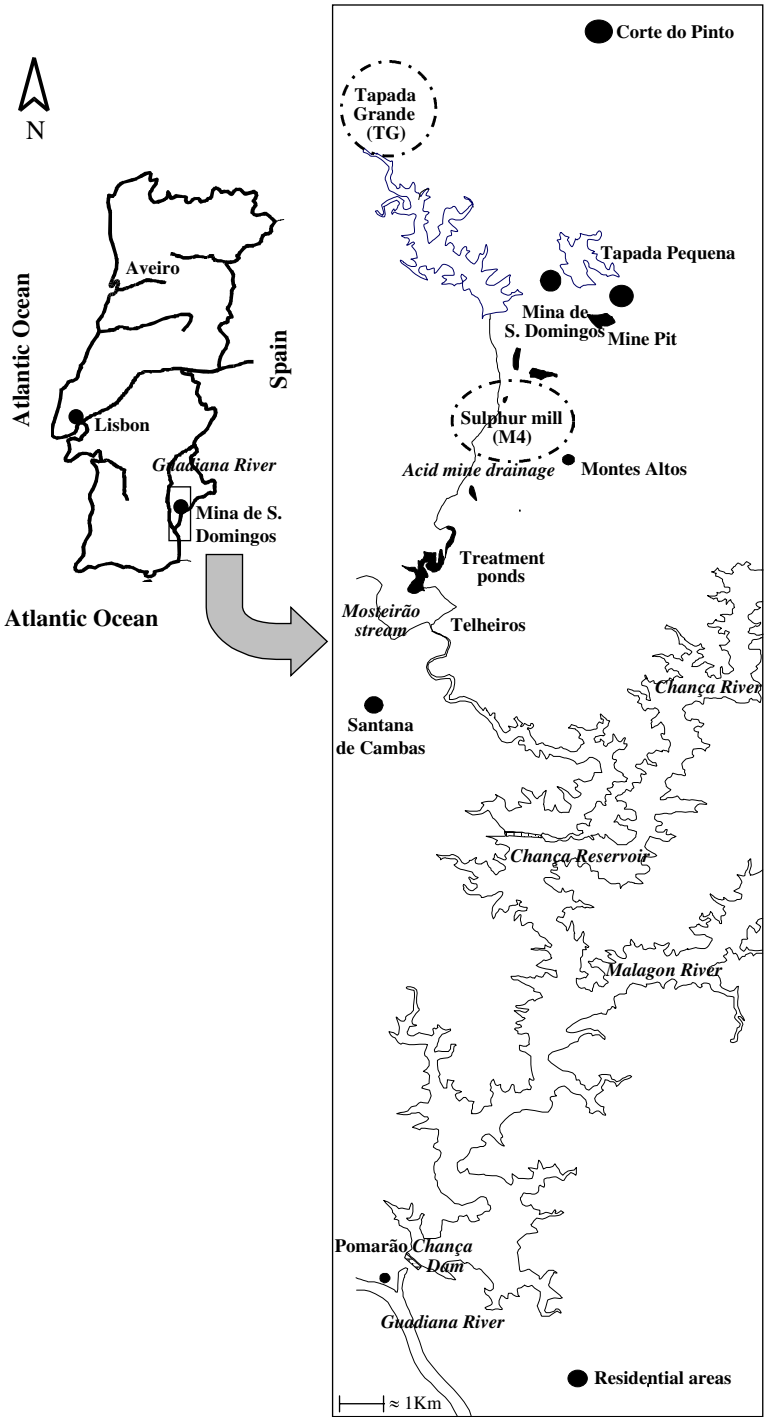

Fig. 1. Map of the study area located in Southeast Portugal.

(1999b, in press) suggested a potentially greater bioavailability of metals in the vicinity of mine. The assessment of soil enzyme activities in the St. Domingos mine area, to evaluate the effects of soil contaminants on its microbial community and subsequently on soil functions, also suggested a great bioavailability of metals in the mining area (Pereira et al., in press). A previous study on metal bioaccumulation (Pereira et al., 1999b) recorded higher levels of Cd (max. value 2.08 mg/kg wet weight) and Fe (max. value 647 mg/kg wet weight) in the liver of rats captured near the old sulphur mill (M4) than in those captured in the north part of the Tapada Grande lake (TG) (Fig. 1). In opposition, Zn concentrations (max. value 331 mg/kg wet weight) were higher in livers of rats captured at TG. The authors did not analyse other metals recorded in the soil and water compartments.

Table 1
Total As and metal concentrations (mg/kg), pH, conductivity (Cond., μS/cm) and organic matter (OM, %) at TG and M4 sites in the St. Domingos mine area (Pereira et al., in press)

	Fe	Cu	Cr	Zn	Pb	Ni	Mn	As	Cd	pH	Cond.	OM
TG	47,000	32	231	87	40	44	1608	20	3	6.4 ± 0.2	86.9 ± 67.0	7.68 ± 3.5
M4	49,000	281	114	108	1124	11	156	306	1	4.7 ± 0.7	74.7 ± 39.8	5.8 ± 1.8

pH, conductivity (Cond.) and organic matter (OM) – Average ± SD.

2.2. Sampling procedure

Small mammals were sampled at TG, 3 km apart from the mine pit, and M4, near the old sulphur mill (Fig. 1). The choice of these sites was supported by physical, chemical and soil microbial parameters, which gave some insights about the degree of soil contamination and toxic effects on soil functions (Pereira et al., in press). From April to July 2000, rats and mice were captured in three 5-day periods, using live traps baited with a mixture of canned tuna fish and children's pap. The trapped animals were taken alive to the laboratory, where they were anaesthetised and sacrificed according to legal procedures. Traps were placed on the field some days prior to the capture allowing animals to familiarise with the new object (Fig. 2).

After being weighted, the sex of each animal was determined and standard mammal body measurements were recorded. Liver, kidneys and spleen were dissected and weighed to the nearest 0.1 mg. Eye lenses were removed and preserved in a borate buffered 4% formaldehyde solution. As soon as possible, eye lenses were dried at 60 °C, until constant weight. The weight of eye lenses is considered a good criterion to assess mammals' age because its development is continuous during the animals' entire life by accumulation of insoluble proteins (Bourlière and Spi, 1975; Wheeler and King, 1980). Hair samples were also collected, from the inguinal and lumbar regions and stored in polyethylene bags, closed with a zipper. After collection, samples were immediately transported to the laboratory and stored at −20 °C, prior to analysis.

Fig. 2. Live traps in the field.

2.3. Sample preparation for light and electron microscopy

Small sections of liver, spleen and kidney were collected and fixed for 24 h in Bouin's fluid. Dehydration in graded concentrations of ethanol and sample inclusion in paraffin wax was sequentially performed. Sections (5–7 μm thick) were obtained and stained with hematoxylin–eosin for light microscopic examination. Observations were made and photographs taken using a microscope model Olympus BH2-RFC equipped with an automatic photomicrographic system Model PM-10ADS. The remaining portions of the organs were stored in polypropylene tubes at −20 °C until chemical analysis.

For electron microscopy, small fragments of liver were fixed with 2.5% glutaraldehyde in 0.1 M cacodilate buffer, and then washed in the same buffer. Specimens were postfixed in 2% buffered osmonium tetroxide, dehydrated through graded alcohols, and embedded in Epon 812 (158 EMS®). Thin sections were made with a diamond knife and double-stained with uranyl acetate (Merck®) and lead citrate (17800 EMS®). Observations were carried out using a Hitachi transmission electron microscope, operating at 100 kV.

2.4. Chemical analysis

Tissue samples were oven-dried at 105 °C until constant weight and dry weight was determined. Wet ashing was performed in a sand bath at approximately 60 °C, in closed 50-ml polypropylene tubes using the HNO_3 (extrapure, Merck®)/H_2O_2 (proanalysis, Merck®) procedure. Hydrogen peroxide was added to the samples in 1-ml aliquots until they were clear. The samples were diluted to a final volume with Milli-Q® water obtained from an ultrapure water System (Ultra Clear SG®). Wet ashing was considered an appropriate procedure for the preparation of biological samples for chemical analysis, since it is performed at a relatively low temperature reducing the possibility of analytes loss, especially the most volatile ones (Miller-Ihli, 1989; Hoening and Kesabiec, 1996).

Before chemical analysis, hair samples were cut into small pieces (2–3 mm) and washed five times, according to the method proposed by IAEA (1977 in Subramanian, 1996), following the sequence: acetone–Milli-Q® water–Milli-Q® water–Milli-Q® water–acetone. Hair samples were immersed in 25 ml of acetone or water,

each time, during 20 min with magnetic stirring. Washed samples were oven-dried at 60 °C, during 24 h, and then weighed. The washing step is controversial (Bozsai, 1992; Subramanian, 1996), but it is crucial for the analysis of hair samples in the removal of exogenous dirt.

A GBC Model 932 Plus Atomic Absorption Spectrometer (AAS) was used for flame atomic absorption analysis of Fe, Mg and Zn. For Graphite Furnace analysis of As, Cd, Cr, Cu, Mn, Ni, and Pb, this apparatus was equipped with a GBC-GF3000 Graphite Furnace, deuterium background correction and a furnace autosampler PAL 3000. Standards were prepared using stock solutions Specpur® 1000 μg/ml (Alfa Aesar®, Johnson Matthey Company). Dilutions were made using HNO_3 at 2%. For each element, three standards were used to cover the analytical working range of the instrument. The standard addition method was performed in some spare hair and bovine liver and kidney samples. Recoveries of know amounts of trace elements added to the samples varied between 82 and 108%.

$Ni(NO_3)_2$/$Mg(NO_3)_2$ and diammonium hydrogen phosphate (1%) were used as matrix modifiers to thermally stabilise arsenic and lead, respectively (Krynitsky, 1987).

2.5. Statistical analysis

For the comparison of trace element concentrations between different groups of individuals, one-way ANOVAs were performed. In order to meet the assumptions of this statistical procedure, data were transformed using the equation: $x' = \log(x + 1)$ (Zar, 1996). Ratios were transformed by the equation, $x' = \text{arcsen}(x)$. Pearson's correlation coefficients (r) were calculated to investigate associations between heavy metals and As concentrations recorded in the different tissues and hair. For each element, associations among the concentrations found in the different tissues were also analysed by the same statistical procedure. Statistical analyses were performed with transformed data after normality and the homogeneity of variances have been confirmed. Raw data are presented in Tables 2–5, in order to facilitate comparison with data reported by other authors.

3. Results

3.1. Tissue residues analysis

All the wild rats were males, except one, and all the mice were females, which reduced the variability associated with sex. No significant differences were found ($p > 0.05$) in the body parameters recorded (Table 2), between animals captured at the TG and M4 sites, in either species. An exception was observed in rats from M4, which had significantly lighter spleens ($F = 6.522$, d.f. = 8, $p = 0.038$). Additionally, no significant differences were found between relative weights (organ weight/body weight) of eye lenses, liver, kidney, and spleen for rats and mice from both sites.

Table 2
Body and organs weight and corresponding ratios from rats and mice captured at M4 and TG sites

	M4		TG	
	Average ± SD	*n*	Average ± SD	*n*
Rattus rattus				
Body weight (bw)	87.469 ± 47.56	5	98.959 ± 64.87	4
Kidneys wt (average)	0.361 ± 0.19	5	0.380 ± 0.23	4
Kidneys wt/bw	0.005 ± 0.002	5	0.004 ± 0.0	4
Liver wt	5.154 ± 3.59	5	6.092 ± 1.99	4
Liver wt/bw	0.057 ± 0.02	5	0.062 ± 0.001	4
Spleen wt	0.101 ± 0.07[a]	5	0.237 ± 0.09[a]	4
Spleen wt/bw	0.002 ± 0.001	5	0.003 ± 0.001	4
Mus spretus				
Body weight (bw)	21.236 ± 4.32	8	18.571 ± 7.32	7
Kidneys wt (average)	0.156 ± 0.04	8	0.181 ± 0.12	7
Kidney wt/bw	0.007 ± 0.002	8	0.013 ± 0.02	7
Liver wt	1.006 ± 0.30	8	0.889 ± 0.34	7
Liver wt/bw	0.047 ± 0.01	8	0.049 ± 0.01	7
Spleen wt	0.023 ± 0.01	8	0.021 ± 0.01	7
Spleen wt/bw	0.001 ± 0.0	8	0.001 ± 0.001	7

bw, body weight (g); wt, wet weight (g).
[a] Statistically significant differences between sites.

The heavy metal and As concentrations were recorded in the liver, kidney and spleen of both species (Tables 3 and 4). The liver and spleen were the target organs for essential elements such as Fe, Mg and Zn. The average level of Mg was significantly higher in the liver of rats from M4 ($F = 201.349$, d.f. = 8, $p < 0.001$) than in those from TG, while the opposite was observed for Fe and Zn (Fe: $F = 10.229$, d.f. = 8, $p = 0.015$; Zn: $F = 37.510$, d.f. = 8, $p < 0.001$). Although no significant differences were recorded between sites, the highest average concentration of Fe was recorded in the spleen of rats from M4 (Table 3). The average concentration of Mg in the kidneys was also significantly higher in rats from M4 ($F = 5.932$, d.f. = 8, $p = 0.045$). Considering non-essential elements, the spleen of rats from M4 was also the target organ for the bioaccumulation of As ($F = 9.528$, d.f. = 8, $p = 0.018$) (Table 3).

In the liver of Algerian mice, the average levels of Mg ($F = 9.501$, d.f. = 14, $p = 0.009$) and Fe ($F = 7.690$, d.f. = 14, $p = 0.016$) were significantly higher at M4 than at TG (Table 4). Similar to rats, the highest average concentration of Fe was recorded in the spleen of mice from M4, although no significant differences were recorded (Table 4). The average concentration of Mg in the kidneys was also significantly higher in the mice from M4 ($F = 8.381$, d.f. = 14, $p = 0.013$). The kidneys of mice from M4 presented significantly higher average concentrations of As ($F = 9.530$, d.f. = 13, $p = 0.019$) and Cd ($F = 5.633$, d.f. = 14, $p = 0.034$) (Table 4).

Significantly higher concentrations of As were found in hair of both species from M4 than from TG

Table 3
Concentrations of metals and As on rats from the TG and M4 sites

	Liver		Kidney		Spleen	
	Average ± SEM	*n*	Average ± SEM	*n*	Average ± SEM	*n*
Cr						
TG	0.513 ± 0.221	4	0.345 ± 0.051*	4	1.231 ± 0.332	4
M4	0.117 ± 0.029	5	0.072 ± 0.012*	5	0.185	1
Cu						
TG	12.147 ± 0.956	4	13.998 ± 3.896	4	11.095 ± 2.261	3
M4	15.073 ± 4.320	5	22.104 ± 3.296	5	14.007 ± 2.679	3
Fe						
TG	316.811 ± 31.112*	4	292.538 ± 90.770	4	747.448 ± 251.428	4
M4	189.543 ± 25.510*	5	164.506 ± 24.856	5	1863.135 ± 845.696	4
Mg						
TG	0.016 ± 0.003*	4	0.029 ± 0.011*	5	0.05 ± 0.022	5
M4	0.382 ± 0.02*	5	0.214 ± 0.066*	4	0.353 ± 0.111	4
Ni						
TG	0.060 ± 0.021	4	0.060 ± 0.021	4	0.841 ± 0.301	4
M4	0.266 ± 0.142	5	0.065 ± 0.020	5	0.577 ± 0.330	5
Zn						
TG	0.141 ± 0.014*	4	0.107 ± 0.03	4	0.06 ± 0.015*	4
M4	0.048 ± 0.008*	5	0.076 ± 0.008	5	0.120 ± 0.012*	5
As						
TG	0.003 ± 0.003	4	1.041 ± 0.342	4	0.678 ± 0.309*	4
M4	12.733 ± 9.915	5	2.198 ± 0.602	5	9.993 ± 2.651*	5
Cd						
TG	0.381 ± 0.025	4	0.409 ± 0.076	4	0.506 ± 0.163	4
M4	0.320 ± 0.140	5	1.275 ± 0.489	5	0.747 ± 0.089	3
Pb[a]						
TG	–		0.643	1	2.706	1
M4	5.953	1	3.344	1	16.592–82.745	2

Only concentrations above the detection limit were presented. Fe, Mg and Zn concentrations expressed as mg/g, dry weight. As, Cu, Cd, Cr, Ni and Pb concentrations expressed as μg/g, dry weight.

* Statistically significant difference ($p<0.05$).

[a] Only concentration ranges are presented.

(rats: $F = 59.432$, d.f. = 8, $p < 0.001$; mice: $F = 26.188$, d.f. = 13, $p < 0.001$). The concentrations of Ni ($F = 22.627$, d.f. = 8, $p = 0.002$), Cr ($F = 35.592$, d.f. = 8, $p < 0.001$) and Cu ($F = 22.452$, d.f. = 8, $p = 0.002$) were significantly higher in the hair of rats from TG. Although no significant differences were found, the highest average concentration of Cd was recorded in animals from M4. This observation meets the data obtained for liver and kidney samples (Tables 3–5). These data suggested that hair can only be used as an indicator of metal levels in the kidney ($r = 0.826$; $n = 9$; $p < 0.01$) and liver ($r = 0.697$; $n = 9$; $p = 0.037$) of rats for Cr. However, data for the concentrations of As in the hair of both species were significantly higher in animals from M4, which was in agreement with data from the analysed organs. Lead concentrations were above the detection limit in too few samples to make appropriate comparisons and, therefore, only ranges are presented in Tables 3 and 4.

Interspecies comparisons revealed significant differences between the liver concentrations of Fe ($F = 8.027$, $p = 0.015$), Cu ($F = 5.254$, $p = 0.043$) and Zn ($F = 12.568$, $p = 0.005$). The same was observed for the hair concentrations of Zn ($F = 12.508$, $p = 0.005$), Cu ($F = 8.221$, $p = 0.015$) and Ni ($F = 5.819$, $p = 0.034$). The highest average concentrations were always recorded in Algerian mice.

Significant positive associations were recorded between non-essential and essential elements in the liver of rats (As/Zn: $r = 0.692$, $p = 0.0387$) and mice (Cd/Fe: $r = 0.651$, $p = 0.009$; As/Mg: $r = 0.543$, $p = 0.045$). Although stronger, the same kind of associations was recorded in the spleen of rats (As/Mg: $r = 0.841$, $p = 0.008$) and mice (Cd/Mg: $r = 0.710$, $p = 0.022$). Interactions between essential elements in the liver, kidney and hair were diverse and difficult to predict (Table 6).

3.2. Histopathological analysis

The liver of rats from M4 demonstrated a strong hepatocyte vacuolation. The natural arrangement of these cells in cords was also visibly disturbed, in opposition to the liver of the rats from TG (Fig. 3a and b). There were no signs of morphological injury in the hepatic portal vein of animals from either site (Fig. 3b). Liver specimens from all rats captured at M4 had similar histological changes, except for the liver of one rat that also showed signs of haemorrhage. The ultrastructural observation of rats' hepatocytes confirmed the presence

Table 4
Concentrations of metals and As on mice from TG and M4 sites

	Liver		Kidney		Spleen	
	Average ± SEM	*n*	Average ± SEM	*n*	Average ± SEM	*n*
Cr						
TG	0.361 ± 0.068	7	1.441 ± 0.343	7	14.921 ± 6.127	7
M4	0.651 ± 0.238	8	0.985 ± 0.361	8	8.357 ± 2.961	5
Cu						
TG	38.623 ± 7.796	7	26.059 ± 6.145	7	6.577 ± 3.100*	6
M4	29.034 ± 3.969	8	47.781 ± 13.104	8	21.831 ± 4.670*	6
Fe						
TG	333.456 ± 53.518*	7	219.024 ± 52.043	7	–	–
M4	681.185 ± 126.299*	8	280.537 ± 38.004	8	1919.311 ± 2061.786	4
Mg						
TG	0.250 ± 0.071*	7	0.306 ± 0.061*	7	0.318 ± 0.140*	7
M4	0.548 ± 0.066*	8	0.517 ± 0.122*	8	0.767 ± 0.124*	5
Ni						
TG	0.186 ± 0.073	7	0.359 ± 0.092	7	0.758	1
M4	0.561 ± 0.237	8	2.175 ± 0.823	8	12.526 ± 4.423	7
Zn						
TG	*0.108 ± 0.022*	7	0.093 ± 0.007	7	0.137 ± 0.096*	7
M4	0.094 ± 0.009	8	0.112 ± 0.027	8	1.340 ± 1.167*	3
As						
TG	0.148 ± 0.026	6	0.861 ± 0.267*	6	1.734 ± 0.608	6
M4	1.792 ± 0.946	8	3.887 ± 0.817*	8	–	–
Cd						
TG	0.293 ± 0.087	7	0.379 ± 0.119*	7	0.270 ± 0.042	7
M4	1.014 ± 0.337	8	1.122 ± 0.273*	8	1.191 ± 0.639	8
Pb[a]						
TG	2.562	1	6.316–6.738	2	–	–
M4	0.869	1	19.555	1	–	–

Only concentrations above the detection limit were presented. Fe, Mg and Zn concentrations expressed as mg/g, dry weight. As, Cu, Cd, Cr, Ni and Pb concentrations expressed as μg/g, dry weight.
* Statistically significant difference ($p < 0.05$).
[a] Only concentration ranges are presented.

of large vacuoles in the cytoplasm (Fig. 4). Although less pronounced, hepatocyte vacuolation and an abnormal arrangement of epithelium cells were also observed in the Algerian mice from M4, in opposition to those from TG (Fig. 5a and b).

The kidneys of mice from M4 showed glomerulla swelling (Fig. 6a and b). No lesions were observed in the kidneys of rats from either site (data not shown).

The observation of histological sections of spleen showed cell depletion near the fibrocollagenous capsule in rats from both sites (Fig. 7a and b). However, in the spleen of rats from the vicinity of the mine the distinction between red and white pulp was not clear and capsule thickness increased. Regarding the Algerian mice, the most pronounced histopathological features observed in animals from M4 were a thick fibrocollagenous capsule and the lack of cells in the periphery of the organ (Fig. 8a and b).

4. Discussion

Except for the spleen of rats, the results obtained for body parameters contradict those obtained by other authors (e.g., Halbrook et al., 1993; Shore and Douben, 1994; Nunes et al., 2001b), with wild and laboratory mammals, which usually observe reductions in the weight of body and organs of animals exposed to environmental contaminants. Still, as it was pointed out by Nunes et al. (2001b), reductions recorded in body measurements and traits of wild mammals should not be directly attributed to contaminants, because resources may be available differently between contaminated and uncontaminated areas, leading to a differential growth rate of local populations. Spleens' atrophy may explain the decrease in weight recorded for this organ in rats from M4, when compared to those from TG.

The selection of a reference site is often the most difficult task in field studies because it should present the same physical and chemical properties, when compared to the contaminated sites, and simultaneously demonstrate low toxicity of metals and other contaminants (Indeherberg et al., 1998). In the St. Domingos mine risk assessment process this problem also occurred, because this area is located in the Iberian Pyrite Belt, a metallogenic province that crosses the Southeast Alentejo, towards Spain (Webb, 1958). Therefore, high concentrations of heavy metals are to be expected even at

Table 5
Concentrations of metals and As in the hair of rats and mice from TG and M4

	Rattus rattus		*Mus spretus*	
	Average ± SEM	*n*	Average ± SEM	*n*
Cr				
TG	1.309 ± 0.197*	4	1.673 ± 0.618	7
M4	0.232 ± 0.066*	5	0.579 ± 0.165	8
Cu				
TG	29.272 ± 10.254*	4	10.475 ± 3.081	7
M4	3.856 ± 0.756*	5	21.385 ± 4.731	8
Fe				
TG	324.858 ± 155.768	4	109.269 ± 51.304	7
M4	88.362 ± 43.991	5	97.388 ± 28.117	8
Mg				
TG	0.071 ± 0.008	4	0.481 ± 0.103	7
M4	0.296 ± 0.125	5	0.489 ± 0.079	8
Ni				
TG	0.178 ± 0.024*	4	0.283 ± 0.073	7
M4	0.069 ± 0.008*	5	0.701 ± 0.204	8
Zn				
TG	0.160 ± 0.042	4	0.257 ± 0.032	7
M4	0.095 ± 0.028	5	0.257 ± 0.042	8
As				
TG	0.336 ± 0.203*	4	0.228 ± 0.051*	7
M4	2.456 ± 0.184*	5	3.497 ± 0.546*	8
Cd				
TG	0.228 ± 0.027	4	0.227 ± 0.052	7
M4	0.401 ± 0.214	5	0.432 ± 0.083	8
Pb[a]				
TG	0.031–4.299	2	–	–
M4	19.245–61.290	2	0.460–0.946	2

Only concentrations above the detection limit were presented. Fe, Mg and Zn concentrations expressed as mg/g, dry weight. As, Cu, Cd, Cr, Ni and Pb concentrations expressed as μg/g, dry weight.
* Statistically significant difference ($p<0.05$).
[a] Only concentration ranges are presented.

Table 6
Pearson correlation coefficients for the relationships between elements in the organs of the Algerian mice and rats from the St. Domingos mine area

Rattus rattus		*Mus spretus*	
Liver		Liver	
Fe/Mg	−0.764*	Fe/Mg	0.523*
Fe/Zn	−0.757*	Cr/Ni	0.737**
Mg/Zn	0.924***	As/Mg	0.543*
As/Zn	0.692*	Cd/Fe	0.651**
Kidney		Kidney	
Cr/Mg	−0.693*	Cu/Ni	0.748***
		Cu/Zn	0.600*
Spleen		Spleen	
As/Mg	0.841**	Cd/Mg	0.710*
Hair		Hair	
Fe/Cu	0.930***	Cd/Ni	0.685**
Cr/Ni	0.954***		
Cr/As	−0.945***		
Ni/As	−0.876**		

*$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$.

a great distance from the mine but, those concentrations may not reflect heavy metals toxicity because it depends on their availability, which in turn is determined by several different physical and chemical factors (Esnaola and Millán, 1998; Rieuwerts et al., 1998). Although different methods have been proposed for the evaluation of metals availability in soils (e.g. Sterckeman et al., 1996; Esnaola and Millán, 1998), in situations of environmental contamination, biomonitoring is usually a powerful tool since it can provide valuable information about hazards, their bioavailability and subsequent impact on biota (Talmage and Walton, 1991). In the St. Domingos mine area significantly higher levels of As and Cd were recorded in the kidney of mice from the M4 site when compared to those captured at TG, which suggested the great bioavailability of these elements in the mining area. The same was observed for rats but no significant differences were found among animals from both sites. Arsenical compounds can be present in the organism and the kidney is the major organ for As elimination from the body, which contributes in a great extent for the susceptibility of this organ to As exposures (WHO, 1981; Hunder et al., 1999; Liu et al., 2000). The toxicity of As in mammals was found to be related with levels above 3 μg/g in the liver and kidney (Gupta, 1998) and animal data suggest that As exposure may have chronic effects on the kidneys (WHO, 1981). Liu et al. (2000) recorded that glomerular swelling is one of the degenerative changes that usually occur in mice chronically exposed to As. These changes were also observed in mice from M4. Although no positive correlation between the levels of Cu and As was found in the kidney of mice, the presence of high levels of both elements was recorded in animals captured at M4. Different authors (e.g., Hunder et al., 1999; Ademuyiwa and Elsenhans, 2000) have reported this finding in the kidney of rats, which is probably due to a functional relationship regarding the renal retention of these elements. The lack of a positive correlation in our study may result from interactions with other elements.

Both kidney and liver have been mentioned as the preferential organs for Cd bioaccumulation (Leita et al., 1991; Talmage and Walton, 1991; Alonso et al., 2002) and, once more, we have confirmed this fact for the Algerian mice. The kidney is the organ that first attains its critical concentration of Cd in chronic exposures, and histopathological changes are some of the early symptoms (Świergosz-Kowalewska, 2001). Glomerular dysfunction was also reported as one of the effects on the kidney yielded by long-term exposure to Cd (WHO, 1992). Regarding Pb, although its levels were recorded in very few samples, suggesting that small mammals were not exposed to this element, it should not be ignored that 90% of the total body burden of Pb is found in bone (Talmage and Walton, 1991). Therefore, the biological samples analysed were not the most

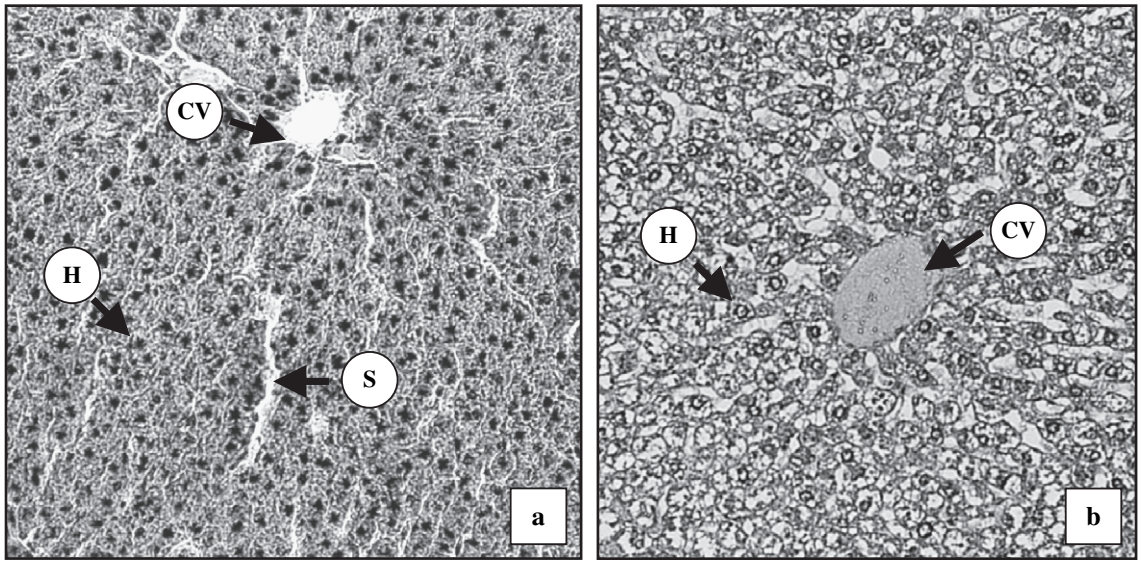

Fig. 3. (a) Liver from a rat captured at TG without signs of morphological injury at the central vein (CV) and in the sinusoidal channels (S) (100×). Epithelia showed normal features; (b) liver from a rat captured at M4 displaying strong hepatocyte (H) vacuolation (100×).

appropriate to assess chronic low level exposures. Additionally, Shore and Douben (1994) have already observed the small capacity of wild rats to bioaccumulate Pb in contaminated environments.

Chromium was mainly recorded in the organs (except for the liver) of rats and mice from TG. This met the data obtained for soil samples, since, the concentration of this metal in TG was about 2-fold what was recorded in M4 (Pereira et al., in press) and it was above the toxic level defined for the soil compartment (MEE and DEPA, 1995). Similar to the observations in our study, Talmage and Walton (1991), in their review of several studies performed with wild mammals, also observed that the bioaccumulation of this metal is frequently higher in reference sites. These observations may be explained by competition mechanisms between essential and non-essential elements regarding metal enzyme complexes. The opposite was observed in the liver of Algerian mice from M4 which bioaccumulated the highest levels of Cr. The strong positive association found between Cr and Ni ($r = 0.737, p = 0.0017$) (Table 6) could have been responsible for such an aspect. Therefore, analogous mechanisms of bioaccumulation for both elements, in the liver of Algerian mice, could explain the high levels of Cr recorded in this organ in opposition to what was observed in kidney and spleen.

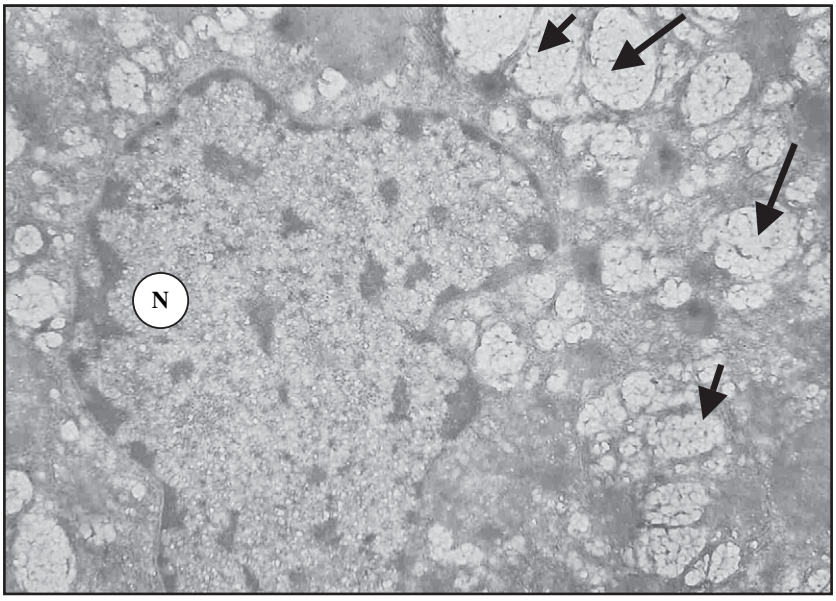

Fig. 4. Ultrastructural detail of a hepatocyte of a rat captured at M4 (77,000×). Arrows show strong vacuolation of cytoplasm; nucleus (N).

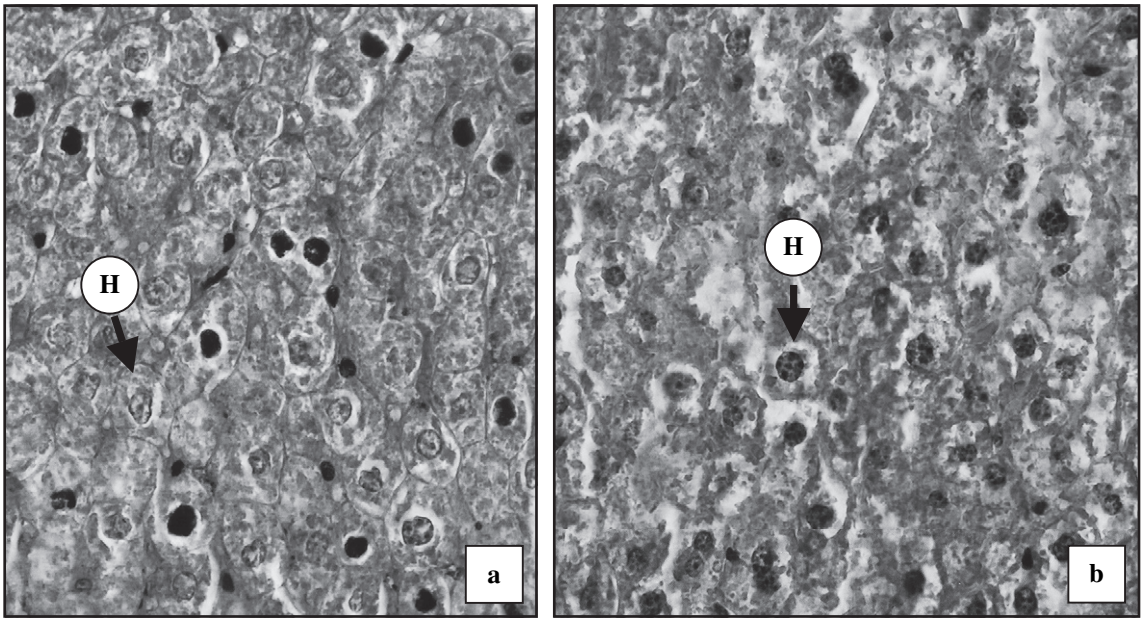

Fig. 5. Liver from Algerian mice captured in St. Domingos mine area. (a) TG (400×); (b) M4 (400×). Arrows show hepatocytes (H).

The liver of rats and mice was the main target organ for some essential elements and which agrees with the role of this organ in homeostatic mechanisms. The liver is the major organ primarily exposed to toxic and essential substances that enter the organism through inhalation or ingestion. Its main blood supply comes from the intestines (McCuskey and Sipes, 1997; Haschek and Rousseaux, 1998). The liver extracts metals from plasma, metabolises, stores and redistributes them in various forms either into the bile or back into the blood stream (Blázovics et al., 2002). The highest levels of Cu, Zn and Fe recorded in the liver of the Algerian mice, from the St. Domingos mine area (TG and M4 sites), were similar to those recorded by Lopes et al. (2002) in mice from the same species captured in a non-contaminated area. The low levels of essential elements recorded in these wild mammals can be explained by the exposure to a mixture of contaminants and the subsequent interactions between them. In spite of the significant associations found for the liver among different essential trace elements (Table 6), these data require confirmation. Several significant positive associations between essential elements and As and Cd were also recorded. When compared with the levels recorded in the other organs, the Fe contents in the spleen of both species were the highest, which results from the main functions of spleen, namely the destruction of aged or damaged red blood cells and the storage of Fe as ferritin or hemosiderin for future recycling (Ross et al., 1995; Junqueira and Carneiro, 1999). The histological changes

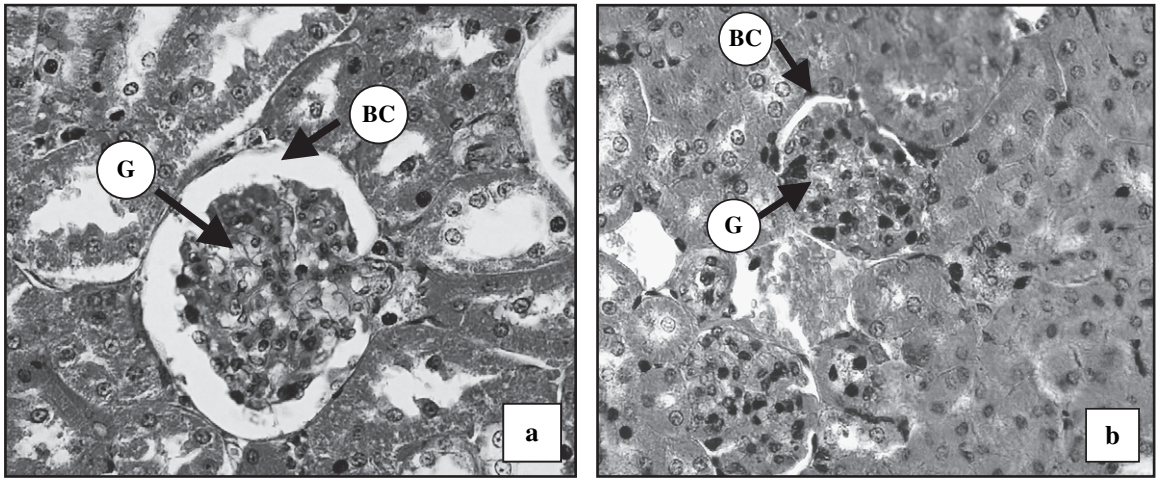

Fig. 6. Kidney from Algerian mice captured in St. Domingos mine area. (a) TG (400×); (b) M4. Arrows show glomerulus (G) and Bowman capsule (BC).

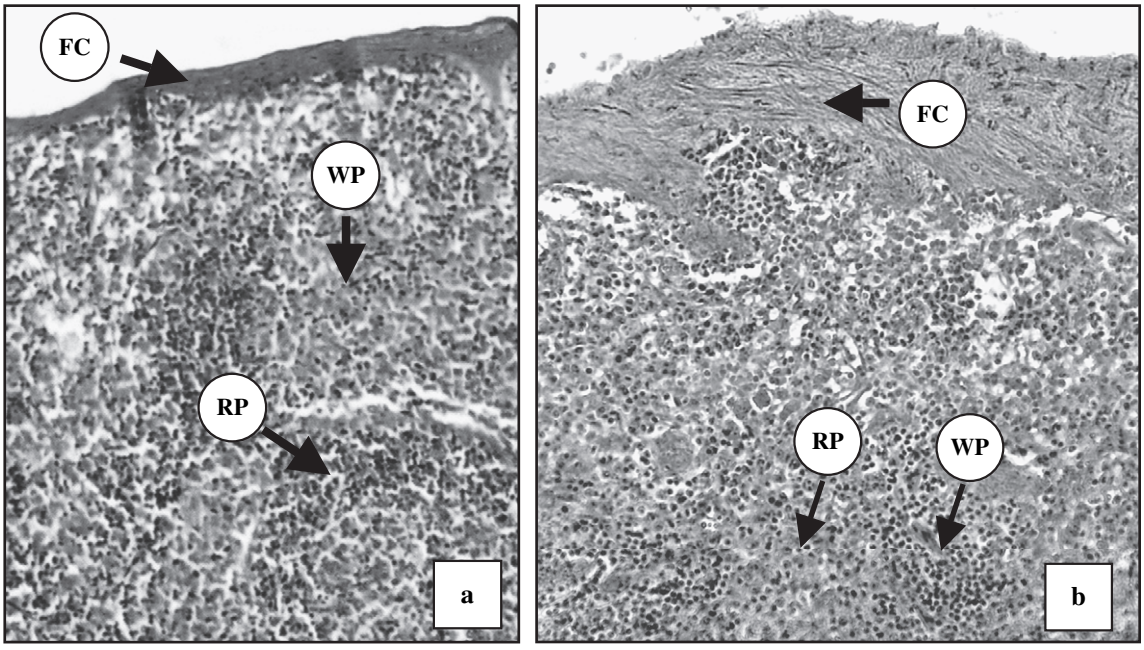

Fig. 7. Spleen from rats captured at St. Domingos mine area. (a) TG (100×); (b) M4 (100×). Arrows show the fibrocollagenous capsule (FC), the red (RP) and the white pulp (WP).

recorded suggested that these and other functions (e.g., the filtration of antigens circulating in the blood, and subsequent immunological responses) may have been impaired in the spleen of rats from M4 because it presented significant high levels of As.

The lack of significant differences regarding the concentration of some metals in the different organs results mainly from the great variability in data. In our study, variability associated with sex and age was reduced. Eye lens wt/bw ratios are an indicator of mammal's age (Bourlière and Spi, 1975). The absence of significant differences between ratios, calculated for animals collected in the two sampling sites, suggested that they have similar ages. For the Algerian mice this is also supported by the work of Mira and Mathias (1996). According to these authors, animals more than 8 g in weight are considered adults. Yet, the reduced number of individuals captured and the likely intermittent exposure of wild rodents in their habitat, due to avoidance behaviours (Shore and Douben, 1994), may also have

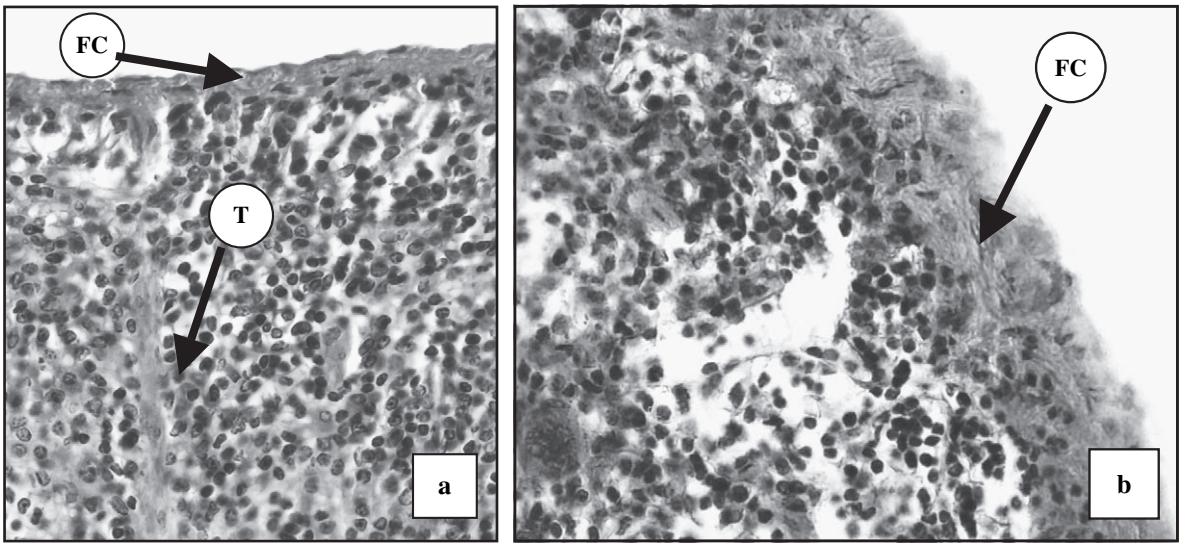

Fig. 8. Spleen from Algerian mice captured in St. Domingos mine area. (a) TG (400×); (b) M4. Arrows show trabecula, (T) and fibrocollagenous capsule (FC).

contributed for data variability. The number of individuals will always be a legal and an economic constraint in this kind of studies with wild mammal species.

Hepatocyte vacuolation observed in animals from M4, but more pronounced in rats, could result from the accumulation of lipids, glycogen or water, which in turn are indicative of disturbances in lipid metabolism, impairment of enzymes involved in the catabolism of glycogen or alterations in structure and permeability of membranes, respectively. All of these effects have been attributed to toxic substances, metals included (Foulkes, 1996; Haschek and Rousseaux, 1998). Severe histological changes (focal necrosis of parenchyma cells, apoptosis, multinucleated giant cells and acute inflammations in the parenchyma portal tracts and around central veins, proliferating nodules and subacute peritonitis) reported by other authors (Habeebu et al., 2000) in animals chronically exposed to Cd, were not observed in rats and mice captured at M4. However, until now few studies have focused on the effects of mixtures of heavy metals. Additionally, there are several cellular detoxification mechanisms, such as the induction of synthesis of proteins like metallothionein, hepatic glutathione and ferritin that could be particularly developed in wild mammals, protecting them from toxic effects of both essential and non-essential metals (Cherian and Ferguson, 1997; Habeebu et al., 2000; Liu et al., 2000). Metallothionein is a family of proteins with a molecular weight of approximately 6.5 kDa, rich in cystein and with seven metal atoms binding to them (Cherian and Ferguson, 1997; Nordberg and Nordberg, 2000). These proteins have been identified in almost all animal *phyla*, from invertebrates to humans, and may be found in many mammal organs where they take part in toxicokinetics and biochemistry of essential and toxic metals, such as Cd, Cu and Zn (Henry et al., 1994; Cherian and Ferguson, 1997; Nordberg and Nordberg, 2000). Thus, intracellular accumulation of metals such as Cd in the liver and the kidney may be explained by their sequestration through metallothionein binding, whose transcription seems to be triggered by the presence of metals in these organs (Cherian and Ferguson, 1997; Storelli et al., 1999). Satarug et al. (2000) recorded a 100-fold increase in hepatic metallothionein levels of Wistar rats after the administration of a low dose of Cd (10 μmol/kg) or a high dose of Zn (200 μmol/kg). Several studies also provided evidence of the protective role of metallothionein proteins in renal tubule cells from toxic injury produced by that metal (WHO, 1992). Even though, mechanisms are not yet clearly understood, hepatic glutathione is a major cellular antioxidant and also performs a variety of protective functions, forming complexes with heavy metals and subsequently enhancing their excretion (Vogiatzis and Loumbourdis, 1998). Lopes et al. (2002) found a great activity of glutathione-*S*-transferases, the enzyme that catabolizes the development of these complexes, in the liver of the Algerian mice, when compared with other wild mammal species. This enzymatic mechanism may also have contributed to reducing the effects on the liver of Algerian mice captured at M4. In brief, the presence of high levels of residues in the different organs may be indicative of exposure, but not of biological effects, since these metals may be complexated in non-toxic forms.

Similar to what happened in other studies, interspecies differences in the bioaccumulation of metals were observed in our study. These differences can be explained by physiological differences, foraging behaviour and differences in foodstuffs, with respect to the bioavailability of elements, or patterns of habitat use (Hickey et al., 2001). The Algerian mouse has proved to be a strong bioaccumulator of essential and non-essential elements. Both species are omnivorous, but rats have more diversified feeding preferences, which may have contributed to a reduced exposure. According to the findings of Torres and Johnson (2001), for a species from the genus *Mus*, arthropods and incidentally ingested soil appeared to be the most significant sources of ingested metals.

Although pronounced histopathological effects were observed in both species, those recorded in the kidney and spleen of the Algerian mice from M4 co-occurred with the presence of high levels of essential and non-essential elements in those organs, especially the most concerning ones (Cd and As). The consistency of the results recorded for both species suggested that histopathological parameters are good endpoints to assess the effects of the exposure to environments contaminated with heavy metals.

The suitability of hair samples as a biological material to assess environmental exposures was also evaluated. The choice of this type of non-invasive method would be very interesting because they are easy to obtain, transport, store and their collection does not require the animals' sacrifice. However, hair samples per se did not allow great conclusions, except for Cr and As. For these elements (especially for Cr) hair may be used to biomonitor the levels in the liver and kidney. The hair is an important excretion pathway of As from the body (WHO, 1981). As a consequence, As may contribute in removing other metals from their binding sites in the hair structure, as it was demonstrated by the significant negative association between As/Cr ($r = -0.945$, $p < 0.001$) and As/Ni ($r = -0.876$, $p = 0.002$) in the hair of rats (Table 6). In opposition to the data obtained for the liver and the kidney, hair samples were useful to confirm the exposure of rats to As and, subsequently, the bioavailability of this element in the M4 site. This conclusion was also supported by the significantly higher levels of As bioaccumulated in the spleen of rats from the M4.

In summary, our study has proved that small rodents inhabiting in St. Domingos mine area are exposed to

different essential and non-essential metals at once, particularly As and Cd. The kidney was the major target organ for the bioaccumulation of both elements and signs of renal toxicity were shown by the histopathological analysis of mice captured near the mining area. However, since high levels of bioaccumulated residues may only reflect the ability to store contaminants in a non-toxic form, more studies are necessary to confirm the cause–effect relationship suggested. This evaluation can be performed exposing Algerian mice to contaminated soil in microcosm field experiments. Within risk assessment studies we believe that it is more important to assess the effects yielded by the exposure to the complex mixture of metals, present at the area, than to evaluate the role that each metal plays on the development of such effects through laboratory tests with individual contaminants. According to our data, Cd and As were the most bioavailable for either species at M4. Although animals from the TG did not present histological alterations in the organs analysed, some essential elements were recorded at higher concentrations in animals captured at this site. Therefore, it would be advisable to choose another sampling site, at a greater distance from the mine, towards the North, as a reference site. However, high concentrations of essential metals will probably be found at that site too, on the soil and biota compartments, because St. Domingos mine is located in one of the greatest European metallogenic provinces, the Iberian Pyrite Belt. This study also gave rise to the suspicion of the potential exposure of livestock, which usually grazes on the area, and to the subsequent exposure of local inhabitants. Some important wildlife species, from the Algerian mouse food webs, may also be exposed to heavy metals in the St. Domingos mine area.

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