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Enhanced levels of hazardous trace elements (Cd, Cu, Pb, Se, Zn) in bird tissues in the context of environmental pollution by Raša coal

Rudarsko-geološko-naftni zbornik
(The Mining-Geology-Petroleum Engineering Bulletin)
UDC: 550.4
DOI: 10.17794/rgn.2022.1.3

Original scientific paper

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Abstract

The mining and burning of Croatian Raša coal resulted in the release of various contaminants into the local environment. They have remained in the local soil, water, and plants, and became available to animals through their consumption. In this study, tissue samples from a total of 12 birds from the Raša area and 8 birds from a control area were subjected to multi-element analysis (Al, V, Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo, Cd, Pb) by ICP-MS. The obtained results showed increased Se and Cd concentrations in some bird tissue samples compared to the control area. The highest concentrations of Cu, Zn, and Se were found in the liver, while the highest concentrations of Cd and Pb were found in the kidneys. Although some hazardous trace elements were elevated in the tissues of game species, the risk of consuming meat and offal of such species has been found to be very low for human health.

Keywords:
hazardous trace elements; Raša coal; birds; selenium; environmental pollution

1. Introduction

Coal is a type of fossil fuel formed by a combination of biological, chemical, and physical decomposition of accumulated plant and animal remains during the geological past. Due to the manner and longevity of its formation, coal contains almost 70 trace elements (**Dai et al., 2016; Dai and Finkelman, 2018; Fiket et al., 2016, 2018**). Coal, as a fossil fuel, has the longest tradition of use worldwide. Even though it is an important and relatively cheap source of energy, it is impossible to ignore its impact on the environment (**Finkelman et al., 2021**). It is because coal combustion by-products, i.e. fly and bottom ash, are enriched with hazardous trace elements (HTEs), which are released into the environment by various routes (**Saikia et al, 2015a, b**). Once these particles reach the water and soil, they become permanently available to the organisms that live there (**Prevendar Crnić et al., 2015**), and crops and plants (**Maqbool et al., 2019; Medunić et al., 2021**).

In Croatia, coal mining was in operation in the eastern part of Istria, in the Raša Bay area (see **Figure 1**). Raša coal mines were intensively exploited from the mid-18th century to the end of the 1990s. This coal was used for energy production in a local coal-fired power plant (**Medunić et. al., 2016**). Raša coal is a special type of coal with a high content of organic sulfur (up to 11%), the so-called SHOS (superhigh-organic-sulfur). Such coals are enriched in the U-Se-Mo-V association (**Dai et al., 2015**). **Medunić et al.** (**2016, 2018a**) report that the soil surrounding the power plant and Raša coal mines is polluted with sulfur and a range of HTEs (selenium and cadmium in particular) as a consequence of former Raša coal combustion activities. Even today, underground Raša coal beds are a source of HTEs that are circulating throughout the local environment (**Medunić et al., 2020**).

Residues of contaminants in sensitive animal tissues are a good indicator of the potential risk they pose to the organisms in which they are measured (**Wayland et al., 2006**). Birds of prey and mammals are at the top of the food chain, so they are the most exposed to high concentrations of HTEs, and therefore, they tend to accumulate HTEs at high levels in their organs (**Kalisinska, 2019**). In terms of the environmental pollution caused by coal, **Lemly** (**2004**) pointed out that during long-term exposure to atmospheric conditions, rain can leach Se and other HTEs from coal and coal refuse. Those elements end up in leachate, which becomes a source of pollution for the surrounding water and local animals.

Some trace elements are essential, and some are non-essential. Essential trace elements are those elements that are required for an organism's growth, development, and normal functioning. However, all essential elements

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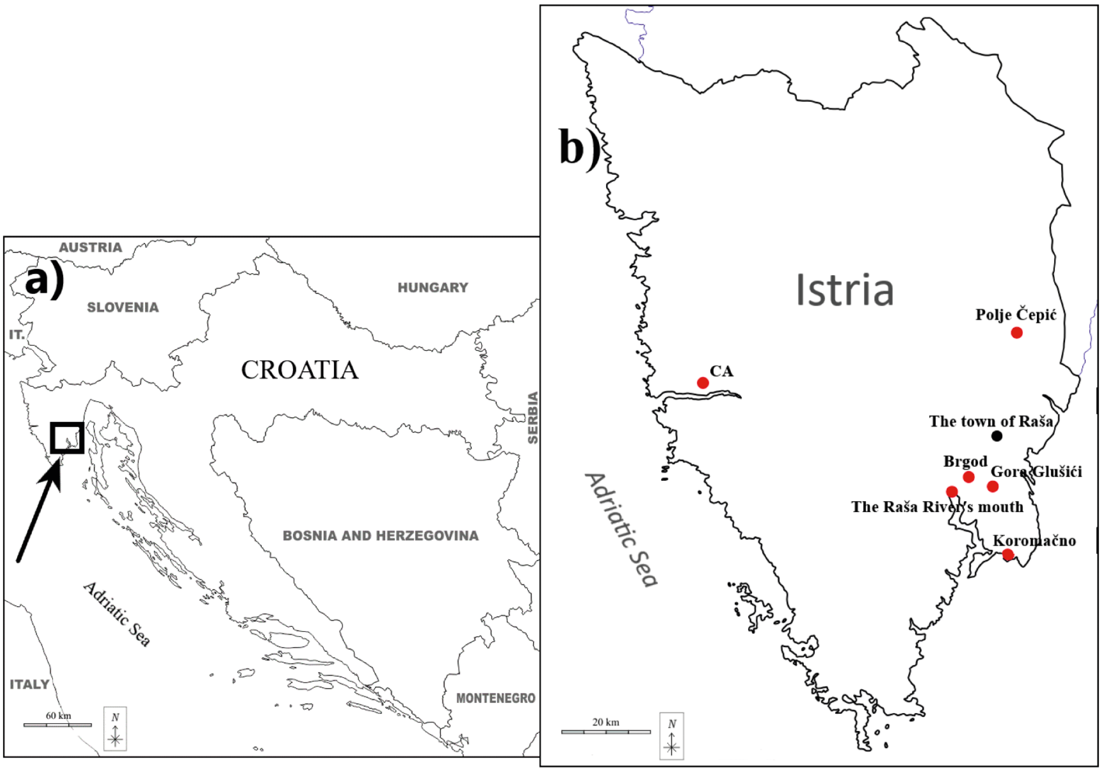

Figure 1: Map of the study area, its geographical position (a) and sampling location names (b)

can be toxic if they enter the living body in a sufficiently high concentration, and over a sufficiently long period (**Kalisinska, 2019**). On the other hand, non-essential elements can be toxic even in a small dose. Once introduced into the body, they easily enter the food chain where their concentrations increase up to a hundredfold, passing from prey to predators, which is called biomagnification. Increased levels of HTEs in the blood indicate short-term exposure, while increased levels of HTEs in the kidneys and liver indicate long-term exposure. However, increased levels of HTEs in the bones indicate the longest exposure because the remodelling of the skeletal system is very slow (**Kalisinska, 2019**). Selenium, zinc, and copper are essential elements, while cadmium and lead are non-essential elements. Cadmium is a known teratogen (possible adverse effect on a fetus), carcinogen (causes cancer), and mutagen (causes a change or damage in the DNA molecule) (**Eisler, 1985**). Lead is known as a cumulative metabolic toxin that accumulates in different parts of the body (**Raikwar et al., 2008**). It also adversely affects the hematopoietic, nervous, reproductive, and circulatory system (**Eisler, 1985**).

Birds are the longest best researched animals in Europe and North America (**Kalisinska, 2019**). They are often used as bioindicators of environmental pollution for several reasons. Their classification, distribution, biology, and ecology are well known. Furthermore, birds are widespread, easy to study, and live in a variety of habitats. They occupy different positions in the food chain, especially its top. Therefore, they are extremely sensitive to changes of HTEs at lower trophic levels (**Egwumah et al., 2017**). Sedentary birds are considered to be very good bioindicators of local pollution (**Lebedeva, 1997**). The aim of this study was to investigate the content of selenium and other HTEs (Cu, Zn, Cd, and Pb) in tissues (muscle, liver, kidney, brain) of birds (pigeon, jay, duck, and magpie) collected from the Raša coal area and surrounding sites in Istria (Croatia), in order to investigate whether or not they also indicate environmental pollution derived from soil and water polluted by coal.

2. Materials and methods

2.1. Site description

The study area is located in the eastern part of Istria (see **Figure 1**). The geological structure of the wider study area consists of sedimentary deposits from the Lower and Upper Cretaceous and Paleogene. Istria is a karst region composed mainly of limestone and other carbonate rocks. The eastern part of Istria, where coal deposits are located, contains Paleogene deposits. Coal seams from the Upper Paleocene to the Lower Eocene lie on massive layers of Upper Cretaceous limestones. Foraminiferal limestones from the Middle Eocene continue on the coal seams. It is followed by the Eocene deep-sea flysch sediment composed of marl, marly limestone and sandstone (**Šarin and Tomašić, 1991**).

Birds were collected from a total of six sites (see **Figure 1**): the mouth of the Raša River, Brgod and Gora

Table 1: Descriptive statistics for comparison of Cu, Zn, Se, Cd, and Pb concentrations in bird tissues from the Raša area (R) and the control area (C)

Sample	**N**	**Mean**	**Median**	**Min**	**Max**	**Lower quartile**	**Upper quartile**	**Standard deviation**
Cu muscle R	12	**5.27**	5.08	4.09	6.98	4.48	5.86	0.972
Cu muscle C	8	**3.91**	4.01	0.762	6.34	3.27	4.80	1.64
Cu liver R	12	**17.7**	4.37	2.74	68.1	3.32	234	25.7
Cu liver C	8	**3.76**	3.29	2.86	6.13	3.17	4.10	1.06
Cu kidney R	12	**4.76**	4.21	3.56	7.28	3.95	5.41	1.31
Cu kidney C	8	**4.13**	4.09	3.26	5.30	3.59	4.54	0.685
Cu brain R	12	**2.89**	2.77	2.33	4.58	2.49	3.02	0.592
Cu brain C	6	**3.15**	3.10	2.37	4.26	2.80	3.25	0.627
Zn muscle R	12	**10.6**	10.3	7.80	14.3	9.76	11.7	1.81
Zn muscle C	8	**9.71**	9.29	6.65	14.9	8.71	10.1	2.36
Zn liver R	12	**34.0**	30.0	20.3	68.4	24.5	41.4	14.1
Zn liver C	8	**26.8**	25.6	19.2	41.0	19.6	32.0	7.91
Zn kidney R	12	**25.7**	25.7	21.6	31.3	23.1	28.1	2.99
Zn kidney C	8	**26.8**	27.7	20.7	32.3	22.9	30.1	4.32
Zn brain R	12	**12.1**	12.2	10.0	14.5	10.8	13.2	1.40
Zn brain C	6	**12.0**	11.6	10.1	14.6	11.2	12.9	1.54
Se muscle R	12	**0.776**	0.406	0.150	3.36	0.308	0.939	0.926
Se muscle C	8	**0.196**	0.192	0.114	0.301	0.150	0.236	0.0623
Se liver R	12	**1.78**	0.721	0.230	7.51	0.544	1.87	2.34
Se liver C	8	**0.517**	0.450	0.198	1.16	0.322	0.616	0.303
Se kidney R	12	**1.67**	1.14	0.598	4.60	0.973	1.84	1.24
Se kidney C	8	**0.947**	0.952	0.552	1.36	0.696	1.18	0.292
Se brain R	12	**0.398**	0.272	0.177	0.971	0.237	0.513	0.274
Se brain C	6	**0.223**	0.235	0.180	0.258	0.181	0.249	0.0346
Cd muscle R	12	**0.00592**	0.00508	0.000784	0.0174	0.00303	0.00644	0.00482
Cd muscle C	8	**0.0127**	0.00290	0.000825	0.0815	0.00202	0.00470	0.0278
Cd liver R	12	**0.389**	0.319	0.0816	1.578	0.153	0.441	0.402
Cd liver C	8	**0.177**	0.160	0.0525	0.417	0.124	0.190	0.108
Cd kidney R	12	**1.60**	1.50	0.286	4.99	0.723	1.73	1.28
Cd kidney C	8	**0.477**	0.463	0.0801	0.940	0.344	0.590	0.253
Cd brain R	12	**0.00262**	0.00217	0.000479	0.00703	0.000926	0.00346	0.00220
Cd brain C	6	**0.00167**	0.00155	0.000481	0.00315	0.000701	0.00255	0.00106
Pb muscle R	12	**0.0364**	0.00358	0.00131	0.398	0.00204	0.00535	0.114
Pb muscle C	8	**0.0396**	0.00292	0.00158	0.292	0.00199	0.00679	0.102
Pb liver R	12	**0.232**	0.0272	0.00886	2.44	0.0217	0.0407	0.696
Pb liver C	8	**0.0666**	0.0142	0.00408	0.430	0.0116	0.0235	0.147
Pb kidney R	12	**0.249**	0.109	0.0275	1.14	0.0512	0.306	0.337
Pb kidney C	7	**0.250**	0.0495	0.0212	1.45	0.0233	0.0797	0.532
Pb brain R	11	**0.0138**	0.0106	0.00117	0.0424	0.00481	0.0200	0.0116
Pb brain C	6	**0.0156**	0.00716	0.00490	0.0606	0.00524	0.00851	0.0221

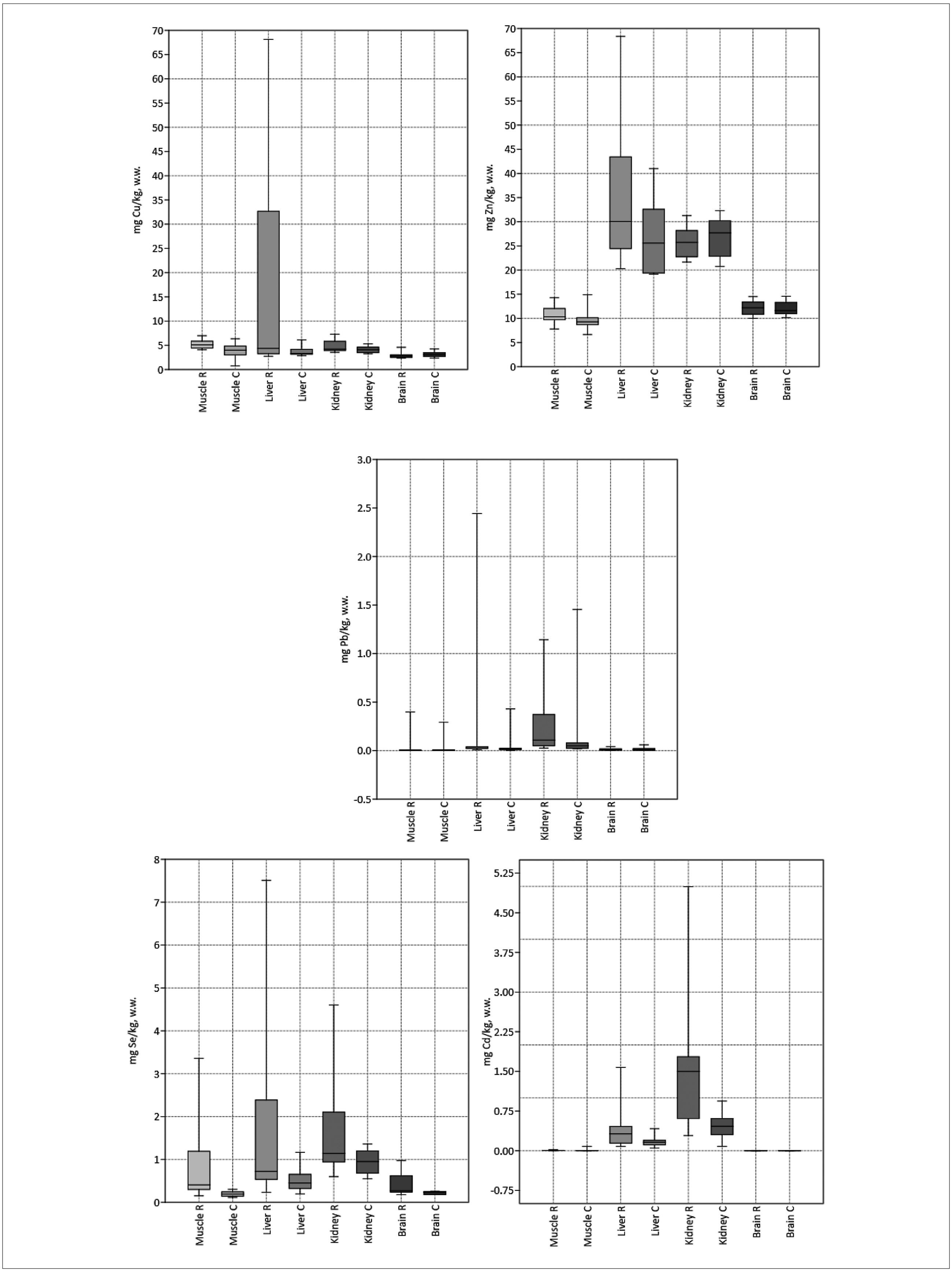

Figure 2: Box plot diagrams compare median, minimum, and maximum levels of Cu, Zn, Se, Cd, and Pb in bird tissues from the Raša (R) area and the control (C) area

Table 2: Mean concentrations of Cu, Zn, Se, Cd, and Pb in tissues of pigeons, mallards, and magpies from the Raša area compared with the literature data (all concentrations are expressed in mg/kg, w.w., literature data expressed in d.w. were recalculated into w.w. considering 70% of water content in bird tissues)

Reference	Location	Bird species	Tissue	Cu	Zn	Se	Cd	Pb
Present study	Raša, Istria	pigeon	muscle	5.12	10.03	0.344	0.00450	0.00350
			liver	3.56	25.7	0.575	0.231	0.0238
			kidney	3.87	26.8	1.01	1.43	0.0728
			brain	2.78	12.9	0.233	0.00270	0.0158
		mallard	muscle	6.56	12.4	2.09	0.00360	0.00360
			liver	59.0	53.7	5.29	0.757	0.0410
			kidney	6.85	23.7	3.55	2.30	0.201
			brain	3.56	10.6	0.840	0.00270	0.0151
		magpie	muscle	4.39	9.65	0.324	0.00290	0.00360
			liver	4.88	33.3	0.747	0.132	0.0294
			kidney	4.43	21.9	1.30	0.310	0.632
			brain	2.33	12.4	0.299	0.00100	0.0106
Begum and Sehrin (2013)	Bangladesh	pigeon	muscle	1.64	43.7		0.00900	0.117
			liver	10.5	71.9		0.330	0.855
			kidney	3.69	12.4		0.279	0.351
Hutton et al. (1980)	London, Chelsea	pigeon	liver		43.9		0.735	6.48
			kidney		50.6		3.69	9.64
Torres et al. (2010)	Santa Cruz de Tenerife, Spain	pigeon	muscle	4.02	12.2	0.338	0.00750	0.111
			liver	3.41	40.9	0.487	0.110	0.291
			kidney	2.84	25.0	0.815	0.678	0.287
Horrai et al. (2007)	Japan	pigeon	muscle	4.56	10.3	0,318	0.00260	
			liver	14.6	39.6	0,624	0,0939	
			kidney	3.93	27.2	1,07	0,339	
			brain	3.09	13.9	0.237	0.00130	
Alipour et al. (2016)	Kani Barazan, Iran	mallard	muscle	2.48	6.18		0.138	0.273
			liver	3.21	17.9		0.315	0.711
			kidney	2.89	9.08		0.201	0.579
Szymczyk and Zalewski (2003)	Poland	mallard	muscle	4.79	10.4			0.136
			liver	7.08	24.3			0.225
Zarrintab and Mirzaei (2018)	Iran	magpie	muscle	3.97	31.8		0.237	0.657
			liver	7.73	32.9		0.588	0.864
			kidney	4.82	21.8		0.456	0.792
Kim et al. (1998)	Japan	white-chinned petrel, black-browed albatross, and grey-headed albatross	muscle	3.03	19.6	2.06	0.810	0.0111
			liver	4.41	44.4	9.06	5.820	0.0357
			kidney	4.53	47.1	13.7	31.8	0.0405
			brain	2.72	15.2	4.44	0.260	0.0147
Abduljaleel et al. (2012)	Malesia	chicken	muscle	2.90	23.7	0.604	0.0477	0.106
			liver	0.465	4.78	0.180	0.0450	0.0630

Glušići (located within a radius of 5 km (air distance) from the source of pollution), the town of Raša (the Raša Bay), Polje Čepić (located about 15 km north), and Koromačno (about 10 km south of the source of the pollution). The Kontija Forest, above the Lim Channel, on the west coast of Istria, was chosen as the control area (CA). CA is about 35 km away from the source of the pollution.

2.2. *Bird sampling*

Samples were taken during December 2019 and January 2020. A total of 20 bird samples from six localities were collected. Five wild pigeons and two mallards were collected from the mouth of the Raša River. One wild pigeon was collected from Koromačno, one mallard from Polje Čepić, one magpie from Brgod, and two jays from Gora Glušići. Six wild pigeons, pheasants and jays were collected from the control area. Al, V, Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo, Cd and Pb concentrations were measured in the muscle, liver, kidney and brain of each bird species.

2.3. Preparation of bird tissue samples

Homogenized tissue samples (0.5 g) were weighed into a Teflon liner with the addition of 3 mL H_2O and 2.5 mL HNO_3 (65%). The wet digestion of the samples was performed using a high-pressure microwave oven Multiwave 3000 (Anton Paar, Graz, Austria). The digestion was performed in three steps: I) 2.5 minutes at 500 W; II) 20 minutes at 1000 W; III) 30 minutes at 1200 W. Following the cooling to room temperature, the digested clear solution was quantitatively transferred to a 50 mL volumetric flask and filled up to the mark with ultra pure water. A mixture of internal standard solution (ISTD) containing In, Bi, and Sc (Inorganic Ventures, Blacksburg, VA, USA) was added on-line using the standard ISTD mixing tee-connector.

2.4. Multi-element analysis of bird tissues

Element concentrations were determined by inductively coupled plasma with a mass detector, Agilent ICP-MS Model 7900 (Agilent, Palo Alto, CA, USA). High purity argon was used throughout (99.999%, White Martins, Brazil). Calibration of the instrument was carried out using certified standards of 99.99% purity for all elements (Se, Cd, Pb, Cu, Zn) at a concentration of 10 mg/L (Environmental Calibration Standard, Agilent Technologies, USA). All data are expressed in mg/kg on a wet basis (w.w.).

3. Results and discussion

Out of 13 analysed elements (Al, V, Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo, Cd, Pb), 5 elements (Se, Cd, Pb, Cu, Zn) were selected for further data analysis as they were found to be elevated in the local Raša environment (**Medunić et al., 2016, 2018a, 2020, 2021**). Their pooled concentrations are shown in **Table 1**. The highest Cu and Se concentrations were measured in the liver, while the lowest was in the brain of all the bird species. The highest Zn concentrations were measured in the liver followed by the kidneys, while the lowest Zn concentrations were measured in the muscle samples. The highest concentrations of Cd were found in the kidneys, while the lowest ones were in the brain samples. Furthermore, the highest concentrations of Pb were found in the liver and kidney samples.

Median, minimum, and maximum values of analysed HTEs per tissue from the Raša area were compared with the respective ones from the control area (see **Figure 2**). Generally, median and maximum values of most HTEs are increased in bird tissues from the Raša area compared with the control ones. The maximum Se, Zn, Cu, and Cd concentrations were found in mallard tissues from the Raša area.

3.1. Comparison of HTEs in analysed bird tissues with literature data

A review of the literature data (see **Table 2**) showed that HTE concentrations were mainly reported for tissues of a single bird species, while this study involved several bird species. In order to facilitate a comparison of HTE levels in tissues of birds included in the present study with the literature data, the mean concentrations of HTEs per tissue of each species were calculated. Thus, the mean concentrations of Cu, Zn, Se, Cd, and Pb in the tissues of pigeons, mallards, and magpies from the Raša area were compared with the relevant research (see **Table 2**).

- *Selenium*

Ohlendorf and Heinz (**2011**) reported Se concentrations (mg/kg, w.w.) in birds' liver, kidneys, and muscles. Liver concentrations of Se from 0.35 to 1.00 are defined as adequate, from 2.00 to 6.00 as high, from 4.00 to 23.00 as toxic. Se concentrations from 0.5 to 1.2 in kidneys are defined as adequate, while concentrations from 1.5 to 5 are defined as high. On the other hand, for Se concentration in muscles the following applies: adequate 0.13-1.3, high 0.4-5.5, toxic 1.3. The mean concentration of Se in the liver of all bird species from the Raša area (1.78 mg/kg w.w.) is between adequate and high values. While the mean Se concentrations in kidneys (1.67 mg/ kg w.w.) and muscles (0.78 mg/kg w.w.) of birds from the Raša area are in the group of high values.

The mean concentrations of Se in the liver, kidneys, and muscles of pigeons and jays are within the range of adequate values. A mean concentration of 1.30 mg Se/ kg, w.w. in kidneys of magpies exceeds the range of adequate values. The highest mean Se values were measured in mallard tissues. Concentrations of 2.09 mg Se/ kg, w.w. in muscles and 5.29 kg Se/kg, w.w. in the liver are considered toxic, while a concentration of 3.55 mg Se/kg, w.w. in the kidneys is considered high. Elevated Se concentrations in both muscle and liver indicate long-term Se exposure. The measured Se values are the highest in kidneys and liver of all bird species except in mallards where the situation is reversed. When the concentration of Se in the kidneys is elevated compared to those in the liver, the dietary intake of Se is considered low. In contrast, when the level of Se in the liver exceeds that in the kidneys, it is evidence of increased intake of Se through diet (**Martinez, 1994**). It is known that Se bioaccumulates very quickly in the food chain of aquatic ecosystems so its concentration and toxicity increase. Aquatic organisms consume Se directly from water, but also from food. When it ends up in water, Se is almost impossible to remove from that system and its impact is visible decades after the source has been removed. Namely, it circulates in water in a way that the plants and animals incorporate Se into their organism and then die and decompose. It then ends up in sediment, and due to the chemical and biological characteristics of the water, it can be released and re-enter the food chain (**Lemly, 2009**). Mallards feed on aquatic plants, invertebrates and small vertebrates. Therefore, elevated Se values in their tissues, compared to other bird species, were expected.

• *Zinc*

The highest mean Zn concentrations were found in the liver (34.0 mg/kg w.w.) and kidneys (25.7 mg/kg w.w.) of birds. Concerning bird species, the highest mean Zn concentration was found in the liver of mallards (53.7 mg/kg w.w.). This value is higher compared to the mean Zn concentration of 17.9 mg/kg w.w. in the mallard¢s liver from the area of Iran, according to **Alipour et al.** (**2016**). **Beyer et al.** (**2004**) reported a mean Zn concentration in mallard¢s liver of 132 mg/kg w.w. This value indicates Zn poisoning in the Tri-State Area (Kansas, Missouri, Oklahoma) in the USA known for Pb and Zn mining and poisoning of wild birds with the same. The lowest mean Zn concentration was found in the liver of pigeons: 25.7 mg/kg w.w., which is lower than the mean Zn concentration in the pigeon¢s liver (71.9 mg/kg w.w.) from a research study on the level of heavy metals in the tissues of pigeons from the area of Bangladesh (**Begum and Sahrin, 2013**).

• *Copper*

The concentration that indicates Cu poisoning in birds depends on several factors. It varies from species to species and depends on the bird¢s diet and the chemical form of Cu (**Demayo, 1982**). The mean Cu concentrations in muscles, liver and kidneys of mallards from the Raša area are as follows: 6.56; 59.0; 6.85 mg/kg w.w. For comparison, **Alipour et al.** (**2016**) reported the following mean Cu concentrations in mallard¢s muscles, liver, and kidneys from the Iran area: 2.5; 3.2; 2.6 mg/kg w.w.

The Cu concentration in the liver is a reliable indicator of animal exposure to a large number of contaminants and also for Cu (**Underwood, 1971**). **Henderson** (**1974**) discusses one of the rare cases of Cu poisoning in mallards. Namely, a group of 100 Canadian geese was poisoned with water containing 600 mg/L of copper (II) sulfate pentahydrate. Necrosis of the upper gastrointestinal tract was diagnosed in all birds following with a Cu concentration of 56 to 97 mg/kg w.w. in the liver. **Beck** (**1956**) concludes that, compared to other birds, mallards have a higher concentration of Cu in the liver. For example, Cu concentration in the liver of chickens and turkeys ranges between 12.7 and 17 mg/kg d.w. (3.81 and 5.1 mg/kg w.w. assuming 70% water content). While the mean concentration of Cu in the liver of musk ducks is 153 mg/kg d.w. or 45.9 mg/kg w.w. (**Beck, 1956**).

The mean Cu concentrations in muscles, liver and kidneys of pigeons from the study area were: 5.12; 3.56; 3.88 mg/kg w.w. **Begum and Sehrin** (**2013**) reported the following mean Cu concentrations in the same tissues respectively: 1.64; 10.5; 3.69 mg/kg w.w. Regarding magpies from the study area, the mean Cu concentrations in muscles, liver and kidneys were: 4.39; 4.88; 4.43 mg/kg w.w. For comparison, **Zarrintab and Mirzaei** (**2018**) reported the following mean Cu concentrations in magpie¢s muscle, liver, and kidney: 3.97; 7.73; 4.82 mg/kg w.w.

• *Cadmium*

Eisler (**1985**) concludes that evidence of Cd vertebrate contamination is a concentration of the same in the kidneys or liver greater than 10 mg/kg w.w. or 2 mg/kg w.w. per whole body, while a Cd concentration exceeding 200 mg/kg w.w. in the kidney or 5 mg/kg w.w. in the whole body can endanger the vertebrate¢s life. **Scheuhammer** (**1987**) states that Cd concentrations >0.9 mg/kg w.w. in the liver and >2.4 mg/kg w.w. in the kidney indicate possible exposure of birds to Cd from the environment. According to the obtained data, most Cd had accumulated in the bird¢s kidneys, which is in line with the fact that Cd acts as a nephrotoxin or a substance that destroys kidney cells and tissues (**Kalisinska and Salicki, 2010**).

The mean Cd concentration in the liver and kidneys of all birds from Raša area do not exceed the threshold of 0.9 and 2.4 mg/kg w.w. However, the mean Cd values in liver (0.757 mg/kg w.w.) and kidneys (2.23 mg/kg w.w.) of mallards are very close to the limit values. Such Cd concentrations are elevated compared to Cd concentrations in the liver (0.315 mg/kg w.w.) and kidneys (0.201 mg/kg w.w.) of wetland mallards in Iran reported by **Alipour et al.** (**2016**). Cd, like Se, accumulates very fast within the aquatic sediment and remains there for a long period of time. That is a possible reason for the slightly elevated Cd values in the liver and kidneys of mallards (**Huckabee and Blaylock, 1973**).

The mean Cd concentrations in the kidneys of pigeons, magpies and jays are as follows: 1.43; 0.310; 1.73 mg/kg w.w. The mean concentration of Cd in the pigeon¢s kidneys (1.43 mg/kg w.w.) is higher than the mean concentration of Cd in the pigeon¢s kidneys (0.279 mg/kg w.w.) measured in Bangladesh (**Begum and Sehrin, 2013**). Also it is lower than the mean concentration of 3.69 mg/kg w.w. in the kidneys of pigeons from central London (**Hutton et al. 1980**). The mean Cd concentration in kidneys of magpies (0.310 mg/kg w.w.) is lower compared to the mean Cd concentration in kidneys of magpies (0.456 mg/kg w.w.) from Iran (**Zarrintab and Mirzaei, 2018**).

• *Lead*

Franson and Pain (**2011**) considered 2 mg/kg w.w. as the threshold level for Pb in kidneys and liver in birds.

Brain Pb concentrations above 1.5 mg/kg w.w. indicate poisoning (**Kalisinka, 2000**). The mean Pb concentrations in the liver (0.232 mg/kg w.w.), kidneys (0.249 mg/kg w.w.) and brain (0.0138 mg/kg w.w.) of all birds from the Raša area do not exceed these threshold levels. **Begum and Sehrin** (**2013**) reported the following mean Pb concentrations in muscles, liver, and kidneys of pigeons from the Bangladesh area: 0.117; 0.855; 0.351 mg/kg w.w. The mean Pb concentrations in muscles, liver and kidneys of pigeons from the study area are lower than these values (0.00350; 0.0238; 0.0728 mg/kg w.w). The Pb concentrations in muscles, liver and kidneys (0.00360; 0.0294; 0.632 mg/kg w.w.) of magpies are lower compared to the mean Pb concentrations in magpies from Iran (0.657; 0.864; 0.792 mg/kg w.w.) (**Zarrintab and Mirzaei, 2018**). Also, the mean Pb concentrations in muscles, liver, and kidneys (0.00360; 0.0410; 0.201 mg/kg w.w.) of mallards are lower than the Pb concentrations in the same tissues (0.273; 0.711; 0.579 mg/kg w.w.) of mallards from Iran (**Alipour et al. 2016**).

3.2. Comparison of HTE levels in tissues of pigeons and jays from the Raša area with the results of the previous research in the same area

Medunić et al. (**2018b**) investigated HTEs in the tissues of pigeons, jays, and black coots from the Raša area. Birds from the Raša area in this study are pigeons, jays, magpies, and mallards. In both cases, the mean concentrations of the trace elements were expressed in mg/kg w.w. and measured by tissues of different bird species from the area under long-term influence of SHOS coal. Therefore, it is possible to compare the mean concentrations of Cu, Zn, Se, Cd, and Pb in the tissues of pigeons and jays obtained by this study with the same data obtained by the study of **Medunić et al.** (**2018b**). These data are shown in **Table 3**. The mean concentrations of Cu, Zn, Se, Cd, and Pb in this study were generally increased in almost all tissues of pigeons and jays compared to the previous paper (**Medunić et al., 2018b**). Copper concentrations are up to 11 times higher, while Se concentrations are up to 5 times higher than previously measured. Lead concentrations in all pigeon tissues are lower than previously measured. Unlike pigeons, Pb concentrations in some jays' tissues are up to 22 times higher than previously measured. Furthermore, the biggest differences were observed in Cd concentrations. **Medunić et al.** (**2018b**) state that Cd concentrations in pigeons' muscles, liver and heart, as in jays' muscles were below the limit of detection. The measured concentrations of Cd in the pigeons' kidneys are 143 times higher, while in the jays' kidneys are 173 times higher than previously measured. However, it should be noted that the measurement data in this paper probably differ from the measurement data of **Medunić et al.** (**2018b**) due to different sample preparation and analysis techniques applied. Generally, the mean concentrations of all elements are higher in the tissues of pigeons and jays from the Raša area compared to the control area. The exceptions are Pb concentrations in pigeons' tissues, which are higher in pigeons from the control area than those from the Raša area. A possible reason for that is the presence of Pb from ammunition in some bird samples.

Table 3: Mean concentrations of selected HTEs in the tissues of pigeons and jays from the studied Raša area, *control area, and **data for muscles, liver, and kidneys of pigeons and jays of the previous research (**Medunić et al., 2018b**)

		Cu	**Zn**	**Se**	**Cd**	**Pb**
PIGEON	muscle	5.12	10.0	0.344	0.00450	0.00350
		*4.62	*10.1	*0.189	*0.00250	*0.0505
		**0.45	**10.7	**0.18	**<LOD	**0.17
	liver	3.56	25.7	0.575	0.231	0.024
		*3.46	*23.4	*0,381	*0.158	*0.0809
		**6.26	**102	**0.5	**<LOD	**0.08
	kidney	3.87	26.8	1.01	1.43	0.0728
		*4.40	*28.5	*0.896	*0.551	*0.288
		**0.96	**24	**0.56	**0.01	**0.09
	brain	2.78	12.9	0.232	0.00270	0.0158
		*3.21	*12.2	*0.228	*0.00150	*0.0200
		-	-	-	-	-
JAY	muscle	4.21	10.2	0.322	0.0152	0.201
		*2.78	*10.2	*0.301	*0.00450	*0.00340
		**2.44	**11.3	**0.25	**<LOD	**15.3
	liver	4.31	29.5	0.635	0.441	1.24
		*3.24	*40.9	*1.16	*0.0525	*0.0264
		**3.71	**22.2	**0.56	**0.09	**0.11
	kidney	4.46	27.5	1.01	1.73	0.657
		*3.35	*22.8	*1.36	*0.0801	*0.0211
		**1.49	**17.5	**0.23	**0.01	**0.03
	brain	2.48	11.9	0.281	0.00290	0.00820
		*2.80	*11.2	*0.246	*0.000500	*0.00490
		-	-	-	-	-

3.3. Statistical comparison of HTEs in bird tissues from the Raša area and control area

Table 4 shows the p-values for individual bird tissues from the Raša area in comparison to the control area tested by the Mann Whitney U test. The significance level was $p < 0.05$. Statistically significant differences were found among Se data for muscles ($p=0.003$), and liver ($p=0.04$), while Cd was significantly different for kidneys ($p=0.01$). It is shown in **Figure 3**.

Kalisinska (**2019**) suggests that elevated HTEs in bird liver and kidneys are indicative of long-term exposure to contamination with HTEs. **Ohlendorf and Heinz**

(**2011**) concluded that Se concentrations in bird muscles, liver, and eggs change in response to Se exposure through the diet. However, changes in Se concentrations in bird tissues are very slow and require a long time to be noticed (**Martinez, 1994**).

The Se and Cd concentrations in bird tissues from the study Raša area were increased, compared to the control area. Presumably, this is a consequence of environmental pollution by long-term mining, processing, and Raša coal combustion across the study area (**Medunić et al., 2016, 2018a, b, 2020, 2021**).

3.4. Potential risk of consumption of game bird meat for humans

Contaminants such as HTEs are transferred from lower to higher trophic levels through food chains. Humans are at the top of the food chain, and are the most exposed to various contaminants in the plant and animal organisms they feed on (**Islam et al., 2015**). Game is considered a suitable bioindicator of environmental pollution by HTEs, such as Cd, Pb, Hg, and As. Their levels in domestic and wild animals differ significantly due to their diet. Food of wild animals depends on seasonal availability, they feed over a large area, and generally live longer than domestic animals that have a limited choice of food. Game meat is used in the diet of hunters and members of their household, so there is a potential risk of intake of HTEs into their body (**Srebočan et al., 2012**). Within the **Ordinance of maximum levels for certain contaminants in food** (**146/12**) in Croatia, there is no data on the maximum permitted amounts of HTEs in muscles and offal of game birds used for the consumption. Therefore, as reference values for Pb categories under the terminology of meat and offal of cattle, sheep, pigs and poultry are used. That is, the categories of meat, liver and kidney of cattle, sheep, pigs, poultry and horses, for Cd concentrations (see **Table 5**).

Table 4: Results (p-values) of the Mann Whitney U test applied to HTEs in bird tissues from the Raša area and the control area (bold are significant at $p < 0.05$)

	muscle	liver	kidney	brain
Cu	**0.05**	0.26	0.37	0.2
Zn	0.13	0.15	0.51	0.96
Se	**0.003**	**0.04**	0.15	0.07
Cd	0.37	0.13	**0.01**	0.6
Pb	0.79	0.11	0.19	0.7

Table 5: Maximum permitted concentrations of Pb and Cd according to the **Ordinance of maximum levels for certain contaminants in food** (146/12) in Croatia

mg/kg, w.w.	Pb	Cd
Muscle	0.1	0.05
Liver	0.5	0.5
Kidney	0.5	1.0

According to the **Hunting Act** (**OG 99/18, 32/19, 32/20**) **of the Republic of Croatia**, the wild pigeon (*Columba livia*) and the mallard (*Anas platyrhynchos*) belong to small feathered game. All mean Pb concentrations in the muscles, liver and kidneys of pigeons and mallards from the contaminated area are lower than the maximum allowed. The mean Cd concentration in

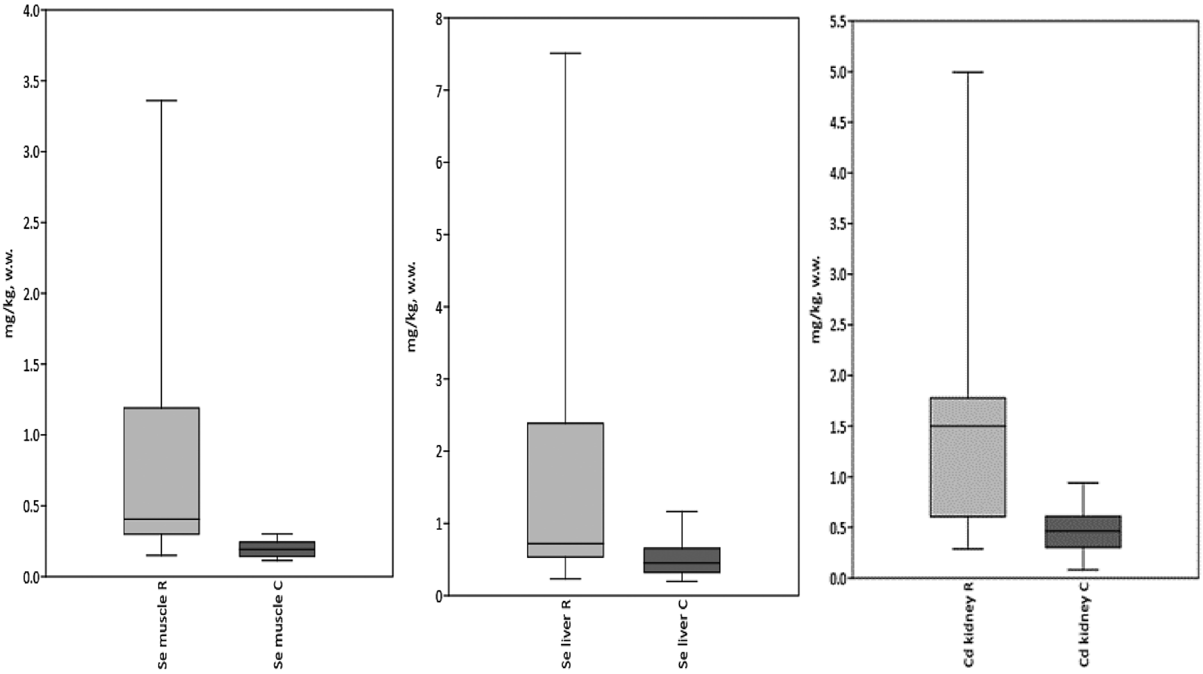

Figure 3: Box plot diagram compares Se concentrations in muscle and liver samples, and Cd in kidneys of birds from the Raša (R) area and the control (C) area

mallard¢s liver (0.756 mg/kg, w.w.) is higher than the maximum allowable Cd value in poultry¢s liver (0.5 mg/kg, w.w.). Also, the mean value of Cd in the kidneys of pigeons (1.43 mg/kg, w.w.) and mallards (2.30 mg/kg, w.w.) exceed the maximum allowable value of Cd in the poultry¢s kidney (1.0 mg/kg, w.w.).

According to the data from the **Statistical Yearbook 2011 of the Croatian Bureau of Statistics**, the average annual consumption of game meat is very low and amounts to 0.3 kg per household member. The assumption is that game meat (muscles) is consumed much more than offal, and that large game (deer, roe deer, wild boar) is generally consumed more than feathered game (ducks and pigeons). Therefore, it is possible to conclude that the risk of consuming the organs of ducks and pigeons with elevated Cd concentrations for people from the study area is very low.

4. Conclusions

In the present study, HTEs such as Se, Cu, Zn, Cd, and Pb were analysed in the tissues of four bird species (pigeon, jay, duck, magpie) from the Raša coal area and the control site near Rovinj city. The aim was to determine whether they were the result of possible pollution of the area by centuries of mining and exploitation of SHOS seleniferous Raša coal. Selenium concentrations in the muscles and liver of birds from the Raša area were found to be elevated compared to bird samples from the control area. The same was found for Cd concentrations in the kidneys of birds from the Raša area. This indicates possible environmental pollution with Se and Cd by long-term mining, processing and combustion of Raša coal in the study area. The highest Cu, Zn, and Se concentrations were found in liver, while the highest Cd and Pb concentrations were found in bird kidneys. Mean Se concentrations in muscles and kidneys of birds from the Raša area exceed adequate reference intervals. The highest mean Se, Zn, Cu, and Cd concentrations were measured in mallard tissues. Mallards are waterfowls, unlike pigeons, jays and magpies, which are more connected to the mainland through their way of their life. Elements such as Se and Cd are known to bioaccumulate in the food chain of aquatic ecosystems, such as the Raša locality, and their impact is visible decades after the removal of pollution sources. Therefore, the concentrations of the selected elements in the mallard tissues are higher than in the tissues of other bird species, which is also due to their diet. Considering that mallard and pigeon are edible species, the concentrations of Cd and Pb in their tissues were compared with the maximum permitted levels of certain contaminants in food. The risk of consuming meat and offal of these species for consumers has been found to be very low.

Acknowledgment

This collaborative study was carried out partly thanks to a financial support (“Potpora VEF-PMF, 2019” led by the leading coauthor (A.P.C.)) granted by the University of Zagreb in 2019. All the co-authors thank **Mr. Mladen Bajramović** (IUR, Raša), and hunters **Mr. Dalibor Kvaternik** (Labin), and **Mr. Emilio Mendiković** (Pazin) for providing help with fieldwork and bird sample collection in 2019.

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SAŽETAK

Povišene razine opasnih elemenata u tragovima (Cd, Cu, Pb, Se, Zn) u tkivima ptica u kontekstu raškim ugljenom onečišćena okoliša

Rudarske i industrijske aktivnosti povezane s raškim ugljenom rezultirale su ispuštanjem raznih onečišćujućih tvari u lokalni okoliš. Te tvari i dalje su prisutne u lokalnome tlu, vodi i biljkama te su dostupne životinjama unosom hrane. U ovome istraživanju uzorci tkiva ukupno 12 ptica iz područja Raše te 8 ptica iz kontrolnoga područja podvrgnuti su multielementnoj (Al, V, Cr, Mn, Fe, Co, Cu, Zn, As, Se, Mo, Cd, Pb) analizi s pomoću metode ICP-MS. Dobiveni rezultati pokazali su povišene koncentracije Se i Cd u nekim uzorcima tkiva ptica u usporedbi s kontrolnim područjem. Najveće koncentracije Cu, Zn i Se nađene su u uzorcima jetre, dok su najviše koncentracije Cd i Pb nađene u bubrezima. Iako su neki opasni elementi u tragovima povišeni u tkivima prikupljenih divljih ptica, analizom podataka utvrđeno je da je rizik od prehrambena unosa (meso i iznutrice) tih vrsta vrlo nizak.

Ključne riječi:
opasni elementi u tragovima, raški ugljen, ptice, selenij, onečišćenje okoliša

Authors contribution

Andreja Prevendar Crnić (Full Professor, PhD, Veterinary Toxicology) provided financial funds for the field work, prepared bird tissue samples for chemical analysis, carried out data analysis, and interpreted dietary data values. **Danijela Damijanić** (mag.oecol.) participated in data analysis, and wrote the entire manuscript. **Nina Bilandžić and Marija Sedak** (Senior scientists, PhD, Veterinary Sciences) conducted chemical analyses of bird tissue samples. **Gordana Medunić** (Full Professor, PhD, Geology) participated in the sampling campaign, and partly participated in data analysis and editing of the manuscript.