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Use of honeybees (*Apis mellifera* L.) as bioindicators of spatial variations and origin determination of metal pollution in Serbia

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Abstract: Honeybees have been proposed and used as bioindicators for the last few decades, because of their nature. Until now they have mostly been used to determine the present pollution and to distinguish the differences between the sampling locations and the sampling periods. With the use of multivariate statistical methods honeybees can also be used to distinguish the origin of this pollution. In this study the concentrations of Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Sr and Zn were measured in the bodies of adult honeybees collected from nine different apiaries in Serbia. With the help of the statistical methods it was established that the least polluted area was the one that has no industrial activities or the intense traffic nearby. The most polluted was the urban region, followed by a region close to thermal power plants and ash disposal site. Using PCA and CA the origin of the analyzed metals were proposed. It was suggested that Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mn and Ni have anthropogenic origin mainly from the intensive agriculture, traffic and coal combustion.

Key words: biomonitoring; honeybees; metal; pollution; PCA; CA.

INTRODUCTION

Pollution can be of anthropogenic or natural origin. Pollution that represents a bigger concern is the one emitted by mankind. The number of humans on the planet is rapidly increasing, which has led to the increase in the anthropogenic pollution as well. Metals are one of the pollutants that have the negative effect on the environment, even more so since they are not bio-degradable and are accumulated in soil, water and air, and have a potentially negative effect on human health.[1–3]

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The metal pollution can have different origin. The combustion of fossil fuels in the thermal power plants (TPP) causes the emission of fly ash and smoke containing particulate matter (PM) rich in toxic metals.[4–8] The industrial areas can also have different industries that emit metal pollution: oil refineries, petrochemical factories, steel manufacturing companies, etc.[9–11] The burning of fossil fuels for the purposes of heating, together with the intense traffic are major pollution sources in urban areas.[12,13] In rural areas major pollution comes from the intensive agriculture. The use of different pesticides, fertilizers, insecticides, herbicides and fungicides can contribute to the accumulation of metals in soil and water.[14]

Considering the negative effects that toxic metals have on human health and that the anthropogenic influence is expanding, with it the emissions of toxic metals too, it is vital to monitor their concentrations in the environment. This means that there is a need for more sampling stations, whose price and application are expensive, which is a drawback for larger areas that need to be monitored. This has led to the research and development of methods that are faster, cheaper and cover larger areas. Consequently the use of bioindicators has been increasing, since they have all of these characteristics. Many different animal and plant spices have been proposed and used as suitable bioindicators.[15–21]

Toxic metals can be accumulated in the bodies of honeybees because they are in general not deadly for bees. These metals can originate from water the bees drink, from the suspended PM in the air, depositing directly on the body of the bee, or from soil that can either be transferred from roots through plants to flowers (pollen and nectar) or can be re-suspended in the air and deposited on the plant organs that honeybees visit.[22] So, honeybees reflect pollution that is present in all aspects of the environment: soil, water and air. This is why in the last few decades they have been increasingly used as bioindicators of pollution, including the toxic metal pollution.[22–29]

The another important part of monitoring the toxic metal pollution is the method used for the detection of these metals. For the detection of metals in the biological samples, some different traditional methods are used, such as ion chromatography (IC), atomic absorption spectroscopy (AAS), inductively coupled plasma optical emission spectroscopy (ICP-OES), inductively coupled plasma mass spectrometry (ICP-MS).[16,30–33] These methods require the complicated sample preparation, that usually involves expensive equipment.[34] This is why in the past few years different new methods for detection of metals in biological samples are being explored, amongst which are X-ray fluorescence (XRF), scanning electron microscopy (SEM), which can be coupled with X-ray spectroscopy (SEM-EDX), and fluorescence nanoprobes.[34–36]

The aim of this study was to compare the concentration of Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Sr and Zn found in bodies of adult honeybees collected during year 2014, from nine different apiaries in Serbia and to assess the origin of these metals.

EXPERIMENTAL

Study area and samples

The samples were adult honeybees obtained from nine different apiaries in Serbia. Two apiaries were located in Belgrade (BG), one at the Faculty of Veterinary Medicine (BGVT) and one at the Faculty of Agriculture (BGPO); two in the city of Pančevo (PA), one in the eastern part of the city (PABA), one in the western part of the city (PAZV); one in the village of Pavliš (PV); one in the village of Mesić (MS); one in the village of Drmno (DR); one in the town of Kostolac (KO); one in the village of Stari Kostolac (STK). At each of the apiaries at least two colonies were sampled. Detailed maps and explanations of sampling locations are provided in the Supplementary material to this paper (Figs. S1 – S4).

Between 5 and 10 g of the sample (50–100 bees) were collected from the outer frame of the hive that was occupied with bees but without brood.[26] Samples were transferred into sterile plastic containers and frozen in the laboratory and kept at –21±3 °C until analyzed.

Honeybee analysis

Samples were measured and dried in the oven at 60 °C until the constant mass was obtained (approximately 96 h). A test portion of dried samples between 0.5 and 0.6 g was taken and digested according to the US EPA SW-846 Method 3052, under high pressure in closed Teflon vessels, with 7 ml of concentrated HNO_3 and 2 ml of concentrated H_2O_2. The mineralization was performed in a closed microwave digestion system (ETHOS 1, Advanced Microwave Digestion System, Milestone, Italy) by heating the samples up to 200 °C (15 min), followed by another 15 min at the same temperature. Each sample was cooled, transferred to a 25-mL volumetric flask and diluted to 25 mL with deionised water.

The concentrations of metals were measured by the inductively coupled plasma-optical emission spectrometry, ICP-OES (iCAP 6500Duo, Thermo Scientific, Cambridge, UK). The multi-element standard solutions were used (Multi-Element Plasma Standard Solution 4, Specpure®Alfa Aesar, Karlsruhe, Germany) for the determination of the elements of interest. The quality control was based on the analysis of blanks (containing 7 ml of concentrated HNO_3 and 2 ml of concentrated H_2O_2 but no analyte, prepared following the whole sample preparation procedure), duplicates and analysis of the standard solutions.

Statistical analysis

The Grubbs test for outliers was performed first, and the outliers were removed. The assessment of the data normality was done by the Shapiro–Wilk test. For some elements, the data were not normally distributed; therefore, they were log-transformed and the normality was reassessed. The results were processed by the one-way analysis of variance (ANOVA), followed by Tukey's honestly significant difference (*HSD*) multiple comparisons test. Differences in concentrations were considered significant if p values were ≤0.05. For the source appointment principal component analysis (PCA) with Varimax, the normalized rotation was used, as well as the hierarchical cluster analysis (CA) with the variables standardized by means of the Z – score and Ward's method with Euclidian distances as a measure of similarity.

RESULTS AND DISCUSSION

Metal concentrations

The range and average concentrations found in honeybees are given in Table I. The highest concentrations were observed for Ca (1167 mg kg^{-1} d.m.) followed, in the declining order, by Mg, Na, Fe, Zn, Mn, Al, Cu, Sr, Ba, Ni, Cr, Cd, and the lowest concentrations was observed for Co (0.104 mg kg^{-1} d.m.).

TABLE I. Range and mean concentrations of analyzed elements detected in the adult bodies of honeybees; the range is presented from measurements of concentrations for each individual colony, and not from average concentrations per apiary (as displayed in Table II)

Element	Range of concentrations, mg kg^{-1} dry mass	$\bar{x}$ / mg kg^{-1}
Al	7–146	36
Ba	0.54–3.97	1.69
Ca	660–1838	1167
Cd	0.03–0.260	0.125
Co	0.022–0.221	0.104
Cr	0.045–0.333	0.144
Cu	11.8–29.2	19.1
Fe	77–227	145
Mg	590–1312	979
Mn	21–78	48
Na	216–630	415
Ni	0.12–1.88	0.74
Sr	0.79–3.71	1.99
Zn	59–179	99

The mean concentrations and the standard deviations for the metals analyzed were calculated for each apiary, and are given in Table II. High standard deviations are present for some of the analyzed elements, which was to be expected. There were at least two colonies sampled at each location. The samples from each colony were prepared and analyzed separately and the mean concentrations were calculated from three repeated measurements of each sample. Considering that the bees from different colonies do not necessarily fly in the same direction, even if they are in the same apiary, the detected metal concentrations can vary between colonies. This is the reason behind the high standard deviations for some of the elements.

Analysis of variance (ANOVA)

Using one-way ANOVA statistical differences between at least two of the analyzed locations were observed for Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni and Sr (S-5 of the Supplementary material). Only for the concentration of Zn found in bodies of adult honeybees there were no statistically significant differences between any of the analyzed locations. This can suggest that the distribution of Zn in the environment is even.

TABLE II. Mean metal concentrations in bodies of honeybees (mg kg^{-1} dry mass) at different apiaries and standard deviations

Element	Location								
	BGPO	BGVT	DR	KO	MS	PABA	PAZV	PV	STK
Al	24±7	25±13	52±47	48±45	48±50	26±11	25.8±6.0	16.8±5.4	74.5±8.1
Ba	1.83± ±0.66	2.15± ±0.79	1.41± ±0.99	1.28± ±0.88	2.78± ±0.62	0.86± ±0.30	1.28± ±0.69	1.16± ±0.72	2.3± ±1.0
Ca	1178± ±155	1173± ±34	955± ±176	915± ±174	1322± ±240	1147± ±249	1294± ±303	907± ±239	1653± ±164
Cd	0.194± ±0.068	0.113± ±0.065	0.058± ±0.014	0.121± ±0.034	0.181± ±0.043	0.072± ±0.033	0.129± ±0.093	0.086± ±0.062	0.20± ±0.11
Co	0.132± ±0.026	0.12± ±0.015	0.099± ±0.040	0.174± ±0.067	0.111± ±0.034	0.056± ±0.010	0.101± ±0.078	0.062± ±0.051	0.149± ±0.056
Cr	0.132± ±0.045	0.169± ±0.047	0.158± ±0.095	0.084± ±0.012	0.149± ±0.068	0.124± ±0.018	0.212± ±0.098	0.090± ±0.042	0.169± ±0.006
Cu	24.9±1.8	26.6± ±2.7	15.6±4.2	16.6± ±1.6	21.8±3.0	15.95± ±0.90	16.4± ±2.4	16.4±3.3	17.2±3.6
Fe	151±31	186±37	114.3± ±7.6	196±45	139±43	113±22	158±54	114±49	203.5±3.0
Mg	1117±123	985±30	899±224	916± ±195	1019±84	979± ±113	1107± ±150	751±193	1097±64
Mn	54.8±9.2	50±10	47.5±2.6	63.4±4.2	61.1±7.7	37.2±4.6	57±21	30.2±5.8	41.7±6.9
Na	432±99	379±34	311±111	399±102	454±101	406±138	531±74	357±84	501±105
Ni	0.783± ±0.038	0.63± ±0.24	0.68± ±0.24	0.42± ±0.23	1.62± ±0.20	0.48± ±0.23	0.55± ±0.31	0.21± ±0.11	0.982± ±0.085
Sr	2.17±0.40	2.10± ±0.73	1.80± ±0.59	1.88± ±0.27	2.18± ±0.14	1.18± ±0.45	2.0±1.2	1.88± ±0.77	3.20± ±0.34
Zn	105±21	94.6±5.6	80±11	115±12	104±12	100±20	108±18	102±46	87.9±8.4

For Al and Fe it was observed that STK had significantly higher concentrations then PV (Table II, Fig. S-5). Higher concentrations of Al and Fe at STK sampling site was expected, due to its vicinity to an ash disposal site. Popović *et al*.[37] already established that the fly ash from thermal power plants "Kostolac" is rich in this element. In our preliminary research the honeybees from this region have proven to have higher concentration of these two elements compared to urban and agricultural-woodland regions.[27]

Ba and Sr had significantly higher concentrations in MS compared to PABA (Table II, Fig. S-5). Ba had also higher concentrations in MS compared to PV, while Sr had higher concentrations in STK compared to PABA. MS is surrounded by agricultural land and vineyards, which use different Ba compounds, that can be the source of the elevated Ba concentrations at this location.[27,38]

The higher, statistically significant concentrations, for Ca were detected in the honeybees from STK apiary, than in those coming from DR, PV and KO. Also higher concentrations can be seen for MS compared to PV (Table II, Fig. S-5). Again high concentrations of Ca in STK can also be associated with its vicinity to the ash dump site of the thermal power plants "Kostolac". After the production ash is suspended in water and after that it is transported to the dump site. Considering that the ionic strength change in the aquatic environments will cause the exchange reactions on the ash particles, and that the fly ash is a significant source of abundant cations, including Ca, it is expected for the concentration of this metal to be higher near the ash dump site.[37,39,40]

The concentrations of Cd were statistically higher in BGPO and MS compared to DR (Table II, Fig. S-5). Cd is present in many artificial phosphate fertilizers and its presence in the agricultural region surrounding MS is not surprising.[41] The elevated presence of Cd in an urban area of BGPO can be due to traffic. It has already been concluded that the soil and the vegetation in urban areas are more burdened by this toxic metal.[42]

Co concentrations were significantly higher at three locations, namely KO, STK and BGPO compared to PV (Table II, Fig. S-5). Coal combustion can have a negative effect on Co concentrations in the environment due to its presence in the fly ash produced.[43] Therefore, KO and STK sampling locations are positioned in the vicinity of the thermal power plants "Kostolac", and Životić *et al.*[44] have concluded that coal from this basin has relatively high content of Co, therefore this can explain the higher concentrations of this element at these sites. Co is also present in higher concentration in urban environments due to intense traffic.[42]

At PAZV sampling site concentrations of Cr were higher than in PV (Table II, Fig. S-5). PAZV is located in an industrial area, and Cr can come from the industrial processes happening at petrochemical industry and oil refinery in the region.

Levels of Cu were higher at BGPO sampling site compared to DR, PABA, PAZV and PV sampling sites, as well as at BGVT in comparison to DR, KO, PABA, PAZV, PV and STK, and at MS sampling site compared to DR (Table II, Fig. S-5). BGPO and BGVT are urban areas located in the capitol of Serbia Belgrade. It has been established earlier that Cu is an constituent of the vehicle brake pads.[45] In our earlier research we have already determined that honeybees from urban regions have higher Cu content then those from other locations. Also it has been proposed that honeybees from MS region have higher Cu content because of the vineyard vicinity that is treated with Bordeaux mixture containing copper sulfate.[27]

Mg concentrations were higher in BGPO and PAZV compared to PV (Table II, Fig. S-5). Considering that Mg is one of the most abundant elements that is essential to honeybees it can be that these locations are naturally richer in this element. This can also be for Na concentrations that are higher at PAZV sampling site compared to DR.

Concentrations of Mn were higher in KO and MS compared to PV and PABA. Also BGPO, PAZV, BGVT, and DR had higher concentrations of this element compared to PV sampling site. It can be concluded that the concentration of Mn are low at PV location (Table II, Fig. S-5). In urban sampling locations (BGVT and BGPO) and sampling locations located near the thermal power plant "Kostolac A" (KO) and "Kostolac B" (DR) Mn concentration are higher due to traffic and power plant emissions.[37,46] Coal burned in thermal power plants "Kostolac A & B" comes from the Kostolac basin. This coal has relatively high concentrations of Mn, which is much higher than Clarke values for brown coals, and this explains the higher Mn content in honeybees sampled at locations near thermal power plants "Kostolac A & B".[44]

Ni had statistically higher concentrations at the MS sampling site compared to PV, KO, PABA, PAZV, BGVT and DR sampling sites. Statistically lower concentrations were found at PV in relation to STK, BGPO, DR, BGVT, PAZV and PABA (Table II, Fig. S-5). Higher concentrations of Ni at MS can be explained because of the agricultural nature of the surrounding environment. Some commercial phosphate fertilizers, as well as the animal manure that are used in this region can be rich in Ni.[47,48] Honeybees in our preliminary research coming from this region also had high concentrations of Ni.[27]

Source appointment

For the source appointment of trace metals the most commonly used multivariate statistical methods are principal the component analysis (PCA) and the cluster analysis (CA).[49] PCA and CA are mostly used to identify pollution sources in street dust and soil.[50,51] For the identification of sources of metal pollution in bioindicators these methods are rarely used.[52] The source appointment of the metal pollution in honeybees using PCA and CA was done only once.[32] The factor loadings greater than 0.70 were considered excellent, while those under 0.30 were regarded as very poor.[53]

It can be observed in Table III that half of the elements, Co, Mn, Cu, Al, Ca, Cr, Zn and Na had loadings higher than 0.70. There were three principal components with eigenvalues larger than 1. These three components explain 70.92 % of total variance.

The first component is mainly characterized by Co, Mn and Cu. To a smaller extend it is also characterized by Cd, Ba and to a small extend Fe and Ni (Table III). This is confirmed by CA where Cu, Co, Fe, Ba, Mn and Cd form one large cluster (Fig. 1). This component can be attributed to anthropogenic sources mainly related to agriculture and traffic. Cu is known to be a constituent of the vehicle brake pads.[45] It is also a part of Bordeaux mixture used for treatment of crops.[27] Ba is used as an insecticide in the form of barium fluorosilicate and carbonate.[38] Cd, as well as Co and Mn concentrations are higher at locations with intense traffic.[42,54]

The second component is characterized by Al, Ca and Cr, and to a lesser extent Ba, Ni and Sr (Table III). The origin of these elements could be related to the

TABLE III. Principal component analysis (PCA) of metals measured in bodies of honeybees (PCA loadings >0.70 are shown in bold)

Element	Component		
	1	2	3
Co	**0.818**	0.335	0.129
Mn	**0.790**	0.077	0.167
Cu	**0.715**	0.100	0.224
Cd	0.648	0.116	0.574
Ba	0.618	0.606	0.173
Fe	0.534	0.450	0.355
Al	0.009	**0.887**	-0.085
Ca	0.196	**0.743**	0.490
Cr	0.228	**0.737**	0.234
Ni	0.557	0.592	0.066
Sr	0.487	0.571	0.262
Zn	0.297	-0.066	**0.821**
Na	0.090	0.372	**0.780**
Mg	0.305	0.524	0.537
Initial eigenvalues	7.173	1.586	1.170
Variance, %	51.24	11.33	8.36
Cumulative variance, %	51.24	62.56	70.92

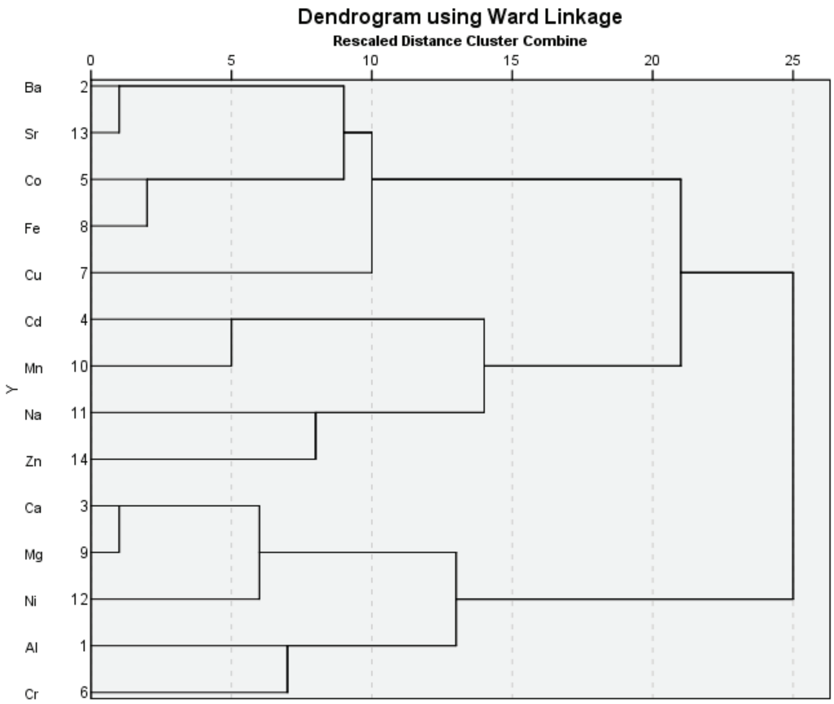

Fig. 1. Dendrogram results from hierarchical cluster analysis for the analyzed elements in bodies of adult honeybees.

emissions and the fly ash produced by thermal power plants “Kostolac”. At different places it was already concluded that emissions Al and Cr are coming from the fly ash of these power plants.[27,40,44,32] This is also confirmed by their separate cluster in CA (Fig. 1). Earlier in the text it was also pointed out that Ca is also an element that has higher concentrations around the thermal power plants, and its ash disposal sites.[39,40]

The third principal component is mainly characterized by Zn and Na, and to some extend Mg (Table III). Considering that these elements are abundant in the environment, and are essential for insects it can be concluded that these metals are from natural origin. Zn and Na form a distinctive cluster in CA as well as they are confirming their mutual source (Fig. 1).

Some of the elements had similar loadings for different components. Ba, Fe, Ni and Sr had similar loading for the first and second component. This means that they have multiple sources. They can originate from agriculture, traffic or industrial processes.

CONCLUSIONS

The data presented above about the concentrations of metals in the bodies of adult honeybees sampled from different apiaries can give us the invaluable data on the pollution of the environment surrounding these apiaries. The chosen method for the sample preparation and the analysis of metals gives good results. This method is widely used with good limits of detection for the metals analyzed in this study. The disadvantages of this method is the complicated sample preparation and costly equipment required.

This study showed that the least polluted location is PV, considering that there is no industry or high intensity traffic nearby this which was expected. The most polluted area was in the urban region, followed by the region located around an ash disposal site of a thermal power plant.

With the help of PCA and CA origin of the metal pollution measured by the use of honeybees can be explained. It was suggested that Co, Mn, Cu, Cd, Ba, Fe, Al, Ca, Cr and Ni, although grouped into two different principal components, both have anthropogenic origin, either from the intense agriculture, traffic or the burning of coal and the disposing of ash from thermal power plants. Zn, Na and Mg have natural origin.

Honeybees were again proven to be excellent bioindicators that can help track metal pollution in the environment and with the help of the multivariate statistics, can also be used to determine the origin of pollution.

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SUPPLEMENTARY MATERIAL

Aditional information and data are available electronically at the pages of journal website: http://www.shd.org.rs/JSCS/, or from the corresponding author on request.

И З В О Д

УПОТРЕБА МЕДОНОСНЕ ПЧЕЛЕ (*Apis mellifera*) ЗА ОДРЕЂИВАЊЕ ПРОСТОРНИХ ВАРИЈАЦИЈА И ПОРЕКЛА ЗАГАЂЕЊА МЕТАЛИМА У СРБИЈИ

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Због својих особина, медоносне пчеле се већ деценијама користе као биоиндикатори. До сада су се углавном користиле како би се одредиле просторне и временске разлике у присутном загађењу. Коришћењем мултиваријантних статистичких метода могуће је користити пчеле и за одређивање извора загађења. У овом истраживању су измерене концентрације Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mg, Mn, Na, Ni, Sr и Zn у телима одраслих медоносних пчела прикупљених из 9 пчелињака у Србији. Употребом статистичких метода утврђено је да је најмање загађено оно подручје на којем нема индустријске активности и у чијој близини нема саобраћајница са интензивним саобраћајем. Најзагађеније подручје је урбани регион, па затим регион у близини термоелектрана и пепелишта. Уз помоћ синтетичких метода (PCA и CA) предложени су извори загађења. Предложено је да Al, Ba, Ca, Cd, Co, Cr, Cu, Fe, Mn и Ni потичу из антропогених извора, превасходно пољопривреде, саобраћаја и од сагоревања угља.

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