Original Research

Biomonitoring of Lead and Fluoride Contamination in Forests Using Chemical Analysis of Hard Tissues of Roe Deer (*Capreolus capreolus* L.)

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Abstract

Our study assesses environmental lead and fluoride pollution in West Pomeranian forests in Poland, on the basis of chemical analysis of antlers and skull bones of roe deer (*Capreolus capreolus* L.) from the surroundings of Szczecin and Drawsko Pomorskie, Poland. Lead was measured by atomic absorption spectrometry, and fluorides by using an ion-selective electrode. Our study indicates greater lead contamination in the forests near Drawsko Pomorskie than in the forests near Szczecin. The cause may be their long-term exploitation as a military area. The roe deer population near Szczecin is exposed to increased fluoride pollution, but the concentration of fluoride in hard tissues of roe deer has diminished considerably in comparison with data collected 10 years prior to our study.

Keywords: roe deer, lead, fluoride, skull bones, antlers

Introduction

Monitoring and protection of the natural environment are crucial issues in enabling proper assessment of the level of exposure of ecosystems/species, thus enabling the choice of proper preventive measures to reduce environmental pollution and the impact on living beings [1].

In Poland's West Pomeranian Voivodship, the main sources of pollution are the urban agglomeration of Szczecin and its neighbouring cities. The "Dolna Odra" power plant, "Pomorzany," and "Szczecin" heat and power stations, plus the "Police" chemical factory emit substantial amounts of gas and dust containing aromatic and aliphatic hydrocarbons, fluorides, mercury, and heavy metals [2, 3]. These pollutants fall into water and soil, accumulate in plants and find their place in animal bodies through food chains [4]. Additional loads for the environment are foreign pollutants and exhaust fumes [5].

Drawsko Pomorskie, located about 100 kilometers east of Szczecin in the area of the Drawsko Lakeland, has no big industrial plants and city agglomerations. Therefore, the environment in this region should be recognized as ecolog-

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ically cleaner in comparison to the forest areas around Szczecin [5].

In order to assess contamination in the environment, hard tissues (i.e. bones and antlers) of free-ranging animals are materials used to assess bone-seeking pollutants such as lead or fluorides. Deer antlers are deciduous bony structures developing on top of permanent frontal protuberances (pedicles) and undergoing periodic replacement [6]. Antler formation takes place during a short period of a couple of months, and in deer species from the arctic and temperate regions antler regrowth is controlled by the photoperiod. After completion of growth, the velvet is shed from the antlers, and the hard antlers are retained for some further months until they are cast and a new set of antlers is produced [6]. The growing antlers are sinks for calcium and phosphate, the main constituents of the bone mineral phase. The forming of antler bone also accumulates substances like fluoride, lead, and other potential environmental pollutants [6, 7]. The concentrations of these contaminants in hard antlers constitute a cumulative measure of environmental exposure, reflecting uptake by the antlers during their limited lifespan. Since antlers grow during a seasonally fixed species-specific period, antlers can be viewed as relatively well "naturally standardized" samples, and as they are regularly collected by hunters, antlers can easily be an acquired research material which may be used for temporal and/or geographical monitoring of forest ecosystems [5, 8-10].

The aim of this study was the assessment of environmental pollution in West Pomeranian province forests by lead and fluoride on the basis of chemical analysis of the antlers and skull bones of roe deer (*Capreolus capreolus* L.) from the surroundings of Szczecin and Drawsko Pomorskie. The roe deer was chosen for the study as it is the most abundant deer species in Poland, and particularly because it has narrower home ranges (of normally less than 100 ha in forest areas) than males of other European deer species; therefore, it is well suited for monitoring environmental pollution at a local level [6, 10-13].

Experimental Procedures

Study Area and Specimens

The research was conducted by using hard tissues, i.e. antlers (n=20) and cranial bones (n=20) of roe deer (*Capreolus capreolus* L.), obtained from regular controlled hunting in the period 2000 to 2006 in the forest areas of West Pomerania. The obtained material originated from the forest areas of Drawsko Pomorskie forest division and forest divisions in the vicinity of the Szczecin agglomeration (Trzebież, Kliniska) (Fig. 1). The Drawsko Pomorskie forest division is situated in the eastern part of the province and lies on high relief early post-glacial ground, with undulating and end moraines. In the coverage of the forest division there are many lakes with diverse areas, and large areas of marshes and swamps. The area is characterized by diversity of habitats and considerable biodiversity. The forest division covers 35,000 ha with a forestation of 49.2%. Scotch pine, which forms 72% of the wood stands, is the predominant tree species. Deciduous and marsh habitats cover 28% of the area. The forest divisions in the neighborhood of Szczecin lie on moraine ground and marginal champains. Poor sandy soils determine the complete domination of pine wood stands. Trzebież forest division with Wkra Forest covers 76,355 ha with a woodiness of 59%. The relief is largely unvaried, diversified merely by

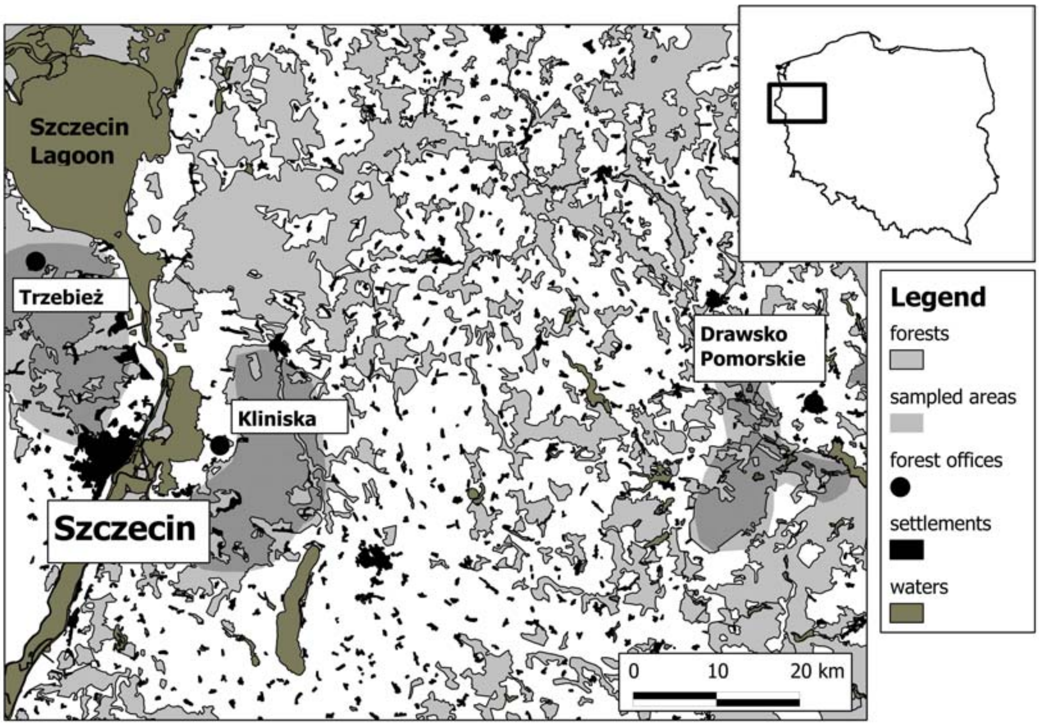

Fig. 1. West Pomerania – the study area.

numerous sand hills. The wood stand is composed mainly of pine (80%), together with alder (7.5%), birch (4%), and beech (4%), respectively. Kliniska forest division covering the Goleniów Forest wood stands is characterized by a similar area (70,000 ha, 32% woodiness) and is situated on the flat landscape of ground moraine with numerous eolian formations. Wood stands consist predominantly of pine (89%).

The age of the examined animals was determined on the basis of tooth appearance (the level of tooth wear, the number of teeth in the jaw [14]) and was estimated to range from 1 to 8 years. The study material consisted of antlers and cranial bones at pedicles (Fig. 2).

Bone and Antler Sampling and Analysis

Before taking the samples, the antlers were thoroughly cleaned with a nylon brush, cleared from soft tissues, defatted in acetone and dried in the open air. Following Tataruch [15], a hole was then drilled into the back of the antler shaft approximately 1.5 cm above the antler-pedicle junction using a tungsten carbide cutter fitted to a hand-held electric drill [15]. Between 1 and 1.5 g of bone powder, comprising both compact cortical and inner trabecular bone, was collected from both antler beams of each specimen. Prior to analysis, the bone powder was thoroughly mixed to ensure homogenous distribution of the particles. The same method was used also for the pedicles, in which holes were drilled approximately 1 cm below the antler-pedicle junction.

For the determination of lead content, 1,000 mg of bone powder was wet ashed in 5 ml of 65% HNO_3. Specimens remained in the acid for 72 hours and were afterwards mineralized by a microwave digester, BM-1S/II, Poland. The solution was then diluted to 10 ml with distilled water.

The concentration of lead was analyzed by atomic absorption spectrometry (AAS-Solar 969) in an air-acetylene flame at 217 nm, because of the favourable signal-to-noise ratio compared to 238.3 nm. Detection limit was 0.2 µg/dL. Average recovery rate of the method was >95%. The arithmetic mean of variation (±SEM) of these determinations was (±10.5%). Internal quality control used 3 standards prepared by the Heavy Metal Toxicology Central Laboratory of the Work Medicine Institute in Łódź, Poland. Control assays were carried out every 10 samples.

For fluoride analysis, 100 mg of bone powder was dry ashed to constant mass by repeated desiccation at 105ºC in a furnace. The ashed samples were than dissolved in 1 ml of 2 M $HClO_4$ using closed plastic tubes. The samples were then shaken in a thermomixer for 1 hour at 95ºC and cooled to room temperature. The solution was then buffered with a total ionic strength adjustment buffer (TISAB II; Orion Research) and measured for fluoride using a fluoride ion-specific combination electrode (Orion Research model 96-09) [16]. Standards for electrode calibration were prepared from Orion reference solution. Individual fluoride values given in the paper are means of three parallel analyses per specimen. The arithmetic mean of variation (±SEM) of these determinations was ±12%. Average recovery rate of fluoride from antler and coronal bone samples using this method of analysis was 98%. Fluoride and lead contents in antlers and cranial bones are expressed as µg/g on a dry mass (d.m.) basis.

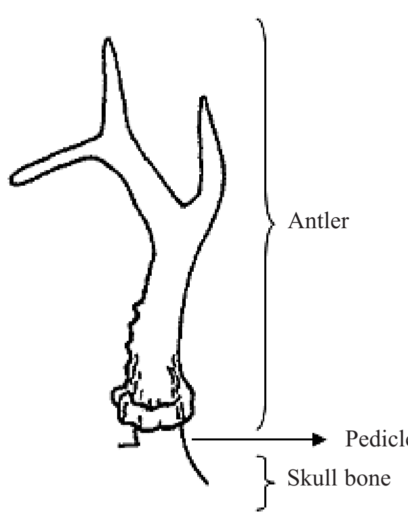

Fig. 2. The study material (antlers and cranial bones at pedicles).

Statistical Analysis

Results were statistically analyzed using Statistica 6.1 software. Arithmetical mean and standard deviation (±SD) were calculated for each of the studied parameters. As most of the distributions deviated from the normal distribution (Shapiro-Wilk test), non-parametric tests were used for the analyses. Correlations between parameters were examined with Spearman's rank correlation coefficient (Rs). To assess differences between parameters, Mann-Whitney tests were used. The level of statistical significance was set at $p<0.05$.

Results

The average content of fluoride in antlers of roe deer from the forests in the vicinity of Szczecin (0.400±0.118 µg/g d.m.) was nearly the same as in antlers of roe deer from Drawsko Pomorskie forests (0.324±0.084 µg/g d.m.) (Table 1).

In skull bones of roe deer living in the neighbourhood of Szczecin the average fluoride content (0.420±0.092 µg/g d.m.) was substantially higher ($p=0.0031$) in comparison with Drawsko Pomorskie forests (0.280±0.072 µg/g d.m.) (Table 1).

Antlers of roe deer from Drawsko Pomorskie forests contained higher amounts of lead (1.093±0.579 µg/g d.m.) than those from the forests near Szczecin (0.808±0.661 µg/g d.m.); however, the difference was not statistically significant (Table 1).

The average content of lead (0.742±0.485 µg/g d.m.) in skull bones of roe deer living near Drawsko Pomorskie was similar to those living near Szczecin (0.604±0.529 µg/g d.m.) (Table 1).

Regardless the area of the origin of the animal, a significant positive correlation was found between the content of

Table 1. Concentrations of lead [μg/g d.m.] and fluoride [μg/g d.m.] in skull bone and antler of roe dear (*Capreolus capreolus* L.) from Szczecin and Drawsko Pomorskie (West Pomeranian, Poland).

Lead concentration [μg/g d.m.]					
Period: 2000-06		Mean	SD	Min.	Max.
Szczecin	Skull bone (n=11)	0.604	0.529	0.080	1.700
	Antler (n=11)	0.808	0.661	0.030	2.110
Drawsko Pomorskie	Skull bone (n=9)	0.742	0.485	0.040	1.450
	Antler (n=9)	1.093	0.579	0.260	1.760
Fluoride concentration [μg/g d.m.]					
Period: 2000-06		Mean	SD	Min.	Max.
Szczecin	Skull bone (n=11)	0.420	0.092	0.280	0.550
	Antler (n=11)	0.400	0.118	0.240	0.630
Drawsko Pomorskie	Skull bone (n=9)	0.280	0.072	0.210	0.430
	Antler (n=9)	0.324	0.084	0.160	0.430

fluoride in the antler and skull bone (Rs=0.48, p=0.028), and between lead content in the antler and skull bone (Rs=0.49, p=0.023), respectively. However, considering the place of living, a significant positive correlation was found only between the fluoride content in the skull bone and antler (Rs=0.69, p=0.019) of the animals from the forests in the neighbourhood of Szczecin, while correlations for other places were statistically non-significant.

Discussion

Three districts of West Pomeranian province, Gryfino district, Police district, and the city of Szczecin, play a crucial part in emitting pollutants to the atmosphere. 88% of total air pollution and 58.8% of dust pollution come from these areas. Abrupt increases in car transportation over recent years and lack of proper road infrastructure is yet another important cause of lead contamination. Western and northwestern winds predominate in the province and are conducive to the flow of pollutants from regions situated on the border of Germany [17] – a country that notes higher emissions of lead than Poland [18]. Lead in soil and dust is accumulated because of the combustion of gasoline with lead tetraethyl and emission from industrial sources [18]. Despite considerable limitations in lead usage, soil and dust remain important sources of risk [19] (Fig. 3), since lead is not biodegradable.

The main source of fluoride contamination in West Pomeranian is the "Police" chemical factory that produces, among other things, superphosphates, which can be a potential source of hydrogen fluoride [19]. The "Dolna Odra" power plant and "Pomorzany" and "Szczecin" heat and power stations are also sources of water-soluble fluorides. The emitted dust can spread great distances depending on the height of chimneys, the directions of winds, the frequency and intensity of precipitation, and the landscape [3]. During the highest intensity of emissions in the years 1981-85, the areas situated near the factories received 1.76 t·km^{-2} of fluorides annually [20]. Such concentrations caused health problems among people and animals, and considerable damage of the wood stands was found even within a 20 km distance from the factory. Emitted fluoride and sulphur compounds inflicted the greatest damage in the wood stands of the Kliniska Forest Division located on the eastern bank of the estuarial part of the Odra River opposite the factory. In recent years, because of innovations in production installations, by strict monitoring of emissions and reducing production, the "Police" chemical factory drastically limited the amount of the emitted fluoride, making merely 15% of the obtained limit in the current period [20, 21].

In our study, despite expected higher concentrations of lead in hard tissues of roe deer living in the vicinity of Szczecin, we found that animals living in the forests near Drawsko Pomorskie contained more lead in their bone structures (Table 1). These differences, however, were not statistically significant.

Higher concentrations of lead in antlers of roe deer from the neighborhood of Drawsko Pomorskie is surprising because Szczecin forests are exposed to higher industrial emissions in comparison with forests of the Drawsko Pomorskie district. Likewise, lead contamination from abroad is higher near Szczecin than in the Drawsko Pomorskie region. The explanation for this discrepancy might be in the presence of the Land Forces Training Center in the forests near Drawsko Pomorskie, i.e. the Drawsko Pomorskie Military Range, one of the largest military ranges of Europe with an area of 36,111 ha [22]. Military vehicles moving within the area of the army range may be an important source of lead pollution of the neighbouring forests. The firing ground is situated in the middle of the forests, so falling dust containing lead penetrates the forest ecosystems directly. Animals inhabiting these surroundings are probably far more exposed to emissions of this element than organisms living in Szczecin forests near the German border. The major route of pollutant intake by the deer was probably the ingestion of plant material contaminated with dry and wet deposits containing lead [23]. In addition, intentional or accidental uptake of (contaminated) soil [24] could have contributed to the body burdens of lead. In this respect, huge amounts of lead bullets that have been accumulating in the soils of the military shooting range may contribute an important path of lead intake in roe deer organisms. Further relevant exposure pathway might be inhalation of contaminated air [25].

Additionally, both lead and particularly fluoride accumulate in antlers not only via the intake of contaminated food during the period of antler growth, but rather also from the skeleton, in which these pollutants had been deposited during the entire life-span of an individual. Therefore, it was expected that both pollutants (and particularly fluorides) [10, 26] would be dependent on the age of the animal analyzed, with significant increases with age, where Kierdorf and Kierdorf (2002) showed that mean lead levels in younger roe deer were significantly lower than those in older deer [27].

Therefore in this research animals were initially divided into younger (1-2 years) and older (4-8 years) groups. The number of younger and older individuals participating in the studies was the same for both study sites. However, the results of statistical analysis revealed no significant correlation between fluoride and lead contents in hard tissues (bone and antler) and age. There was no significant difference in the contents of the analyzed elements in younger and older animals. Therefore, all the animals of the study area were treated as a group representative for the research.

Deer antlers can be a perfect tool for monitoring the natural environment [5, 8, 9]. Hard tissues of roe deer were often used to examine the level of lead contamination of German forests in different time periods [6, 24]. Kierdorf and Kierdorf proved the existence of statistically significant positive correlations between the lead concentration in antlers of roe deer from a specific year and the concentration of lead in air in the same year [6]. Similarly, a highly significant correlation between annual emissions of gaseous pollutants from a large thermal power plant and mean annual contents of lead in roe deer antlers was also found in Slovenia [12]. Concentrations of lead in antlers of roe deer from Lower Silesia, Poland, collected during 1993-94 were studied by Chyla et al. [28], indicating a steady drop in pollution in the examined area.

In our research the lead concentration in antlers of roe deer between the years 2000 and 2006 in West Pomerania was considerably lower (mean: 0.763 μg/g near Szczecin, and 1.088 μg/g in the Drawsko Pomorskie region) in comparison with the majority of other European data. For example, in 1932-98 the average lead concentration in antlers of roe deer from the surroundings of Köln (Germany) oscillated between 0.4 and 12.0 μg/g, and the authors noted a considerable reduction of lead contamination of the environment primarily in the 1980s and '90s [6].

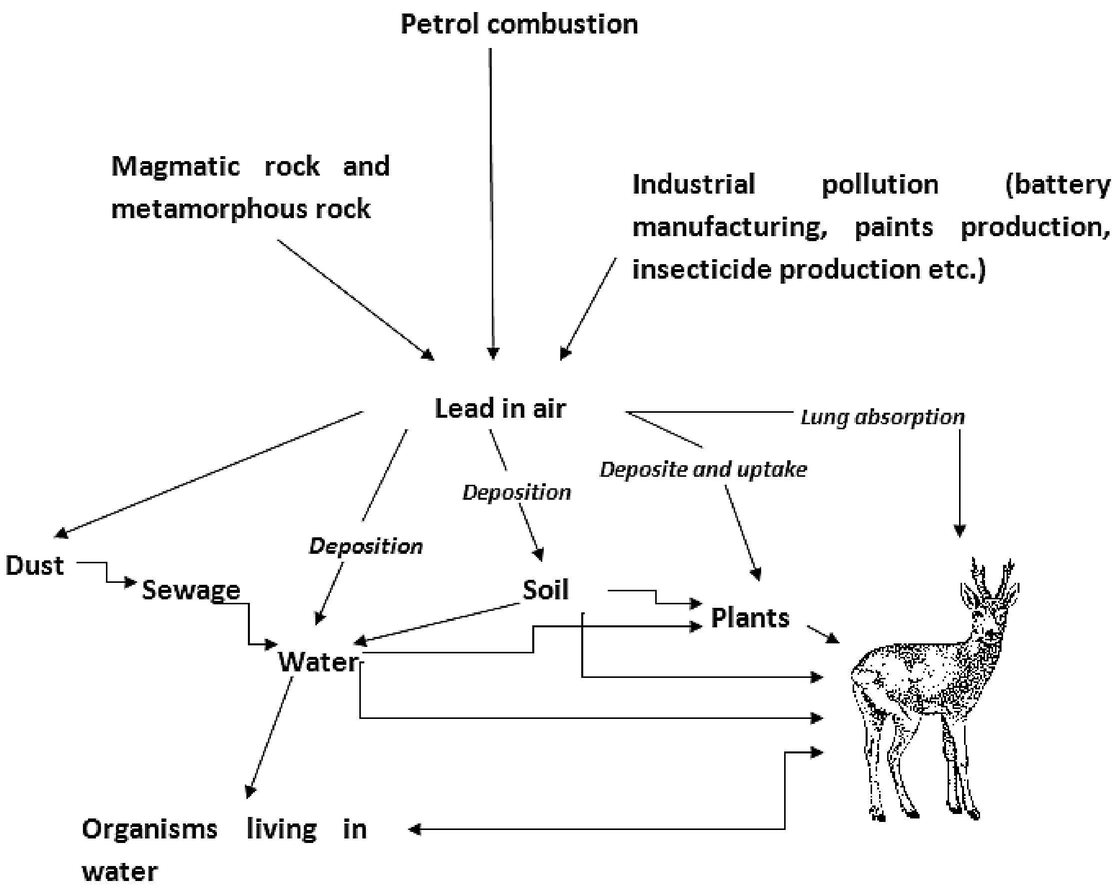

Fig. 3. Lead circulation in the environment [19].

However, the highest measured levels of lead in roe deer antlers were determined in the vicinity of a large lead smelter in northern Slovenia, where average contents in 1925-2005 reached as a high as 54.7 μg/g, with a maximum of 554 μg/g in the mid-1980s, i.e. in the period of the highest emissions from the smelter [13]. In the same paper, Pokorny et al. also presented data of lead content in antlers of roe deer in 1970-2007 in the only Slovenian military testing ground, where the maximum reached up to 6.77 μg/g [13]. Kierdorf and Kierdorf presented data of lead concentrations in roe deer antlers collected in the industrialized area of Siegen (western Germany) in 1948-2000. Lead concentrations ranged between 0.3 and 166.3 mg/kg dry weight. Authors showed a marked decline in lead exposure of the roe deer inhabiting the study area in the investigated period [29]. The same authors carried out research in different regions of Western Germany in 1948-2000 and showed that lead concentrations in antlers ranged between 0.3 and 166.6 mg/kg dry weight, as well as a steady decline in antler lead concentration occuring in the last two decades of the 20th century [30].

Similar research was conducted in Russia using skulls and antlers of wild forest reindeer [31]. The animals were shot by hunters between 1986 and 1990 from North Karelia and Archangielsk regions. The range of lead concentration in skull bones of the examined animals was 3.0-146.0 μg/g, and in antlers 5.1-65.4 μg/g. The lead concentration obtained from this region was different from those in cervides from the most polluted forest region of southern Poland, and contained mean lead concentrations of 14.8 μg/g (range 3.8-33.3 μg/g) [7]. Much lower concentrations (range: 0.2-3.2 μg/g) were found in deer antlers collected in 1985 in an unpolluted region of Poland [25]. However, elevated heavy metal concentrations in the tissues of reindeer in comparison with other cervides may be also explained by dietary habits [31], as reindeer feed mainly on lichens, which have been shown to accumulate substantial amounts of trace elements [31, 32]. When comparing data for roe deer with results from other species, it should be also considered that roe deer grow their antlers largely during winter, whereas in other deer species from temperate regions (except for the Pere David's deer, *Elaphurus davidianus*), antler growth takes place during spring and summer [6]. It was shown for areas exposed to atmospheric pollution that trace element levels in plants eaten by deer were markedly higher in autumn compared to spring [33]. During antler growth in winter, roe deer therefore probably take up more trace elements per unit of body weight with their diet than e.g. red deer (*Cervus elaphus* L.) from the same area, which grow their antlers in spring/summer [6]. Apart from variability in the season of antler formation, the particularly specific feeding strategy of roe deer is "concentrate selector," which means that the species primarily feed on those plants or their parts that are rich in nutrients and also trace elements [34]. Additionally, most of the lead ingested by deer is present on the plant surface as a consequence of dry and wet

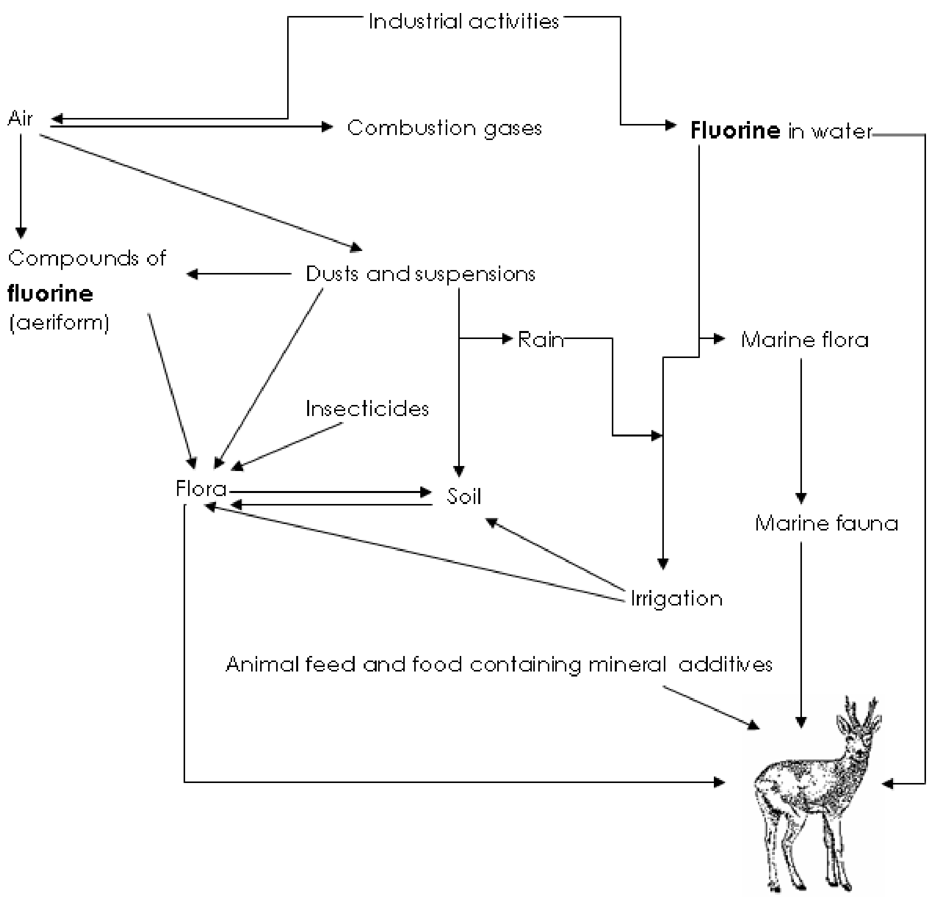

Fig. 4. Fluorine circulation in the environment [45].

deposition [30]. This should be the main reason for elevated contents of toxic substances in either roe deer bone structures or soft tissues in comparison with other ungulate/deer species [34]. However, Robbins et al. and Gordon and Illius showed that many of Hoffman's physiological and nutritional interpretations are not supportable [35, 36].

Fluoride is essential for living organism (Fig. 4) and is built into the bones, teeth, and antlers of deer in the form of fluorapatite. Both a surplus or deficiency of fluoride cause degenerative changes in hard tissues [19]. In this research the average concentration of fluoride in skull bones of roe deer inhabiting forests near Szczecin was significantly higher than those from the forests near Drawsko Pomorskie. However, such dependence was not found for antlers, which may be caused by its annual antler shedding.

Similar monitoring of fluoride content in roe deer bone tissue was performed in various regions of Germany, where the content of fluoride in roe deer acquired in the years 1932 to 1998 and 1948-2000 was analyzed [6]. In the antlers of roe deer inhabiting Siegen forests (western Germany) collected in 1948-2000, the highest concentration of fluoride compounds was found from 1960-69 (2096 μg/g) and the lowest from 1990-2000 (323 μg/g). The study confirmed the decrease in the content of fluorides in roe deer antlers with a simultaneous reduction in the number of sources emitting pollution [37]. Very similar findings were obtained also in Slovenia, where fluoride content in roe deer antlers had started to continuously decline after the reduction of emissions from a large thermal power plant [26, 38].

In a study conducted in Germany, using the mandibles of roe deer aged 1-11 years (collected between the years 1995 and 1998), Kierdorf and Kierdorf confirmed a decrease in the emission of fluoride to the natural environment, which influenced the decrease of the deposits of this element in the bones of roe deer in that examined period [6], similar to studies carried out by the same authors on antlers of deer from Germany used for a historical biomonitoring study [39, 40].

Research conducted in northwestern Poland in 1993-97 proved that the content of fluoride in roe deer mandibles was dependent on the age of the animal analyzed and on the area of investigation [16]. The average fluoride content in roe deer jaws from Drawsko Pomorskie region amounted to 346 μg/g, from the region of Łobez 467 μg/g, and from the region of Trzebież 662 μg/g [16]. For red deer antlers from four areas in Poland, Samujło et al. recorded mean concentrations between 326-601 μg/g d.m. [41]. In a study carried out in an area similar to Western Pomerania, using mandibles of red deer collected in 1996-97, Zakrzewska et al. observed a very high concentration of fluoride (Szczecin: 200.92-632.70 μg/g; Łobez: 119.32-222.14 μg/g). The content of fluoride increased proportionally to the age of the animal and inversely to the distance from the source of emissions [42]. However, in our study the statistical analysis of the results showed no statistically significant correlations between fluoride content in hard tissues (bones and antlers) and age.

In both the aforementioned and our study, higher concentrations of fluorides are noted in the skull bones of roe deer from the vicinity of Szczecin. Recently, the concentration of the examined contaminants drastically decreased, which may suggest a greater environmental awareness.

Hard tissues of mammals are the place of long term deposits of lead and fluoride. Their concentrations in bones, increasing throughout the animal's life, are a reflection of exposure intensity [43, 44]. It should, however, be indicated that in some life periods deposits of lead and fluorides in bones may undergo mobilization, e.g. in the lactation period, which increases the risk of endogenic contamination [43]. Moreover, during antler growth bone minerals are mobilized from the postcranial skeleton of deer by bone resorption to meet the high mineral requirements of forming antlers [6]. It can be assumed that in the course of this process skeletally deposited contaminants like fluoride and lead are mobilized and transported to the mineralizing antlers [6, 15]. That means that the concentrations of these substances in antler bone would not solely reflect uptake by the animal during the antler growth period, but would to some extent also be influenced by the lifetime exposure of the individual [6]. Antler shedding may also be considered as one of the ways of eliminating heavy metals and fluoride from the system.

Conclusions

This present study again confirmed the usefulness of roe deer antlers and skull bones as monitoring units for assessing temporal changes in environmental pollution by fluoride and lead. Our study indicates higher lead contamination of the forests near Drawsko Pomorskie than of those in the neighborhood of Szczecin. The cause may be their long-term exploitation as a military area. Forests near Szczecin are exposed to a greater risk of fluoride contamination, but the concentration of fluoride in hard tissues of roe deer diminished considerably in comparison with data collected 10 years prior to our study, which is the consequence of many efforts and mitigation measures for reducing emissions of toxic substances into the environment.

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