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Metal contamination in vertebrates from the Tjan-Shan mountains

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Abstract. More than a half of the area of Kyrgyzstan is covered by high mountains. These receive air pollutants transported on long distances by general air circulation. In our study we collected and analysed bone samples from the Kyrgyz Mountains by XRF spectrophotometry. We found northern valleys to be the most contaminated. This might be due to contaminants coming probably from the north. Moreover, we found some western valleys contaminated too, which might be caused by western flows of general atmospheric circulation. We also found more polluted localities in higher altitudes and humid areas with a high level of precipitations. Analysing the occurrence of heavy metals in different parts of skeleton, we found out that they tend to be stored mostly in teeth. No significant correlation between a heavy metal bone compound and animal domestication was found. We discovered that concentrations of P, Ca, Fe, Zn, I and Sr are higher in domestic than in wild animals, which is probably due to their richer diet.

Key words: heavy metals, bone contamination, Kyrgyzstan, wild and domestic animals, XRF spectrophotometry

Introduction

The territory of Kyrgyzstan is mainly represented by mountain masses (about 65%), which, by preventing the penetration of cyclones and anticyclones, serve as a natural barrier for air pollutants (Aidaraliev *et al.* 2002). Knowing that these pollutants tend to be deposited in high altitude mountain areas (Zechmeister 1994), it was necessary to do research on this topic, as only very little is known about the environment pollution in Kyrgyz mountains. During recent years there has been a project running concerning the water quality in this area. There were many studies done thanks to this project dealing mainly with the heavy metal pollution of waters, soils, mosses, and finally of animals by analysing the found bones. The environmental contamination is nowadays a serious issue. Most of the sources of heavy metals are of anthropogenic origin from our industrial activities including mining, but also normal everyday activities such as the use of cars, which in the past were running on leaded petrol for a long period (Tataruch 2005). Heavy metals have various toxic effects on organisms, which are further described in this study. Each of human activities might have a serious impact on our environment. That is why we should be careful of what we do and take responsibility for our planet.

Generally, it is known that there is a strong correlation between orography, the amount of wet deposition, and rainfall composition (Fowler *et al.* 1988). High levels of precipitation seem to be a main source of heavy metal fallout at higher altitudes. Stronger and more frequent winds than at lower altitudes must also be considered for several heavy metals (Zechmeister 1994). The deposition of lead probably depends initially on the element contents of air masses, and it is intensified by the high wind speed in the Alpine areas (Janiga 1998). According to Clough (1975), small particles show the deposition closely related to the wind speed. In the Tatras, the wind speed increases above the tree line, mainly in the high altitude Alpine areas as well as the precipitation amount increases with the altitude (Konček *et al.* 1973). Kyrgyzstan, being a country almost without trees, is highly at risk from the heavy metal pollution transported by wind. Not only the wind speed, but also the prevailing direction of the wind plays an important role in the heavy metal transport in mountains. It was found out that in high altitude habitats, where the chamois occur, western and north-western winds mainly prevail in the West and Central Tatras (Konček *et al.* 1973). The study by Janiga *et al.* (1998) proves this fact. The lead concentration in nasal bones of the chamois *Rupicapra rupicapra tatrica* reflected a wet deposition pattern and prevailing direction of winds in the Tatras, with higher concentrations in the Western Tatras than in the eastern Belianske Tatry Mountains. Furthermore, Janiga (2004) found out that the amount of lead and aluminium is higher with increasing altitudes, in case of lead it is very obvious. It is related to the dry and wet deposition of metals in the form of aerosols on the mountains. This was assessed from 45 samples of the chamois food. Another study confirming the fact of correlation between the heavy metal pollution and altitude is the study by Zechmeister (1994). He examined mosses at different altitudes in the Austrian Alps and he discovered that in Achenkirch the heavy metal concentrations show a clear increase with altitude in most metals. He found fairly high concentrations in mosses collected at the valley bottom. This is probably due to intensive traffic and local house fires (Bolhar-Nordenkampf 1989). Concentrations in the highest areas were mostly as high as, or even much higher than in the valley bottom. Excluding the sampling points at the valley bottom, a very distinct, and for many cases significant, increase of the heavy metal concentration with increasing altitude could be seen. At one point, there was a sharp rise in heavy metal concentration mainly due to a small scale local metal processing industry. Another site was also in the vicinity of an extensive area of local steelworks. Nevertheless, the highest As, Co, Cr, Fe, Ni and V concentrations could be found on the top of the mountain, which is the sampling point furthest from the steelworks. Maybe this was a result of the emission caused by high chimneys, as well as of long range transport. The obvious increase of heavy metal concentration with the ascending altitude could be connected in many cases with various factors. With the high amounts of precipitation in the northern and eastern Alps, however, the main source of heavy metal deposition seems to be the rain. As in the Alps the precipitation rises more or less constantly with increasing altitudes, a correlation is obvious. However, it was discovered that it is not the chemical composition of the precipitation, which is important. In the Tatras, the concentration of lead is evidently lower in the precipitation than in the gutter water (Tuzinský and Chudíková, 1991). The study by Šoltés *et al.* (1992) also states that the amount of lead in air dust tends to be greater than the amount of lead in the precipitation water. Therefore, we assume that particles of lead are brought by air masses, and then they are washed from the rocks to their base. Hajdúk (1988) found that the lead content in the soil samples close to rocky walls was higher than in the samples at the distance ranging from 2 to 8 metres from the wall. The lead content in the soil near the trunk of beech trees close to the gully was twice as high as at the opposite site of the trees. The fact that the heavy metal compound in soil is not natural, but has an anthropogenic impact is proved by two studies (Ewers and Schlipkoter 1991; Tataruch 1995). By monitoring soil they found out that more heavy metals (lead, cadmium) are found in the upper levels of soil than in the lower ones, which caused by imissions.

In vascular plants, lead is absorbed mainly by leaves, and only to a limited extent by roots (Kabata-Pendias and Pendias 1984). Bednárová and Bednár (1978) experimented with many vascular plants in the Tatra Mountains, and found that approximately 50 % of lead is physiologically absorbed by the tissues of plants, while the other 50 % may be washed away. Tataruch (1995) also claims in his study that most of the heavy metal compound in plants results from the deposition on the surface of plants, whereas the amount of lead absorbed by plants from soils with natural lead concentrations is very small. The animal's burden of lead is mainly caused by two sources; one is the respiratory absorption of contaminated air, the other is the gastrointestinal absorption of lead by an uptake of contaminated food plants. While grazing, deer ingest some soil too. Lead contained in the soil might add to the body's total burden. A similar study was done in the Tatra Mountains by Chovancová (1990). She found out that some of 17 plant species with higher concentrations of lead in their tissues may be components in the diet of chamois. As previously mentioned, the Alps and Tatras are a barrier for the long range atmospheric transport of pollutants, which causes a long term pollutant exposure of chamois, so they have no signs of acute poisoning (Janiga *et al.* 1998). Another study stating similar facts is the study by Froslie *et al.* (1985). They mention that high positive correlations between lead levels in herbivorous or granivorous animals and lead deposition data are usually explained by the intake of lead from the surface of food plants, and by the intake of contaminated soil.

An important question, which arises from these facts, is where the heavy metals are stored in the body of animals. It was discovered that skeletal lead in mammals may represent 90 percent of the total body lead content as lead tends to be stored in bones. This having been said indicates that lead in avian bones provides a good index of chronic exposure, while lead in kidneys, liver, and blood provides a good index of acute exposure (Cibulka 1986). In the research by Chessa *et al.* (1998), cadmium and zinc concentrations have been determined in muscle, meat, liver, kidney, bone, milk, blood and wool of sheep in south-west Sardinia, where there is a pollution source due to geological proprieties as carbon-field and ores processed since the Roman era for lead and zinc mines, and anthropogenic activities as lead and zinc smelting-refinery. The accumulation of heavy metals in these tissues has been further confirmed in this study, as well as the suitability to perform biological monitoring by detecting heavy metal tissue-levels has been recognized. Even in the monitoring study performed on lead and cadmium concentration traces in sheep milk, the mean concentrations founded were several times higher than in the case of sheep milk from the agricultural region (control). The research by Sivertsen *et al.* (1994) focused on hepatic concentrations of eleven different elements in reindeer, moose and sheep, and compared them to levels in uncontaminated reference areas. Analyses of kidneys from the same animals were restricted to nickel, which is the main metal contaminant in the border area, and is known to concentrate in renal tissues. When comparing their results with reports on the pollution of air and vegetation, it was concluded that for all elements showing higher levels in reindeer and moose from South Varanger compared to reference areas, the effect most probably was a result of the atmospheric transport of industrial pollution from the nearby Russian towns Nikel and Zapoljarnij.

The study of Tataruch (1995) tried to find out where the minerals needed for the formation of antlers come from. He admitted that only very little is known about the exact mechanism of the formation of antlers, especially about the exact source of minerals needed. Histological investigations by Meister (1956) on bones of white-tai-led deer showed hints of mobilization of calcium, the main constituent of antlers from bones during the period of antler growth. Tataruch suggests in his study that it seems plausible that lead, which reacts similarly to calcium in animal organisms, is also mobilized from the bones, especially from the metabolically active trabecular bone.

He proved that during an annual period of growth minerals needed for the formation of antlers are mobilized not only from the skeleton, but are also derived from the diet. This would mean that lead levels in antlers represent the food burden during the period of 4 months of the antler growth, but also the amount of lead previously accumulated in the deer's skeleton. Trace elements, both essential and non-essential, present in food plants are also stored in antlers. Contents of trace elements in the antler proved to correlate significantly with concentrations of these elements in local feeding plants (Anke and Bruckner 1973). These authors also assumed that antlers should indicate a contamination of the biotope by pollutants such as lead and strontium-90.

The study by Janiga *et al.* (1998) also focuses on the animal's heavy metal uptake from the environment. By analysing the nasal bones of chamois, their research showed that even if animals normally have an age-dependent lead accumulation, which means that lead is only accumulated until the first year of age, and thereafter a stable level is reached, chamois probably may not have the age-dependent lead accumulation after reaching adulthood, since the group of the oldest animals (11 -16 year old) tended to have the largest amount of lead in their bone tissue (Janiga *et al.* 1998). Most of the lead entering the body leaves the body in urine, faeces, sweat and as dead skin cells slough off. The lead that remains in the body tends to accumulate in bones, where it can be stored for decades (Tiwari *et al.* 2013). It was discovered and proved by many studies that Pb is mostly accumulated in calcified tissues (Appleton *et al.* 1999), while kidneys are the most Cd-contaminated organ, lower levels are found in the liver and skeletal muscles were the least contaminated. This fact was proved in the study by Kottferová and Koréneková (1997). They analysed tissues of four species, the roe deer (*Capreolus capreolus*), red deer (*Cervus elaphus*), European hare (*Lepus europaeus*), and wild boar (*Sus scrofa*). For all of these species they concluded that mean values in kidneys and the liver exceeded the highest permissible limit for Cd, but they did not record any exceeded permissible levels for Pb either in internal organs or in muscles, which was also proved by Bukovjan (1991). Therefore, if we want to study the contamination of various heavy metals in the environment, we need to take samples from different body parts of the animal, as it was done in the research by Chessa *et al.* (1998), already mentioned earlier, in which they took different biological tissues of the sheep bred in a contaminated environment, and compared concentrations of lead, cadmium and zinc in milk, blood, wool, liver, kidney, bones and muscles from the risk area as well as from the control area, and they also proved that bones are good indicators of lead pollution, but not the cadmium pollution.

In many studies it is claimed and proved that teeth are a sensitive and reliable marker of the environmental pollution by heavy metals. Once formed, these tissues are not subject to a significant turnover, and it is suggested, therefore, that they provide a permanent, cumulative and stable record of a heavy metal exposure during the teeth development. The bone, in contrast, is subject to a turnover regarding its mineral phase (Budd *et al.* 1998). Appleton *et al.* (1999) studied teeth of bank vole and concluded that heavy metals are sequestered by the mineral phase of teeth, during their formation. The other study by Curzon and Cutress (1983) mentions that there is considerable evidence showing that elements become incorporated into the mineral phase of dental tissues. Teeth accumulate and retain Pb over time, and so they are possibly more reliable and sensitive indicators of the biological impact and accumulation of Pb pollution. The Pb concentration is almost 2 μg/g higher in teeth than in bones (Appleton *et al.* 1999). Gdula-Argasinska with her team, which included Appleton, did a similar study in 2004 to Appleton *et al.* from 1999. They showed that the lead concentration in teeth recorded a significant decrease in recent years, probably due to combined effects of industrial closures together with other factors, such as the gradual decrease in the use of leaded gasoline in Poland. Results of the study confirm again that molar teeth of bank voles trapped in the wilderness are sensitive and accurate indicators of the exposure to environmental pollution by heavy metals, and reflect their decreased deposition related to improved environmental conditions. The study by Wesenberg *et al.* (1979) proved that there is a difference even between different types of teeth. They measured Pb and Cd levels of incisors, molars, epiphyses, diaphyses and kidney cortex. Results confirmed that the kidney cortex is a primary target for Cd, but there is a positive significant correlation between Cd levels in molars and the kidney cortex, so they suggested that rodent molars indicate a degree of Cd absorption, whereas incisors do not, as they found no level of Cd in them.

The study by Sivertsen *et al.* (1994) proved evident interspecies differences between the amount of heavy metals found in the liver and kidneys of wild animals and domestic animals. Regarding concrete results of this study, the group of scientists discovered cadmium levels being somewhat higher, and lead and aluminium levels considerably higher in reindeer compared to moose and sheep in all areas. Reindeer seem to take up elements from atmospheric deposition to a higher degree than other ruminants, as for all the elements showing higher concentrations in South Varanger samples than in samples from western Finnmark, the effect was strongest in reindeer. For some elements such as arsenic, copper, cobalt and chromium, the difference between the areas could only be observed in this species. In contrast, no reflection of atmospheric depositions was found in sheep organs, not even for nickel. Reindeer generally take up more elements from atmospheric deposition than moose and domestic species. Most probably this is due to a high level of lichens in their diet. Lichens accumulate metals from atmospheric deposition to a higher degree than other wild forage plants (Eriksson *et al.* 1990). Sheep, on the other hand, take much of their feed from ensilage and hay. This is especially true in Finnmark, where winters are long and sheep grazing seasons short. Grass for ensilage grows in a short time on relatively small areas, and is thus much less exposed to airborne deposition than to the uncultivated vegetation. The highest hepatic selenium levels found in reindeer and moose in South Varanger would be considered in sheep or cattle indicative of a toxic selenium burden (Osweiler *et al.* 1985). In wild Cervidae, however, hepatic selenium concentrations are known to vary naturally over a wide range, without any sign of toxicity reported (Froslie *et al.* 1987).

Our study focuses on the amount of heavy metals and other elements in bones in relation to the altitude and geographical position of samples. Through maps with highlighted locations of the heavy metal occurrence, we want to discover which parts of the country are the most polluted, and if it has any relation to the ways of heavy metal deposition in mountains. Moreover, it examines the values of heavy metals in domestic animals, while comparing them with wild animals, and if the values vary in different parts of the animal's skeleton. We wanted to find out in which part heavy metals are present at their greatest concentration, and if the information from our research matches with previous pieces of research.

Material and Methods

We were collecting samples in different locations in Kyrgyzstan, along the rivers in the valleys of mountain regions (Fig. 1). For each sample we measured the exact altitude and position, we determined the species where the sample belonged to, and also a kind of bones with the help of the book concerned with the anatomy of domestic animals - *Atlas topografickej anatómie hospodárskych zvierat III* (Popesko 1988). We found different kinds of bones: legs, teeth, mandibles, skulls, vertebrae, ribs, bladebones, pelvic bones and horns. We collected and measured 150 samples altogether, some of them were part of the same skeleton, but we took measure of different parts of it to examine where exactly in skeleton the concentration of heavy metals prevails. 47 of the samples were wild animals, 101 were domestic animals and 2 samples were not clearly identified. Wild animal bones come from Siberian ibex (*Capra sibirica*), golden marmot (*Marmota caudata*), and Marco Polo sheep (*Ovis ammon polii*). Regarding the domestic animals, we found samples of sheep, goats, cows, horses and yaks. Before the XRF analysis, we properly cleaned all the samples found. As XRF is a surface analysis technique, where X-rays penetrate a very short distance into most metal samples (in case of bones it penetrates only a few mm in depth) we carefully grinded the upper layer containing accumulated impurities from the environment during the period of bone decomposition so that we would get a pure bone element content analysis without misleading results from the impurities, even if we could not absolutely prevent it. This issue is further described in the discussion.

Instrumental analysis

For analysing samples, we used the hand-held XRF spectrometer DELTA CLASSIC (USA). This method is widely used in many recent studies: Šoltés and Gregušková 2013; Boivin *et al.* 1996; Cheburkin and Shotyk 1995… In XRF Spectrometry, high-energy primary X-ray photons are emitted from a source (X-ray tube or radioisotope), and strike the sample. Primary photons from the X-ray source have enough energy to knock electrons out of the innermost, K or L, orbitals. When this occurs, atoms become unstable ions. Electrons seek stability; therefore, an electron from an outer orbital, L or M, moves into the newly vacant space at the inner orbital. As the electron from the outer orbital moves into the inner orbital space, it emits the energy known as a secondary X-ray photon. This phenomenon is called fluorescence. The number of element-specific characteristic X-rays produced in a sample over a given period of time, or the intensity, is measured, which determines the quantity of a given element in that sample (Innov-X Systems 2005).

For analysing our samples we used the fundamental parameters analysis for metal alloys as we were working with a solid material. We placed the analyser on the cleaned bone surface so that the bone covered the whole surface of the analysing window. In case of the same bone type we always placed the analyser on the same part of the bone, where the bone was the flattest to ensure it would cover the whole analysing window, and to get comparable results. For example, for all leg bones, we placed it in the middle part of the bone.

The following elements were determined: Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, W, Hf, Ta, Re, Pb, Bi, Zr, Nb, Mo, Ag, Sn, Sb. Analytical modes utilize a Factory Grade Library consisting of a set of minimum and maximum values for each element in an alloy. If the content of an element was under the minimum or over maximum value, it was cited as not detected in data results. For the accuracy of obtained results, each sample was measured 3 times, and then an average value of the three measures was created. The element content was measured in the unit PPM, which means Parts per million, and this unit is commonly used as a measure of small levels of pollutants in air, water, body fluids, etc. Transformed to the metric system 1 part per million is equal to 1 mg/kg.

Statistics

After the measures, we received a raw data matrix, which needed a modification for further processing in statistic programs. We took the average value calculated of the three measures, and worked with these data. Moreover, we included further data in the table- date, location, altitude, animal species and bone type. We sorted samples into categories according to the way of the bone ossification in 5 bone types: limb bones (including pelvic bone and blade), body bones (ribs and vertebrae), teeth, jawbone and skull bones; according to animals, we firstly divided them into the domestic/wild category, then more precisely into 3 categories: wild, small domestic (sheep, goat) and large domestic (cow, horse, yak).

Then we sorted elements after the number of samples, where we measured these elements and we used different methods for their analysis. The first group was represented by elements which were not detected at all; the second group associated elements which were detected in only a few samples. To analyse these, first two groups we made a direct analysis from the matrix without any use of statistic programs as there were too few values. We focused on a bone type where these elements most occur, on the comparison between a wild and domestic animal contamination, and on the correlation between localities and the heavy metal bone content. For the third group of elements found in more than 10 samples, but less than a half of the samples, we used contingency tables. For each element we did two contingency tables, with a number of positive and negative samples for domestic and wild animals, and for different types of bones. Afterwards we analysed these tables with the chi-square test method. This test verifies if differences between observed and expected frequencies are only random (variables are independent), or if they are too high to be just random, so they are statistically significant (variables are related). To analyse frequencies of these elements in wild/domestic animals, we used simple 2x2 tables, where we found out if the result was significant and variables were related or not. To analyse the frequencies of these elements in different bone types, we first did the ratio of positive and negative samples for different bone types in selected elements, and according to the sum of positive and negative samples we calculated the number of expected positive samples. Then we used observed versus expected frequencies test, and it analysed if the difference between observed and expected positive samples was significant and in which type of bone the frequencies of this element were the highest.

The last group of elements were those measured in all or almost all samples, which we analysed through the statistical graphics system STATISTICA ver. 8. At first the raw data matrix was standardized to avoid possible data eccentricity to the maximum extent, and to get the most accurate results. Then we calculated a correlation matrix out of the standardized matrix. The correlation matrix was the basis for the calculation of principal components. We used the principal component analysis for the identification of the impact of various factors on the distribution of elements in samples. Calculated eigenvectors show us the correlation between measured variables. They also determine potential factors that influence the data variability. For each measure the so called factorial scores were calculated, and they were compared for chosen categories (wild animals/domestic animals, small/large animals, localities, bone types). They were compared by the analysis of variance ANOVA at the level of high significance $p<0.05$. In cases of very significant results, we used the Tukey's HSD (honest significant difference) test for the further specification of correlation. Furthermore, we focused on two elements present in the majority of samples- Mn and K. We deleted samples without the occurrence of these elements from the original matrix, and analysed their individual correlation with a bone type, domestic/wild animals and localities.

Results

From toxic metals harmful for living organisms Cd and Sb were not detected at all, and in only a few (less than 10) samples higher concentrations of Hg, Pb, As, Ni, Cu and Cr were found. Lead was found in 4 out of 150 samples, mercury as well as arsenic and selenium in three samples, nickel in 7 samples, copper in 2 and chrome in one sample. Cadmium, antimony and tin in samples were not detected at all. We analysed different types of bones contaminated by these toxic metals. The most

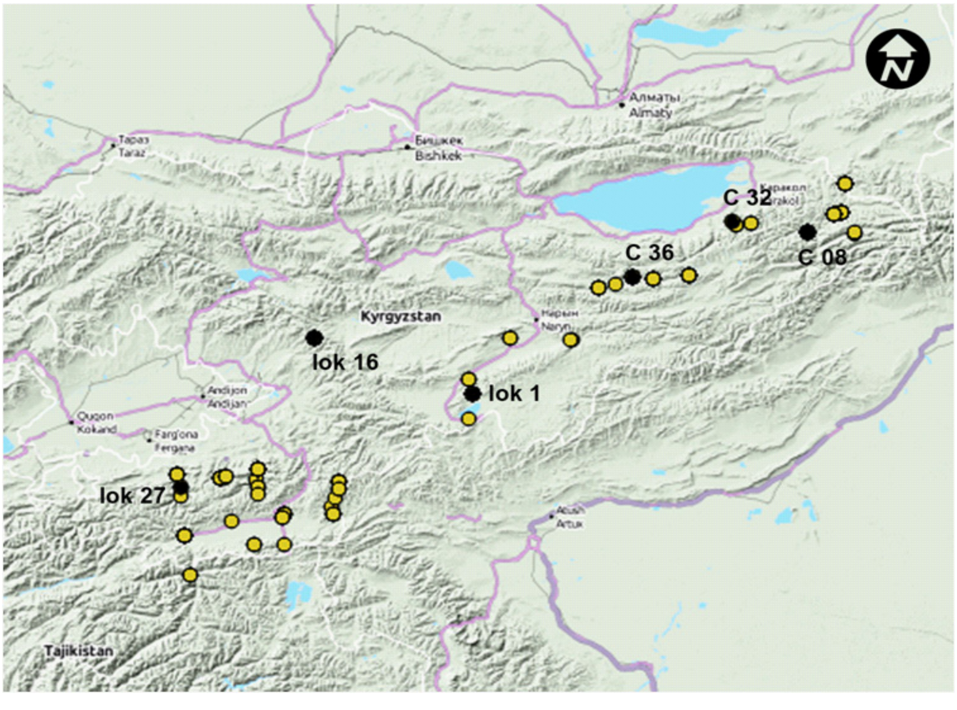

Fig. 1. Localities with detected the highest values of heavy metals (see Results).

Eigenvectors of correlation matrix, Active variables only

Variable	Factor1	Factor2	Factor3	Factor4	Factor5	Factor6	Factor7
Altitude	0.062055	-0.085734	**0.573974**	**0.627833**	0.220921	**0.465102**	0.007318
P	**-0.662257**	0.051306	0.209068	0.012660	0.019406	-0.197348	0.689631
Ca	**-0.678139**	0.081061	0.125555	0.042751	-0.069700	-0.062922	-0.712143
Fe	0.131268	**0.424015**	-0.186427	**0.674344**	-0.224300	**-0.513002**	-0.001841
Zn	0.105557	**0.631140**	0.201331	-0.242743	**0.683375**	-0.142150	-0.062076
Sr	-0.218527	**0.438414**	**-0.551616**	0.106351	-0.024104	**-0.656845**	0.111451
I	0.146854	**0.461641**	**0.481156**	-0.280827	**-0.654268**	0.161094	0.030479
variance	29%	**19%**	**17%**	15%	**9%**	8%	

Table 1. Eigenvectors of the principal component analysis.

contaminated types of bone were teeth. 2 of 4 lead contaminated samples, 2 of 3 mercury contaminated samples, 2 of 3 selenium contaminated samples, both chrome and copper contaminated samples, all 7 nickel contaminated samples, and 6 of 10 cobalt contaminated samples were teeth. However for As there was no clear tendency of accumulation in a specific bone type, all of the three contaminated samples had a different bone origin. Regarding wild/domestic or small/large animals, there was no significant correlation found for these elements with a low occurrence in samples. In case of altitude, it was considerable if the bone was lying at the original place, where the animal died, or it was transported by the river flow in lower altitudes, so we were unable to analyse it properly. To analyse localities with the occurrence of these metals, we created a map, where we marked bone sampling localities, and we highlighted those with the occurrence of heavy metals. We found three most polluted locations with the occurrence of 5 to 6 different heavy metals, and these were lok (occurrence of Ni, Hg, Cr, Co, As), lok16 (occurrence of Pb, Mn, Cu, Cr, Co, Se), C08 (occurrence of Pb, Hg, Cu, Ba, Ni, Mn). Moreover, we found three rather polluted localities with the occurrence of 3-4 different heavy metals. These were C36 (occurrence of Mn, Ba, As, Se), C32 (occurrence of Mn, Hg, Ba), lok 27 (occurrence of Ni, Se, Ba, and in the same valley on the locality 28 there was also Mn found) (Fig. 1).

Analysing the contingency tables for wild/domestic animals with the method of 2x2 tables, we did not discover any significant tendency of a prevailing number of samples with higher levels of the selected element in wild or domestic animals except for rubidium. This was the only element with a significantly ($p<0.05$) higher number of positive samples (where Rb was measured) in wild than in domestic animals. Using the statistical method of observed vs. expected frequencies for different bone types, we obtained significant results ($p<0.05$) for all the elements observed. We found out that sulphur occurs at greater frequencies in teeth and limbs than in other bone types Chlorine is the most frequent in teeth as well as silver, titanium and cobalt. Zirconium is also much more frequent in teeth, but also in body bones (ribs and vertebrae). Molybdenum was found to be the most frequent in teeth and skull bones, while barium in body bones and mandible. Rubidium was the most frequent in skull and body bones.

Principal component analysis.

We used the technique for the evaluation of the elements which were found in each sample. The relations among the elements and other factors are shown in Table 1. Seven main factors are referred.

Analysing these factors through their factorial scores and their correlation with domestic/wild animals, small/large animals, localities and bone type comparing the variables in these categories, we got these results represented in the form of graphs. The first and most important factor (variance 29%) was the decrease of P with the decrease of Ca. This effect was more evident in wild than in domestic animals as shows the graph below (Fig. 2a). The same factor analysed in correlation with small/large animals is shown in the following graph, this factor showed to be more evident in small domestic than in large domestic animals (Fig. 2b).

To better explain the following graphs, it is necessary to know what letters F and p stand for. F-test value (F) is a "between-group variability" divided by "inner-group variability", and p-value (p) is the provability of getting a result from data as, or more, extreme, if the null hypothesis is true.

There was no proven correlation between factor 1 and different localities. In this case no significant result was discovered (p greater than 0.05) due to a great variance of values at single localities.

Factor 1 showed to have an important role in correlation with the bone type. The effect of increasing P together with increasing Ca is strongest in teeth. In skull bones and in body bones it works oppositely, P and Ca tend to decrease. For a further analysis we used the Tukey HSD test, which specified results from the graph below. In teeth, and in a small extent also in mandible and limbs, P and Ca tend to increase (Fig. 2c).

Moreover, we got a second factor (variance 19%), which shows that with increasing Fe there is an increase of Zn, Sr and I. Fig. 3a shows that in domestic animals the increase of these elements is more evident than in wild animals. Fig. 3b shows that small domestic animals accumulate Fe, Zn, Sr and I more rapidly than larger animals.

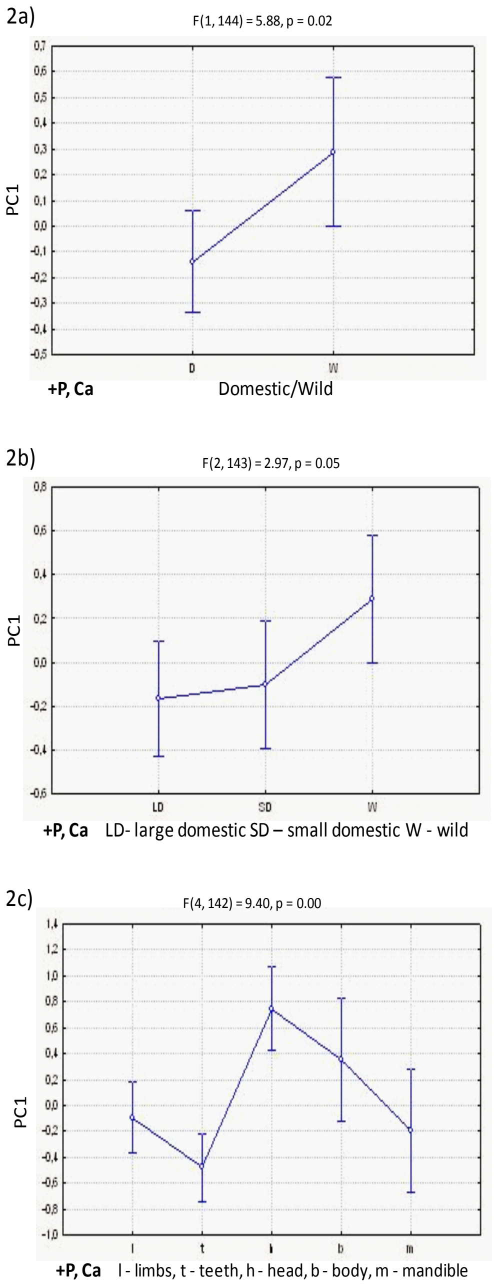

Fig. 2. Contents of P and Ca in the bones and its dependence on animal origin (a, b) and bone type (c).

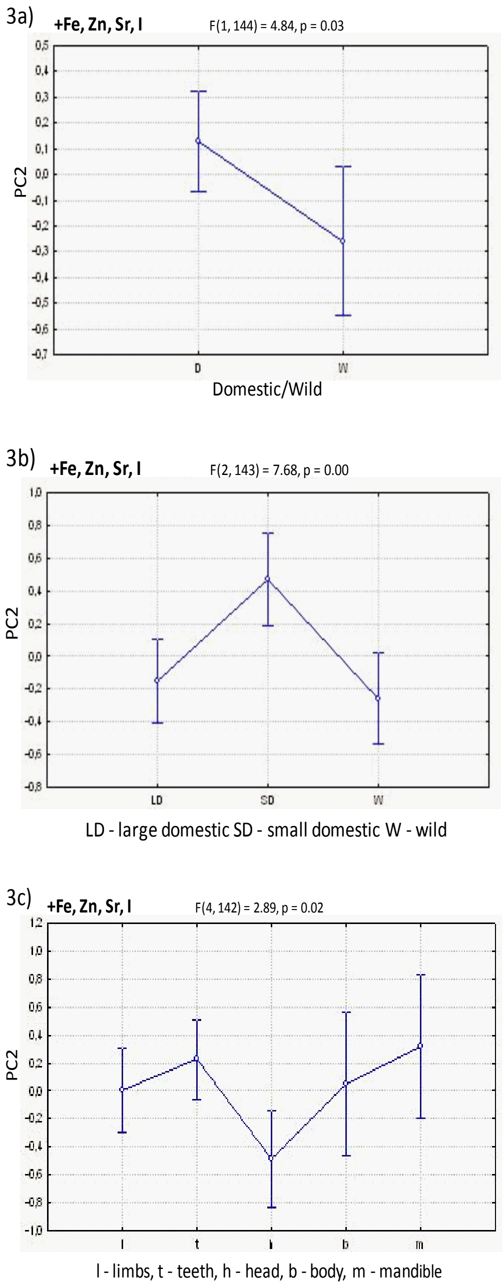

Fig. 3. Mutual increase of Fe, Zn, Sr and I in dependence on animal origin (a, b) and bone type (c).

According to the bone type we found that the effect of increasing Fe, Zn, Sr and I is the strongest in mandible and these elements tend to decrease in skull bones (Fig. 3c).

Fig. 4a shows the correlation between factor 3 (variance 17%) and domestic/wild animals. This factor represents the increase in altitude with increasing the level of iodine and decreasing the level of strontium. This factor was discovered to be much more evident in wild animals than in domestic ones, these seem to accumulate more Sr than I. Fig. 4b shows that it is also a little bit more significant in large domestic than in small domestic animals, small domestic animals have more strontium and less iodine than the large ones.

Concerning bone types, this factor mostly affects teeth (t) and at least the mandible (m). For further precision we did the Tukey HSD test, which showed that mandible and legs have more Sr and less iodine, while teeth and head have more iodine and less strontium. In results of the Tukey HSD test, body bones are in both categories, so have an equal quantity of iodine and strontium (Fig. 4c).

Factor 4 represents the increase of Fe with increasing altitude (variance 15%), and this factor is more evident in wild than in domestic animals (Fig. 5a).

Small domestic animals have less iron than the large ones; it is represented in Fig. 5b. Fig. 5c shows that factor 4 is the most evident in body bones, little bit less, but still quite evident in skull bones, and the least evident in mandible. The Tukey HSD test showed us that neither teeth, nor legs are influenced by this factor.

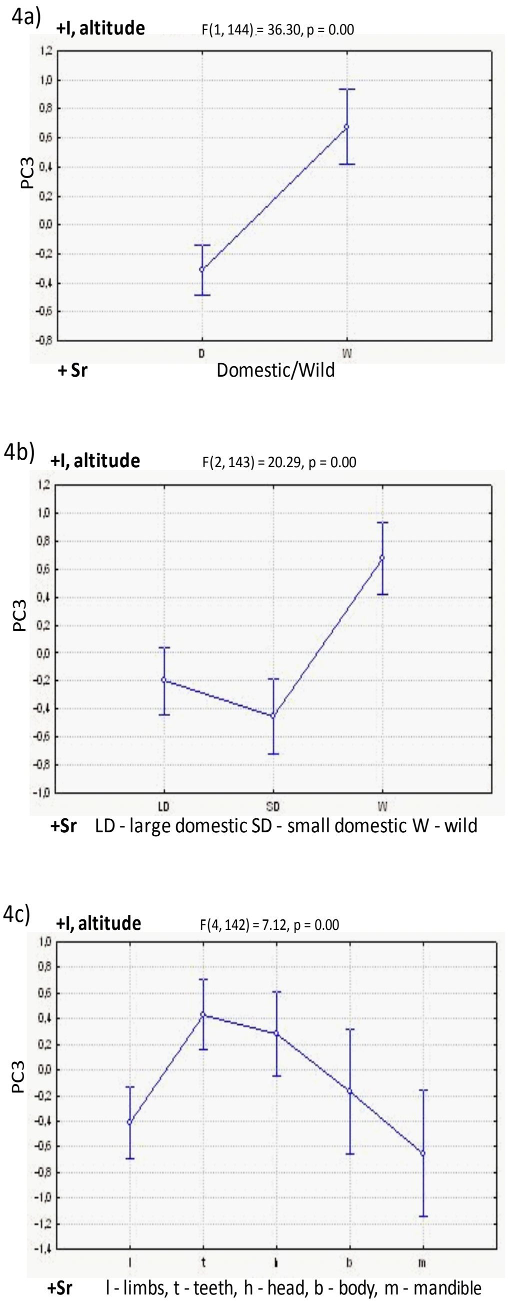

Fig. 4. Antagonistic relation between I (altitude) and Sr in dependence on animal origin (a, b) and bone type (c)

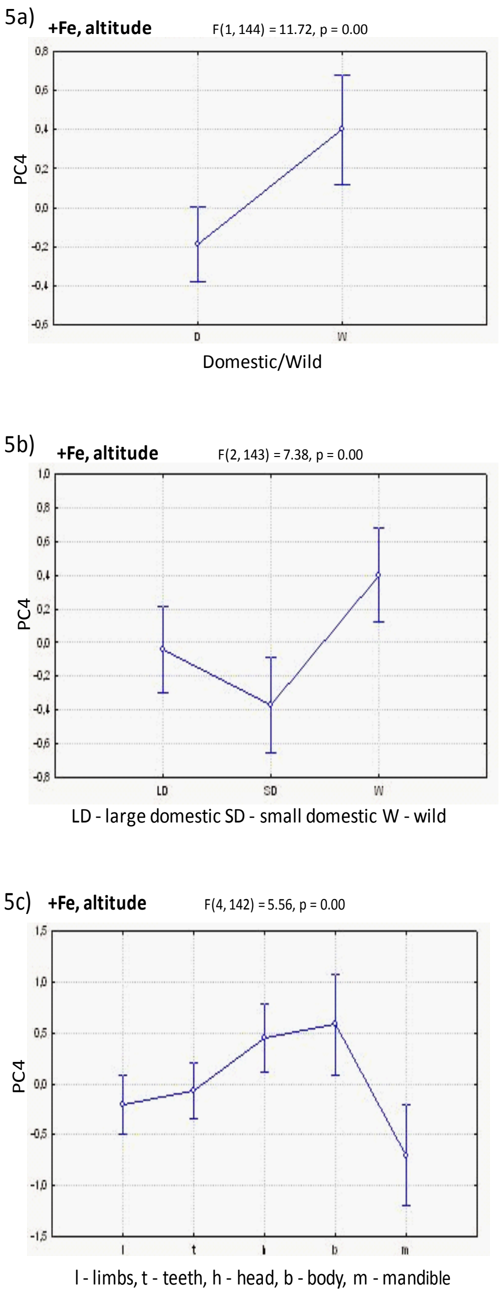

Fig. 5. At higher altitudes the amount of Fe is higher in the bones of wild animals (a, b) and in the body bones (c)

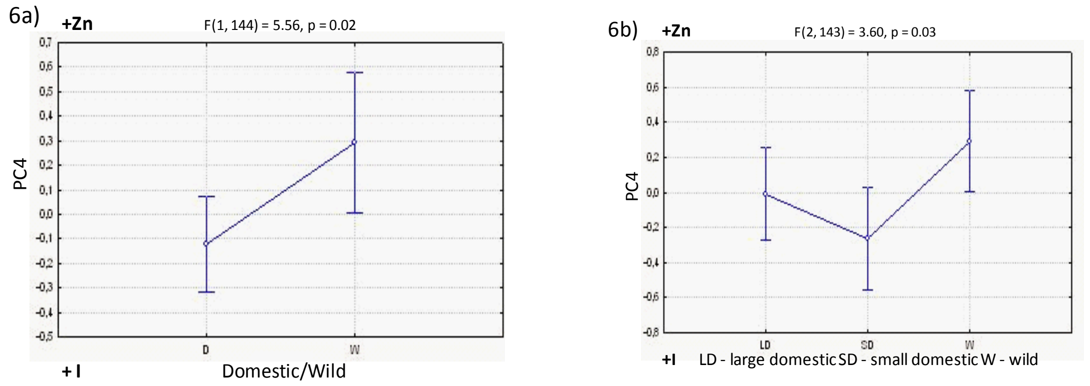

Fig. 6. Antagonistic relation between the Zn and I concentrations in the bones of domestic/wild (a,) and denending on size animals (b).

Factor 5 (variance 9%) means a decrease of I with increasing Zn. It is more significant for wild animals than for the domestic ones (Fig. 6a), and also more significant for large domestic than for small domestic animals (Fig. 6b).

No significant correlation between factor 5 and bone type was found, in both cases p was greater than 0.05, which is not a suitable value for the statistical analysis. In case of factor 6 or 7, no correlations were found at all.

Moreover, as Mn and K were not found in all the samples, but in majority of them, there was enough data for statistical analyses. We created a new matrix, where we excluded samples without the occurrence of these elements so that we could analyse them. We found out that neither Mn, nor K bone content is correlating with the bone type (p=0,66 for Mn and p=0,749 for K). These elements did not show any significant correlation with localities or domestic/wild animals.

Discussion

The fact that cadmium in samples was not detected at all confirms that this element is not stored in bones but rather in inner organs, especially kidneys as many researches show (Chessa *et al.* 1998; Appleton *et al.* 1999). Lead, in contrast, tends to be accumulated in bones (Janiga *et al.* 1998; Tiwari *et al.* 2013; Appleton *et al.* 1999). It was partly proved by our results as we found lead in our bone samples but in only 4 out of 150, which is too few to make further conclusions.

Generally, it is known and proved by many studies that heavy metals are mostly stored in teeth, so they are a sensitive and reliable marker of environmental pollution (Appleton *et al.* 1999; Budd *et al.* 1998; Curzon and Cutress 1983). The Pb concentration is almost 2μg/g higher in teeth compared with bones (Appleton *et al.* 1999). Our study proves this theory for lead, mercury, cobalt and nickel, selenium, chrome and copper contamination. More than 50 % of Pb, Hg, Co and Se contaminated samples were teeth, and in case of Ni, Cr and Cu even 100% of contaminated samples were teeth. We found many other elements, which also tend to accumulate mostly in teeth. These are Ti, Mo, Zr, Ag, S, Cl, P, Ca, Fe, Zn, I. The concentration of Ca is much higher in teeth than in other bone types, which is due to the fact that calcium in a tooth is not a metabolically active compartment (Suttle 2010).

From all heavy metals only for As and Mn there was no clear tendency of accumulation in a specific bone type. In case of As it might be caused by the small number of contaminated samples. Schroeder *et al.* (1966) declare in their study that without exceptions, all human tissues contain manganese in concentrations remarkably constant throughout most of life. In lower concentrations, it is an essential element for the function of animal organs and tissues. We suppose that this is why manganese was found evenly distributed in the skeleton in our study as well.

Moreover, barium was found the most frequent in body bones and mandible. There is one study stating this fact, the study by Kyle (1986). He studied human bones and teeth and analysed them by X-ray fluorescence to find the bone element content. In his study he cited that compared to bones, teeth contained lower concentrations of minor elements Al, Fe, Sr, S, Ba and Zn, similar concentrations of Na, K, Mg and Cu, and a higher concentration of Cl. In our study barium was also found to be less frequent in teeth than in body bones and mandible, chlorine was also found the most frequent in teeth, and potassium did not show any correlation with a specific bone type. Only our Cu and S bone concentrations do not match with the Kyle's research. Both elements were found mostly in teeth, but for Cu the difference might be caused by a low number of positive samples. Kyle (1986) cited that Fe and Sr are found in lower concentrations in teeth than in other bones. We partly proved it for Sr as, according to factor 3, strontium has the lowest concentrations in teeth, even if, according to factor 2, there is a slight increase in the strontium content mostly for teeth. Fe, according to factor 2, also slightly increases mostly in teeth, which is the opposite of the Kyle's results, but according to factor 4, in principle component analysis Fe does not show any significant increase or decrease in teeth.

Moreover, many scientists claim in their studies that with the increasing altitude the heavy metal content increases as well. For example, Zechmeister (1994) examined mosses at different altitudes in the Austrian Alps, and discovered that heavy metal concentrations show a clear increase with altitude in most metals. This is caused by high levels of precipitation that seem to be the main source of heavy metal fallout at higher altitudes (Zechmeister 1994). Not only the amount of precipitation, but also the wind speed increases above the tree line (Konček *et al.* 1973). In addition, as small particles of pollutants show a deposition closely related to the wind speed (Clough 1975), the stronger winds, the more intensive deposition of pollutants. In Kyrgyzstan, in the nival zone glacier winds blow all day long (Aidaraliev *et al.* 2002). Half of the most polluted localities in our study were located in altitudes of almost 3000 meters above sea level and more. Another was located above 2000 meters, and the remaining two were probably influenced by other polluting factors. The correlation between the amount of precipitation and heavy metal pollution is obvious. In the Alps, the precipitation rises more or less constantly with the increasing altitude, together with the rising heavy metal pollution as well as it happens in Tatras (Zechmeister 1994; Konček *et al.* 1973). However, precipitations in Kyrgyzstan are influenced by a variety and complexity of its relief forms. In relation to altitude, it was discovered that the precipitation amount rises only up to 3000-3500 meters above sea level, in higher altitudes it remains constant, or it even decreases in some areas. A large amount of precipitation is observed in some areas, which do not depend on altitude. One of these areas is Eastern Isyyk-Kul (Aidaraliev *et al.* 2002). Two of six most polluted localities (C08, C32) were found in Eastern Issyk-Kul (Fig. 1). These localities were apparently more humid than the other ones, so we assume that the increased precipitation amount caused increased pollution by heavy metals. Moreover, in Kyrgyzstan humid landscape zones are located on the slopes with northern expositions with sufficient humidity, or close to riversides, lake basin… (Aidaraliev *et al.* 2002). This fact might further explain why the majority of polluted localities were valleys with a northern exposition. Furthermore, mountain meadows are located between 3000 and 3600 meters above sea level, and are characterised by snow cover for more than half a year, any by the presence of permafrost grounds (Aidaraliev *et al.* 2002). The melting of snow and ice releases heavy metals stored in it (Janiga 2008). This might be one of the reasons why two most polluted valleys were found over 3000 meters above sea level, one of them directly under the glacier, affected by the water flowing from there.

We have not mentioned and analysed yet the local sources of pollution, which also affect the heavy metal content in the environment. Zechmeister (1994) stated in his study that even if heavy metal concentrations show a clear increase with altitude, there were also fairly high concentrations in mosses collected at the valley bottom. He explained that it is probably due to intensive traffic and local house fires (Bolhar-Nordenkampf 1989). One of the polluted localities was found at a lower altitude (lok 16), but this locality was close to the main road with many surrounding villages and intensive grazing. Moreover, polluted bones found on this location might have the origin from higher altitudes, but they could have been brought to lower altitudes by the river flow, which should be also taken into account.

Another factor found significant on localities was the decrease of strontium with the increasing altitude and increasing iodine content. It is known that the iodine content in soils does not affect very much its content in plants or animals because plant species differ widely in their ability to absorb and retain soil iodine (Suttle 2010). On the other side iodine from soil gets into drinking water and correlates with iodine concentrations in local plants of an area (Fuge 2005). From these facts we can assume that geology and the soil iodine content affect its concentration in plants, and ingesting these plants affects also animals. We were not able to find any study focusing on the correlation between altitude and the iodine or strontium content, so we cannot make any conclusion, this issue would need a further study for a proper analysis. However, the locality with the lowest content of iodine and highest content of strontium situated in low altitudes was not far from a gold mine and surrounded by villages. Mining might affect the strontium content, but to our knowledge there is no study concerning this topic, so no further conclusions can be made.

The last factor found significant in its correlation with localities was an increase of iron with increasing altitudes. The high iron concentration is attributable to soil contamination, which is most likely to occur on soils prone to waterlogging (Suttle 2010). In high altitude areas melting snow often floods the soils, so these areas have a higher tendency of waterlogging and higher iron concentrations. The locality with the highest iron concentration was C36, locality 2900 meters above sea level, situated on the confluence of several tributaries with the main stream. Soils here are permanently waterlogged when the water level rises. The locality with the lowest iron level was situated in a low altitude, and was poor on vegetation, which might have been caused by insufficient humidity related to the low iron content.

In this study we found that domestic animals accumulate more P and Ca than wild animals and the increase of Fe, Sr, I, Zn is also higher in the bones of domestic that wild animals. This might be caused by a richer diet of domestic animals compared to the wild ones. Firstly, domestic animals are fed during the winter period, so they do not have to suffer from nutrient deprivation. Suttle (2010) reports in his study that animals may adjust to a suboptimal mineral intake by reducing the concentration of minerals in tissues or products. Finding out that wild animals have lower concentrations of minerals in their bones than the domestic ones, we came to the evidence of their poorer diet. Another important fact influencing the mineral bone content is that lactating animals eat more food per the unit of body weight than non-lactating animals, and therefore have relatively high maintenance requirements (Suttle 2010). As domestic animals are being daily milked, they have to eat more food to have enough reserves. We can assume that this is also one of the reasons why they have more nutrients in bones than wild animals. Furthermore, wild animals are generally living in higher altitudes, where soils tend to be poorer in the mineral content, so plants contain fewer minerals. The primary reason for mineral deficiencies in grazing animals, such as those of phosphorus, sodium, cobalt, selenium and zinc, is that soils are inherently low in plant-available minerals (Alloway 2004).

Wild animals living in higher altitudes were discovered to have a higher concentration of iron in the function of altitude and a higher concentration of iodine in the function of increasing altitude and decreasing strontium (justification of these facts can be found in the previous part of the discussion focusing on localities). Moreover, it was found out that wild animals had the highest concentration of Zn in the function of the lower I concentration and the domestic animals vice versa. An important source of zinc emissions is the burning of coal and other fossil fuels, metallurgy, mining, the application of artificial fertilizers and pesticides. By metallurgy, zinc is released in the atmosphere in large quantities (Alloway 1995). The long-range transport of zinc emissions might have caused its deposition in higher altitudes, so wild animals living in higher altitudes are more exposed to these pollutants. Moreover, lichens are known to accumulate metals from atmospheric deposition to a higher degree than other wild forage plants (Eriksson *et al.* 1990), and this might be one of the reasons why animals in higher altitudes, where lichens mostly occur and are the main source of food for them, are more contaminated by zinc.

Regarding the comparison between small and large domestic animals, we found out that large animals have a higher accumulation of Ca and P. The study by Suttle (2010) explains this fact by stating that net calcium requirements for growth differ greatly between species, with the horse having a far greater need than the sheep. This difference is attributable to both a higher proportion of bone in the equine skeleton (0.12 versus 0.10), and a higher proportion of dense or compact bone in the equine skeleton. On the other hand, concentrations of Fe, Zn, Sb and I were found higher in small domestic animals. As these elements are not main bone constituents as it is the case for Ca and P, the size of the skeleton might not affect their absorption in tissues. We suggest that if small and large animals would have the same intake of these elements, in small animals they would be logically stored in higher density. It seems that large domestic animals get in higher altitudes than the small ones, and they have higher concentrations of iron and iodine in the function of altitude (reason for a higher iron concentration in higher altitudes can be found in previous parts of the discussion). Large domestic animals have an equal concentration of Zn and I, while small domestic animals have less Zn in favour of I. Therefore, we suggest that small and large animals have a different composition of elements in bones.

We did not discover any significant correlation between the bone concentration of heavy metals (excluding zinc described in the previous paragraph) and wild or domestic animals, even if we expected wild animals to be more contaminated according to the study by Sivertsen *et al.* (1994). They found out that reindeer generally take up more elements from atmospheric deposition than the domestic species due to the high level of lichens in their diet, which are known to accumulate metals from atmospheric deposition to a higher degree than other wild forage plants (Eriksson*et al.* 1990). Sheep, on the other hand, take much of their feed from ensilage and hay, and grass is generally not so contaminated. As the domestic animals in Kyrgyzstan spend during the grazing season all the time outside, probably without getting any supplementary food in the form of hay, they are exposed to contaminants comparably to wild animals. This might be the reason why we found approximately the same heavy metal concentrations in domestic and in wild animals.

Rubidium was the only element of the low occurrence in our samples that was found significantly more frequent in wild than in domestic animals. Elements which are absorbed in body through digestion do not have only a plant origin. While grazing, deer also ingest some soil, and the lead content in soil might add to the body's total burden (Tataruch 1995). We suggest that not only lead, but the other elements from soil are also absorbed through the intestines in organisms. Rubidium has an abundant occurrence in the earth's crust (Nyholm and Tyler 2000), and so it gets in soil and is ingested by animals. It is probable that wild animals ingest more soil than the domestic ones as these are fed by hay at least during the winter period, which does not contain soil.

There is an important issue that should be taken into account as it might have affected our results to some extent. It is the level of bone decomposition in terrain, when we found the sample. In addition to general environmental influences, microorganisms play an important role in the destruction of interred skeletal remains. Fungi are known to penetrate actively through hard tissues (Piepenbrink 1986). Grupe and Piepenbrink (1989) showed in their study that microorganisms cause a substantial contamination of the bone by carrying soil metals into the specimen, where they become fixed to the mineral matrix within a short time. Thus, microorganisms can play a major role in the early stages of dead bone decomposition. Three different cleaning methods these scientists applied to the bones prior to analysis failed because it was not possible to remove the fungal tissue quantitatively. They concluded that any excavated bone specimen exhibiting signs of a prior invasion by microorganisms is unsuitable for the trace element analysis, especially in cases where the soil is specifically enriched with the elements under study. However, in our study we were mainly assessing the contamination of the whole environment through the bone element content, so even if there were some trace elements absorbed from soil, it would only confirm the contamination of this location. What is more, we compared bones in approximately the same decomposition rate. Even if some animals were younger and some older, for comparing wild and domestic animals we had almost equal numbers of old and young bones for both categories, so it was possible to compare them. This method of bone sampling was also used in many other studies (Tataruch 1995; Janiga *et al.* 1998; Kiedorf and Kiedorf 2000a; Pokorný 2004; Pokorný 2006). In the studies on bones, authors used slaughtered or hunted animals in order to take fresh bones. However, in our study we preferred non-invasive methods as they are suitable enough for the assessment of the metal concentration in the environment. Moreover, in our case it was not possible to get to the bones of recently killed animals, as we were collecting samples in high altitude valleys, and it was not our intention to kill animals there.

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